Investigations into the possible role of polysialic acid and sialyltransferase activity in neural plasticity in the domestic chick

Thesis

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INVESTIGATIONS INTO THE POSSIBLE ROLE OF POLYSIALIC ACID AND SIALYLTRANSFERASE ACTIVITY IN NEURAL PLASTICITY IN THE DOMESTIC CHICK.

by

Andrew Edward Bedder BSc(hon).

A thesis submitted in partial satisfaction of the degree of Doctor of Philosophy.

Brain and Behaviour Research Group
The Open University
Milton Keynes
MK7 6AA

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<tr>
<th>Abbreviation</th>
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<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACh</td>
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<td>AMCA</td>
<td>7-amino-4-methylcoumarin-3-acetic acid</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<td>A-NCAM</td>
<td>Adult NCAM</td>
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<tr>
<td>Anti-MenB</td>
<td>Anti-meningococcus B</td>
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<tr>
<td>Asn</td>
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<tr>
<td>ATP</td>
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<td>BrdU</td>
<td>Bromo-deoxyuridine</td>
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<td>BSA</td>
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<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
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<td>Cytidine monophosphate N-acetyl neuraminic acid</td>
</tr>
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<td>ELISA</td>
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<td>Fab</td>
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<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<td>IEG</td>
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<td>ITM</td>
<td>Intermediate term memory</td>
</tr>
<tr>
<td>kDa</td>
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<tr>
<td>LHRH</td>
<td>Luteinizing hormone-releasing hormone</td>
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<td>LTP</td>
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<td>MCID</td>
<td>Microcomputer imaging devise</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MSD</td>
<td>Muscle specific domain</td>
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<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
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<td>NeuN</td>
<td>Neuronal nucleus</td>
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<tr>
<td>NeuNAC</td>
<td>N-acetyl neuraminic acid</td>
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<td>Definition</td>
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<td>polyST</td>
<td>Polysialyltransferase</td>
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<td>PSA</td>
<td>Polysialic acid</td>
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<td>PSA-NCAM</td>
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<tr>
<td>PSD</td>
<td>Post synaptic density</td>
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<td>Phosphotungstic acid</td>
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<tr>
<td>ROD</td>
<td>Relative optical density</td>
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<tr>
<td>SCN</td>
<td>Suprachiasmatic nuclei</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SON</td>
<td>Supraoptic nucleus</td>
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<tr>
<td>SPM</td>
<td>Synaptic plasma membrane</td>
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<tr>
<td>SROD</td>
<td>Standardized relative optical density</td>
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<tr>
<td>ST</td>
<td>Sialyltransferase</td>
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<tr>
<td>STM</td>
<td>Short term memory</td>
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<tr>
<td>TBS</td>
<td>Trizma buffered saline</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<td>2-DG</td>
<td>2-deoxyglucose</td>
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<td>2-dgal</td>
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<tr>
<td>VASE</td>
<td>Variable alternatively spliced exon</td>
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<td>W</td>
<td>Water</td>
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## ABBREVIATIONS FOR CHICK BRAIN STRUCTURES

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<tr>
<td>AA</td>
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<td>Archistriatum intermedium, pars dorsalis</td>
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<tr>
<td>Am</td>
<td>Archistriatum mediale</td>
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<tr>
<td>AL</td>
<td>Ansa lenticularis</td>
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<tr>
<td>ALA</td>
<td>Nucleus ansae lenticularis anterior</td>
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<tr>
<td>An</td>
<td>Nucleus angularis</td>
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<td>APH</td>
<td>Area parahippocampalis</td>
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<tr>
<td>Bas</td>
<td>Nucleus basalis</td>
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<tr>
<td>BCS</td>
<td>Brachium colliculi superioris</td>
</tr>
<tr>
<td>BO</td>
<td>Bulbus olfactorius</td>
</tr>
<tr>
<td>CA</td>
<td>Commissura anterior</td>
</tr>
<tr>
<td>Cb</td>
<td>Cerebellum</td>
</tr>
<tr>
<td>Cbi</td>
<td>Nucleus cerebellaris internus</td>
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<tr>
<td>CG</td>
<td>Ciliary ganglion</td>
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<tr>
<td>CHCS</td>
<td>Tractus cortico-habenularis et cortico-septalis.</td>
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<tr>
<td>CO</td>
<td>Chiama opticum</td>
</tr>
<tr>
<td>CP</td>
<td>Commissura posterior</td>
</tr>
<tr>
<td>CT</td>
<td>Commissura tectalis</td>
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<tr>
<td>CTrU</td>
<td>Uncrossed dorsal cochlear tract</td>
</tr>
<tr>
<td>CTrX</td>
<td>Crossed dorsal cochlear tract</td>
</tr>
<tr>
<td>DC</td>
<td>Diocoele</td>
</tr>
<tr>
<td>Dien</td>
<td>Diencephalon</td>
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<tr>
<td>DLA</td>
<td>Nucleus dorsolateralis anterior (rostralis) thalami</td>
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<tr>
<td>DMA</td>
<td>Nucleus dorsomedialis anterior</td>
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<td>Full Name</td>
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<td>-----------</td>
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<tr>
<td>E</td>
<td>Ectostriatum</td>
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<tr>
<td>EGL</td>
<td>External granular layer (of cerebellum)</td>
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<td>EW</td>
<td>Nucleus of Edinger-Westphal: nucleus nervi oculomotorii pars accessoria</td>
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<tr>
<td>FA</td>
<td>Tractus fronto-archistriaticus</td>
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<tr>
<td>FLM</td>
<td>Fasciculus longitudinalis medialis</td>
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<td>FPL</td>
<td>Fasciculus prosencephali lateralis</td>
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<tr>
<td>FU</td>
<td>Fasciculus uncinatus</td>
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<td>GCL</td>
<td>Ganglion cell layer (of retina)</td>
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<td>GL</td>
<td>Granular layer (of cerebellum)</td>
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<tr>
<td>GIL</td>
<td>Glia limitans</td>
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<td>GLv</td>
<td>Nucleus geniculatus lateralis, pars ventralis</td>
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<tr>
<td>HA</td>
<td>Hyperstriatum accessorium</td>
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<td>HIS</td>
<td>Hyperstriatum intercalatum supremum</td>
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<td>Hyperstriatum dorsale</td>
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<td>HM</td>
<td>Nucleus habenularis medialis</td>
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<td>Hp</td>
<td>Hippocampus</td>
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<td>HV</td>
<td>Hyperstriatum ventrale</td>
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<td>HVv</td>
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<td>ICo</td>
<td>Nucleus intercollicularis</td>
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<tr>
<td>ICT</td>
<td>Nucleus intercalatus thalami</td>
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<tr>
<td>IGL</td>
<td>Internal granular layer (of cerebellum)</td>
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<td>IH</td>
<td>Nucleus inferioris hypothalami</td>
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<td>Imc</td>
<td>Nucleus isthmi pars magnocellularis</td>
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<td>IMHV</td>
<td>Intermediate medial hyperstriatum ventrale</td>
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<td>IN</td>
<td>Nucleus infundibuli hypothalami</td>
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<tr>
<td>Inf</td>
<td>Infundibulum</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer (of retina)</td>
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<tr>
<td>INP</td>
<td>Nucleus intrapeduncularis</td>
</tr>
</tbody>
</table>
IO  Nucleus isthmo-opticus
Ipc  Nucleus isthmi pars parvocellularis
IPL  Inner plexiform layer (of retina)
Is   Isthmus
La   Nucleus laminaris
LAD  Lamina archistriatalis dorsalis
LFS  Lamina frontalis superior
LFSM Lamina frontalis suprema
LH   Lamina hyperstriatica
LMD  Lamina medullaris dorsalis
LPO  Lobus Parolfactorius
LS   Lemniscus spinalis
LSO  Organum septi laterale
MC   Mesocoele
mcc  Nucleus magnocellularis cochlearis
ME   Eminentia mediana
Mes. Mesencephalon
ML   Molecular layer (of cerebellum)
MLd  Nucleus mesencephalicus lateralis pars dorsalis
MM   Nucleus mamillaris medialis
MnV  Nucleus motorius nervi trigemini
MnVIIId Nucleus motorius nervi facialis pars dorsalis
MnVIIv Nucleus motorius nervi facialis pars ventralis
MnX  Nucleus motorius dorsalis nervi vagi
MnZ  Mantle zone
MZ   Marginal zone
N    Neostriatum
nBOR Nucleus opticus basalis; nucleus ectomamillaris
      (Nucleus of the basal optic root).
NC   Neostriatum caudale
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NFL</td>
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<td>NH</td>
<td>Neurohypophysis</td>
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<td>Olfactory nerve</td>
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<td>Nervus opticus</td>
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<td>OFL</td>
<td>Optic fibre layer (of retina)</td>
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<td>Nucleus nervi oculomotorii pars ventralis</td>
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<tr>
<td>ONL</td>
<td>Outer nuclear layer (of retina)</td>
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<td>OPL</td>
<td>Outer plexiform layer (of retina)</td>
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<td>Nucleus ovoidalis</td>
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<td>Glandula pineale</td>
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ABSTRACTS BASED ON THIS WORK

Mapping the Anatomical Distribution of Polysialic acid in the Late Embryonic and Early Post-Hatching Chick.  
*A.E.Bedder, S.P.R.Rose and R.C.Bourne.*  
*British Neuroscience Association Abstracts (1999), 15, 7.16, p43.*

Mapping the Anatomical Distribution of Polysialic Acid During the First Two Weeks of Chick Embryological Development.  
*A.E.Bedder, S.P.R.Rose and R.C.Bourne.*  
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ABSTRACT

Polysialic acid (PSA) is an unusual linear homopolymer of α2,8 linked N-acetyl-neuraminic acid residues that occurs in the vertebrate nervous system as a post-translational modification of the neural cell adhesion molecule, NCAM. During development NCAM tends to shift from a highly polysialylated to a lightly polysialylated form - a transformation that is believed to reflect the progress from developmental neural morphoplasticity to a more stable, mature character. Immunocytochemistry, with a specific anti-PSA antibody, is used here to map the distribution of PSA in the brain, retina and optic nerve of Ross Chunky chick embryos at embryonic days 3 (E3), 5 (E5), 9 (E9), 13 (E13), 17 (E17) and 19 (E19) and post hatching days 1 (P1) and 7 (P7). During the first two weeks of development, PSA occurred ubiquitously throughout the central nervous system, although there was some down-regulation between E9 and E13 at the levels of the third and eighth cranial nerves. Between E17 and P7, PSA was down-regulated in most of the cerebellum, pons, medulla and most fibre bundles. PSA was down regulated by the optic nerve between P1 and P7. PSA down-regulation was also observed in the internal tectal nuclei, and a few discrete regions of the diencephalon and telencephalon including the nucleus rotundus and the ectostriatum. In the telencephalon, high levels of PSA were detected as late as P7 in the intermediate mediale hyperstriatum ventrale (IMHV) and the lobus parolfactorius (LPO), regions known to be important in memory formation. Multiple immunofluorescence was used to compare PSA
expression with labelling by specific antibodies that detect galactocerebroside (GalC), a myelin-specific molecule; glial fibrillary acid protein (GFAP), that is specific to fibrous (type II) astrocytes; neuronal cell bodies and neurofilaments. These studies showed that there is no consistent relationship between PSA expression and the onset of myelination. It was shown that PSA is expressed by brain surface astrocytes in most, but not all, brain regions as late as P7 but that PSA is down-regulated by many white matter astrocytes as early as E13. Overall, these results point to significant PSA down-regulation in several chick brain regions close to the time of hatching.

Transient increases in PSA levels have been reported in rats 12-24 hours after acquisition of a step-down avoidance task, following training in a Morris water maze. PSA levels were investigated in the brains of day-old chicks at 12 hours after training on a passive avoidance task in which birds pecked either a dry bead or a bead coated in the aversant methylantranilate. PSA levels were estimated by using a grey level analysis of immunolabelled brain sections in the left and right IMHV and the left and right LPO. No differences between trained and control birds were detected. Since altered synthetic enzyme activity may mediate changes in PSA levels, sialyltransferase (ST) activity was assayed at 6, 12 and 24 hours after passive avoidance training. Activity was assayed, using CMP-(14C)-NeuNAc as sialic acid donor and fetuin as exogenous acceptor, in the left LPO. No differences in ST activity between groups were evident at any of the time points investigated.
AIMS

The present study investigates polysialic acid (PSA), a carbohydrate modification of the neural cell adhesion molecule, NCAM, that is believed to be involved in neural plasticity (Rutishauser and Landmesser, 1996). Data relating to PSA are presented in two contexts: firstly, in the developing brain of the embryonic and early post-hatching chick and, secondly, in memory formation in chicks of one day old.

PSA is expressed at high levels during embryonic development but is progressively down-regulated until it is detected in only a few discrete regions in the adult brain (Rutishauser and Landmesser, 1996; Bonfanti et al., 1992). In the first part of the present investigation, immunocytochemistry was used to map the anatomical distribution of PSA in the retina, optic nerve and brain of the chick during embryonic development and in post-hatching chicks of up to 7 days old. This study therefore aims to discover where PSA is expressed and how that expression changes during the course of development. PSA expression was compared with immunoreactivities for specific markers that detect neurons, fibrous (type II) astrocytes, myelin and neurofilaments by using multiple immunofluorescence. The functional implications of PSA expression patterns are then discussed and, in particular, the occurrence of PSA is considered in relation to morphogenetic events such as the onset of myelination.
PSA has also been implicated in memory formation in adult rats (Doyle et al., 1992a). In the second part of the current study, day-old chicks were trained on a passive avoidance task in which they learn to avoid a small bead (Cherkin, 1969). Immunocytochemistry was then used quantitatively to look at PSA levels in the intermediate medial hyperstriatum ventrale (IMHV) and the lobus parolfactorius (LPO), areas that are believed to be involved in memory formation (Kossut and Rose, 1984; Rose, 1991). This study therefore aims to see if there are any differences in PSA levels between birds that have learned the passive avoidance task and untrained birds. If PSA levels increase during learning, then increases may also be expected in the activity of the sialyltransferase (ST) enzymes that synthesize PSA. To test this hypothesis, ST activity was assayed in control birds and in birds that had been trained on the passive avoidance task. Finally, alternative strategies to investigating a possible role for PSA in memory formation in the chick are considered.
1.1. Overview and perspective.

Sialic acids are modified sugars that were first isolated from bovine submandibular salivary gland and thus acquired their name from the Greek word meaning saliva. Subsequently found to exist in numerous forms, the main type that occurs in the vertebrate nervous system is N-acetylneuraminic acid (NeuNAc) (fig. 1.1). NeuNAc commonly occurs as the terminal monomer units of glycoproteins and glycolipids joined by $\alpha2-3$ or $\alpha2-6$ ketosidic linkages to which further NeuNAc residues may be attached via $\alpha2-8$ linkages. In some structures additional residues, joined by $\alpha2-8$ attachments, extend outwards forming linear homopolymers of polysialic acid (PSA) (for reviews of PSA see Troy, 1992; Rutishauser and Landmesser, 1996).

PSA was first identified in the vertebrate nervous system by Finne in 1982. Around the same time, a recently purified cell adhesion molecule that eventually universally took the name neural cell adhesion molecule (NCAM) was found to contain a large amount of sialic acid (about 30% by weight) (Hoffman et
al., 1982). Hoffman et al. (1982) speculated that this sialic acid may be in the form of PSA. However, when NCAM was purified from adult tissue it was found to contain a much smaller proportion of sialic acid (only about 10% by weight) (Rothbard et al., 1982). The terms embryonic-NCAM (E-NCAM) and adult-NCAM (A-NCAM) were adopted to refer to the molecule in its heavily and lightly sialylated forms respectively. Although Zuber et al. (1992) have reported the presence of PSA in association with the α sub-unit of the sodium channel in the rat brain, NCAM seems to be the main carrier of PSA in the vertebrate nervous system.

![Fig. 1.1. Structure of N-acetylneuraminic acid.](image)

Further studies showed that, when incorporated into liposomes, E-NCAM is less adhesive than A-NCAM (Hoffman and Edelman, 1983; Sadoul et al., 1983). Furthermore, removal of PSA by a specific endoneuraminidase (endo-N) that cleaves PSA chains with a degree of polymerization of at least 8, increases the rate at which chick brain membrane vesicles aggregate in culture (Rutishauser et al., 1985). PSA cleavage has also been shown to increase the rate at which cultured F11 cells adhere to a substrate of tissue culture plastic, polyornithine or
polyornithine and laminin (Acheson et al., 1991). Thus polysialylation came to be viewed as a mechanism that transformed NCAM from a mediator of cell-cell and cell-substrate adhesion into a promoter of de-adhesion. Taken together, these findings pointed to a role for PSA in the morphoplasticity of embryological development. Subsequent studies, however, revealed the presence of E-NCAM in areas of the adult nervous system previously reported to possess a high degree of morphoplastic potential (Miragall et al., 1988; 1990; Theodosis et al., 1991; Seki and Arai, 1993). The terms E-NCAM and A-NCAM now seemed too restrictive and PSA started to be seen as a marker of morphoplasticity in general (Bonfanti et al., 1992; Rutishauser and Landmesser, 1996; Bruses and Rutishauser, 2001).

As a modification of NCAM, an understanding of the function of PSA must begin with a brief review of that cell adhesion molecule.
1.2. **Neural Cell Adhesion Molecule (NCAM).**

Intercellular contacts are mediated by a large number of glycoproteins, that fall into several different families, and are operationally termed cell adhesion molecules (CAMs). This designation is, perhaps, somewhat misleading, since CAMs may also generate or modulate intracellular signals (Williams et al., 1994; Doherty et al., 1995). For such types of reason the term cell recognition molecule may be preferable to CAM. However, the latter term has gained widespread use and will be employed here.

NCAM is perhaps the best characterised CAM and has been widely reviewed (see, for example: Cunningham et al., 1987; Rutishauser et al., 1988; Doherty et al., 1995; Fields and Itoh, 1996). It is an abundant cell adhesion molecule belonging to the immunoglobulin (Ig) superfamily. Although possessing a similar organisation, the extracellular domains of molecules within this group have varying numbers of Ig and fibronectin-III (FN-III) domains. NCAM, for instance, has 5 Ig and 2 FN-III domains while the neural cell adhesion molecule L1, another member of the same superfamily, has 6 Ig and 5 FN-III domains (fig. 1.2; see Fields and Itoh, 1996).
Fig. 1.2. Overall structures of the three main size classes of the neural cell adhesion molecule, NCAM, that are found in the nervous system. All possess 5 immunoglobulin (Ig) domains (represented by semi-circular lines) and 2 fibronectin III (FN-III) domains (filled rectangles). NCAM 180 (a) possesses a large cytoplasmic domain and NCAM 140 (b) has a much shorter cytoplasmic domain. NCAM 120 (c) is attached to the membrane via a glycosyl-phosphatidyl inositol (GPI) anchor. Potential glycosylation sites are indicated by the filled circles.
There are 3 main size classes of NCAM that occur in the nervous system. These size classes differ from one another in the size of their intracellular domains and mode of membrane attachment. Trans-membrane forms may have a large cytosplasmic domain or a small intracellular domain. On sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) these two NCAM isoforms have apparent molecular masses of 180 and 140 kDa respectively. A glycosyl-phosphatidyl inositol (GPI)-anchored isoform migrates on SDS-PAGE with an apparent molecular mass of around 120 kDa.

The structure between the 2 FN-III domains of the NCAM polypeptide may be modified by the employment of up to 4 small exons, of 3, 15, 42 and 48 base pairs in humans. A 37 amino acid sequence produced by expression of all 4 exons is found only in skeletal muscle and has thus been termed the muscle specific domain (MSD). A 30 base pair exon called the variable alternatively spliced exon (VASE) may be spliced between the 2 exons encoding the fourth Ig domain. Expression of this exon rises from less than 3% of NCAM transcripts during early developmental stages to around 50% of all transcripts in the adult CNS, a shift that correlates with the increased stability and/or reduced plasticity of neural connections later in development.

To the isoform variations resulting from alternative splicing must be added those arising from alternative glycosylation. NCAM carries 7 potential asparagine-linked N-glycan sites, of which 3 may become polysialylated (Cunningham et al., 1983; Crossin et al., 1984; Kudo et al., 1996)(fig. 1.3).
Fig. 1.3. Polysialic acid structure associated with N-glycan in the neural cell adhesion molecule, NCAM. PSA is linked to the NCAM polypeptide via a triantennary complex-type N-asparaginyl link. The first sialic acid on each branch is α2,3 linked while subsequent residues are attached via α2,8 linkages.

NCAM may act as both a promoter of morphological plasticity and a plasticity inhibitor and/or stability promoter, depending on the isoform employed (Doherty et al., 1995). These contradictory functions may be rationalised by a consideration of structural differences between isoforms. These may be of several types: [1] mode of membrane attachment; [2] structure of the cytoplasmic tail (Doherty et al., 1992); [3] variations in the extracellular domains (Liu et al., 1993; Walsh et al., 1992) and [4] degree and type of glycosylation (Zhang et al., 1992). The known structural variations of the molecule described above offer ample scope for this.

The function of NCAM (and indeed other CAMs such as L1 and N-Cadherin) in neural plasticity seems to require not only
NCAM-mediated inter-cellular adhesive interactions, but also the activation of second messenger systems intracellularly. Binding of NCAM-specific antibodies has been shown to raise intracellular calcium levels \textit{in vitro} (see Kater and Mills, 1991). This elevation in intracellular Ca\(^{2+}\) itself seems to be the result of complexes of interactions in second messenger signalling pathways (Williams et al., 1994). \textit{In vitro}, NCAM-mediated neurite outgrowth seems to depend on diacylglycerol (DAG) lipase (though not phospholipase A\(_2\) (PLA\(_2\)))(Williams et al., 1994). This suggests a role for arachidonic acid (AA), generated from hydrolysis of DAG by phospholipase C (PLC), itself possibly requiring activation of the fibroblast growth factor receptor (FGFR). Williams et al. (1994) suggest the following sequence of events: CAM activation--FGFR activation--phosphorylation and activation of PLC-->DAG generation-->hydrolysis by DAG lipase--AA produced-->activation of N and L type Ca\(^{2+}\) channels-->Ca\(^{2+}\) influx. However, alternative pathways involving, for example, phosphorylation of mitogen-activated protein kinases and the transcription factor cyclic AMP response-element binding protein, have also been proposed (Schmid et al., 1999). Second messenger signalling will not be considered further. It is clear from the foregoing, however, that NCAM involvement in the modulation of intercellular relationships does not seem to arise purely out of its intrinsic adhesive properties.
1.3. Molecular interactions.

PSA is a large, hydrated and negatively charged molecule that might de-stabilise cell-cell adhesion through steric impediment and/or charge repulsion (Yang et al., 1994). An obvious hypothesis is that PSA might attenuate or completely block homophilic interactions between NCAMs on apposing membranes. Also, the clustering of NCAMs at regions of cell-cell contact, which may enhance intercellular adhesion (Bloch, 1992), might be inhibited by PSA. However, Kadmon et al., (1990a) have suggested an assisted homophilic interaction model in which NCAM binds L1 in a cis manner, thereby increasing the trans-membrane homophilic adhesiveness of the latter. L1/NCAM interaction seems to require the presence of N-glycans on the latter since assisted L1 homophilic adhesion is attenuated following NCAM treatment with N-glycanase (Kadmon et al., 1990b). Not surprisingly, however, PSA seems to have the opposite effect since its presence reduces L1 mediated cell-cell adhesion (Tang et al., 1994). One would suspect that the presence of PSA on NCAM may prevent L1/NCAM association. However, the mechanism proposed by Kadmon et al. (1990a) cannot be universal since NCAM is expressed by L1 negative cells. Stellate and basket cells of the cerebellum, for example, express both PSA and NCAM but not L1 (Hekmat et al., 1990).
Yang et al., (1992) have demonstrated that the environment of the cell membrane is a very crowded place indeed. Thus, the presence of PSA may have more general consequences for a whole range of membrane molecules and trans-membrane interactions. Furthermore, enzymatic removal of PSA by a bacterial endoneuraminidase, that cleaves polysialosyl chains of at least 8 sialic acid units, reduces intercellular distances from around 50nm to about 40nm (Yang et al., 1992). PSA may, therefore, attenuate cell-cell adhesion by preventing CAMs on apposing membranes from coming into contact with each other. The modulation of inter-cellular distances may be a function specific to PSA, since it was not found following cleavage of heparan or chondroitin sulphate (Yang et al., 1992). It cannot be excluded, however, that the presence of other substances, such as membrane bound hyaluronic acid, may also alter inter-membrane distances (Yang et al., 1992).

The possibility that PSA may promote cell-cell de-adhesion by increasing intercellular distances raises the interesting suggestion that, depending on the types of CAMs present, it might also promote adhesion between CAMs that form stronger contacts at greater distances (Yang et al., 1992). PSA may, therefore, serve to re-structure connections, creating a primed state upon which other factors may come into play. That is, PSA may enable de-adhesion, or indeed adhesion, rather than being a component in the process itself. Yang et al. (1992) have also suggested that if PSA increases intercellular distances, it may serve to facilitate access to cell surface receptors by large diffusible molecules such as, for example, nerve growth factor (NGF) or brain derived neurotrophic factor (BDNF).
Finally, the de-adhesive function of PSA may be mediated by less direct mechanisms. Polysialylation may alter NCAM-mediated intracellular signal generation, possibly by means of a conformational change in the NCAM polypeptide (see section 1.2). Cell-cell de-adhesion may occur after the activation of second messenger systems.
1.4. Regulation.

1.4.1. Synthesis: sialyltransferases.

Sialyltransferases (STs) transfer N-acetyl-neuraminic acid (NeuNAc) from cytidine monophosphate N-acetyl neuraminic acid (CMP-NeuNAc) to terminal positions of glycoproteins and glycolipids, generally attaching them via α2-3, α2-6 or α2-8 ketosidic linkages. At least 10-12 enzymes are needed to account for all of the known sialoglycoproteins and gangliosides. However, if specificity, or preference, reaches as far as the protein backbone that figure could reach much higher. Furthermore, different isoforms with differing activities may replace one another in a temporal sequence (Breen and Regan, 1988a and b).

To date, at least 9 sialyltransferases have been cloned and sequenced, including at least five α2-8 STs (Livingston and Paulson, 1993; Sasaki et al., 1994; Nakayama et al., 1995; Yoshida et al., 1995a and b; Kono et al., 1996). Their predicted amino acid sequences suggest an overall structural similarity to all other glycosyltransferases cloned so far. All possess a type II membrane topology with a short NH₂ terminal cytoplasmic domain, a membrane spanning region, a short, typically proline rich, stem region and a large catalytic domain. The stem region is susceptible to proteolytic cleavage which yields a catalytically active enzyme. A conserved sequence, termed the sialylmotif (Livingston and Paulson, 1993), found in the catalytic domain is not shared by any other
glycosyltransferases studied to date and has been used to clone novel STs using a PCR-based approach. This region is shared by STs with different acceptor substrate specificities and it has therefore been suggested that this forms the CMP-sialic acid binding site (Datta and Paulson, 1995).

Of the α2-8 STs cloned to date, 2 have polysialyltransferase (polyST) activity. An ST cloned from hamster by Eckhardt et al (1995), designated polysialyltransferase-1 (PST-1), shows very high amino acid sequence identity to two other STs cloned by Nakayama et al. (1995) (see also Nakayama and Fukuda, 1996) from human fetus and by Yoshida et al. (1995a) from mouse, termed PST and ST8Sia IV respectively, suggesting that these may be counterparts. Similarly high levels of homology are evident between a polysialyltransferase termed STX cloned from newborn rat brain (Livingston and Paulson, 1993), human STX (Scheidegger et al., 1995; Angata et al., 1997) and ST8Sia II (mSTX) cloned from the brain of the three day old mouse (Kojima et al., 1995b). Again, these enzymes are probably homologous. For the sake of brevity, these enzymes will simply be referred to here as PST and STX irrespective of the animal from which they were cloned. The term polyST is used here to refer to polysialyltransferase activity.

Since glycosyltransferases are generally highly specific, it is possible that two, or even three, enzymes may be required for PSA synthesis: an initiase to attach the first α2-8 linked sialic acid, a polymerase required for polycondensation and possibly a terminase to cap the chain. However, while such a mode of synthesis does occur in some bacteria (see Troy II, 1992), there is evidence to suggest that, in vertebrates, PSA may be
synthesized by a single enzyme (Eckhardt et al., 1995; Muhlenhoff et al., 1996). PSA expression is detected in various PSA-negative cell lines (such as COS-1, CHO-2A10, HeLa and 3T3) when transfected with PST, PST-1 or ST8Sia IV cDNA (Eckhardt et al., 1995; Nakayama et al., 1995; Yoshida et al., 1995a). However, these studies are not conclusive. It is possible that the transfected enzyme attaches only the first α2-8 linked NeuNAc, and another ST, already present in the cells but unable to function in the absence of an initiase, is responsible for polycondensation. Conversely, the transfected enzyme may be a polymerase acting on a single α2-8 linked NeuNAc transferred by an endogenous enzyme. On the other hand, it would seem unlikely that cells that do not express PSA should produce some of the enzymes required for its synthesis. However, it may be the absence of just one of the necessary enzymes that accounts for the lack of PSA in the first place. It is worthy of note that the glycolipids, GD3 and GT3, which differ only in that the latter has two α2,8 linked NeuNAcs as opposed to one in the former, may both be synthesised by a single enzyme (Nakayama et al., 1996). That PSA in the vertebrate nervous system is synthesized by a single enzyme would seem the most likely possibility.
1.4.2. Modulation of polysialyltransferase activity.

With the general tendency towards reducing PSA expression during the course of development, a corresponding decrease in polyST activity may be anticipated. Such a fall in PSA synthase activity has been detected in chick embryonic brain between 6.5 and 19 days of incubation, with the decrease being particularly rapid between embryonic days 6.5 and 11 (Oka et al., 1995). Similarly, raised polysialyltransferase activity may be expected to precede the increases in PSA expression detected, for example, during learning. This hypothesis will be investigated in chapter 3.

If PSA levels are modulated by changes in polyST activity, the question is posed: how is enzyme activity regulated? In principle there are several possibilities: different isoform usage, changes in the rate of gene expression, differential splicing, co-valent or non-covalent modifications such as phosphorylation or association with regulatory proteins or ions. All may occur.

ST activity may be inhibited by phosphorylation by protein kinase C (PKC). In 1995, Gu et al. showed that the activities of 4 partially purified STs (LacCerα2-3ST, GM3α2-8, GD3α2-8ST and GM1α2-3ST) are decreased in vitro when ATP is included in the reaction mixture and fall still further with the inclusion of both ATP and PKC, the effect of ATP alone presumably being due to the presence of endogenous protein kinase. This inhibition seems to be reversible, since the activity of the PKC
phosphorylated GM1α2-3ST is partially restored following incubation in the presence of a phosphatase. However, Rafuse and Landmesser (1996) have reported that inhibition of PKC reduces PSA expression by cultured chick myotubes, suggesting that phosphorylation may enhance, rather than inhibit, PSA synthase activity. Gallagher and Regan (1998) have also reported modulation of polyST activity by PKCδ. While these studies point to a modulation of ST activity by PKC, it is not certain whether the effect is direct or mediated by intervening steps. Furthermore, it is unclear whether ST phosphorylation, if it occurs, enhances or inhibits enzyme activity. Indeed, ST phosphorylation may lead to both increased or decreased enzyme activity depending on the particular ST isoform, the site of phosphorylation or the presence of particular ions. These questions remain to be addressed.

The rate of PSA synthesis may be modulated not only by changes in the ST enzyme but, also, by changes in its substrates. This does not simply mean changes in substrate concentrations. For example, polysialyltransferase activity towards NCAM is increased by the presence of an α1-6 linked fucose to the N-glycan core (Kojima et al., 1995a). Thus, in principle, polysialylation may be modulated by changes in fucosyltransferase activity. Kojima et al. (1995a), have shown that STX has different activities towards different proteins, suggesting that this enzyme may recognize part of the protein backbone. It is therefore possible that differential splicing of the NCAM polypeptide may modulate polyST activity.
1.4.3. Electrochemical activity and other factors.

The first contractions of the chick iliofibularis muscle evoked by electrical activity occur around the fifth day of embryonic development (Dahm and Landmesser, 1988). These contractions can be blocked by in ovo injections of d-tubocurarine (dTC). This blockade completely abolished PSA expression at the myotube surface, although total NCAM levels are hardly altered (Fredette et al., 1993). PSA expression by sciatic nerve branches within the muscle is also unaffected (Fredette et al., 1993). This suggests that, at least in muscle, PSA expression may be activity-dependant.

Closely related to electrical activity is neurotransmission. Increases in PSA expression have been detected in the rat hippocampus following training on a step-down avoidance task (Doyle et al., 1992a; see chapter 3). Dopaminergic and muscarinic antagonists both elicit amnesia for this task and also abolish memory-related increases in NCAM polysialylation (Doyle and Regan, 1993; see also chapter 3). Furthermore, activity of the N-methyl-D-aspartate (NMDA) glutamate receptor seems to be required for PSA expression that may be required for oligodendrocyte-type II astrocyte (O-2A) progenitor migration (Wang et al., 1994, 1996; but see Ono et al., 1997b discussed in chapter 2). These studies not only point to a role for neurotransmission in the regulation of PSA expression, but also suggest a link between polysialylation and Ca$^{2+}$ flux. Blockade of voltage gated Ca$^{2+}$ channels has been
shown to reduce PSA expression by cultured chick myotubes (Rafuse and Landmesser, 1996). One can speculate that Ca\(^{2+}\) may modulate polysialyltransferase activity either directly (ST activity is enhanced by divalent cations (Breen and Regan, 1988a; Oka et al., 1995)) or through activation and/or inactivation of phosphatases and/or kinases (discussed above) (Gu et al., 1995; Rafuse and Landmesser, 1996). However, many biochemical steps may intercede between Ca\(^{2+}\) flux and polysialylation. The precise steps involved from Ca\(^{2+}\) influx and PSA expression at the membrane surface require further investigation.

In contrast to the activity-dependent PSA expression discussed above, cultured hypothalamic cells (both neurons and astrocytes) that express PSA in vivo (see section 2.1.3), seem to do so constitutively (Pierre et al., 2001). In cultured hypothalamic cells from which PSA has been enzymatically cleaved, re-expression of PSA at the cell surface occurs about 4 hours after termination of enzyme treatment (Pierre et al., 2001). The re-appearance of PSA at the cell surface is unaffected by treatment with tetrodotoxin (which blocks Na\(^{+}\) channels), Mn\(^{2+}\) (which blocks Ca\(^{2+}\) channels) or glutamate receptor antagonists (Pierre et al., 2001). However, Pierre et al. (2001) found that cooling cultures to 20\(^{\circ}\)C, which inhibits the constitutive but not the regulated pathway, did prevent PSA re-expression at the cell surface (Burgess and Kelly, 1987). This study therefore suggests that PSA expression by some cells may be independent of electrical or chemical activity. It is possible that both constitutive and regulated modes of PSA expression may occur. Polysialylation may, therefore, be
controlled differently by different cell types and/or by different tissues. Furthermore, the regulation of PSA expression may be modified during the course of development.
2.1. Introduction.

2.1.1. Overview of methods of studying polysialic acid.

The expression and function of PSA have been investigated using immunocytochemistry with specific antibodies (of which there are now quite a few (Sato et al., 1995)) and Western blotting with both anti-PSA and anti-NCAM antibodies. The latter are able to detect PSA since polysialylated NCAM appears as a broad smear on an immunoblot rather than the discrete bands observed with lightly sialylated NCAM. However, Probstmeier et al. (1994) have reported discrepancies in the results obtained using these two methods. Probstmeier et al. (1994) compared these two methods directly using early mouse embryos. Using immunocytochemistry, PSA was detected from E8 (1-7 somites) onwards. Using immunoblotting
with the same antibodies, however, no PSA could be found at this stage with only weak staining at E8.5 (8-12 somites), E9 (13-20 somites) and E9.5 (21-29 somites). The reaction became strong at E10 and E11. Several studies that have employed these methods are discussed in the following sections.

The function of PSA has also been investigated using homozygous NCAM knock-out mice that, lacking NCAM, *de facto*, lack PSA-NCAM (Cremer et al., 1994; 1997). A number of deficits have been reported in these animals including a reduced spatial learning ability (Cremer et al., 1994); disrupted hippocampal development (Cremer et al., 1997); reduced size of the olfactory bulb (Cremer et al., 1994); reduced phosphorylation of tyrosine receptor kinase (trk) B (Muller et al., 2000) and a shortened circadian rhythm (Shen et al., 1997)(some of these findings are discussed below and in later sections). There is, however, a remarkable paucity of large scale deficits found in these animals. NCAM is a very abundant molecule comprising perhaps as much as 1% of total brain protein. It is, perhaps, surprising that animals lacking NCAM develop at all. This suggests that most of the nervous system is able to compensate for the absence of NCAM, presumably by making use of other, perhaps related, molecules. The defects observed in NCAM deficient mice may, therefore, only occur in systems that are unable to compensate for the absence of NCAM.

The gene deletion approach has also been used to look at one of the enzymes responsible for PSA synthesis, PST (Eckhardt et al., 2000). When comparing PST-knockout mice with wild-type animals, Eckhardt et al. (2000) reported no detectable
differences in PSA expression in the brain during development or in the size of the olfactory bulb. However, in adult animals PSA levels were lower in the brains of PST-knockouts than in wild-type mice. These results therefore suggest that STX, or as yet unidentified polyST enzymes, are predominantly responsible for PSA synthesis during development, while PST is the dominant polyST enzyme in the adult. Furthermore, these results give no indication that the animals are able to compensate for the loss of PST by making use of other enzymes such as STX. PST-deficient mice may, therefore, offer a useful means of discriminating between the functions of NCAM protein and PSA, as well as between the functions of different polyST enzymes. The production of STX knockout mice would also seem potentially useful for studying the specific functions of the different PSA synthase enzymes.

Much use has also been made of an endoneuraminidase (endo-N) that specifically cleaves PSA (Vimr et al., 1984; Finne and Makela, 1985; Hallenbeck et al., 1987). This enzyme has enabled enquiries into the role of PSA by looking at the consequences of its removal. Some of these investigations are considered in the course of the following discussion (when considering the consequences of PSA cleavage one cannot but be surprised at the paucity of defects found in NCAM knockouts in vivo).
2.1.2. Studies on the role of Polysialic acid during development.

*Axon growth, pathfinding and target innervation.*

PSA has been investigated in connection with the development of both central and peripheral pathways. In the central nervous system, the expression and function of PSA has been investigated in the cortico-spinal tract (CST) of the rat (Daston, et al., 1996). As they develop, axons of the CST initially grow past their targets in the hindbrain and spinal cord and remain within the tract. They subsequently sprout collateral branches, behind the advancing growth cone, that exit the CST and grow towards their targets (a mode of growth termed interstitial). In the cervical regions of the tract, PSA levels are low during the first two days after birth but increase on the fourth post-natal day (P4) when collateral branches are extending in the spinal grey matter (Daston et al., 1996). When PSA is cleaved by endo-N, collateral branching of CST axons is delayed and reduced, although fasciculated fibres within the tract itself seem to be unaffected (Daston et al., 1996). This result is consistent with the view that PSA plays an enabling role in axon branching and/or de-fasciculation. However, PSA is absent in the CST in the brainstem between P0 and P4 when collaterals of CST axons are extending into the basilar pons (Daston et al., 1996). On the basis of this result, Daston et al. (1996) suggest that PSA modulates axonal responses to specific
environmental signals that are present in the spinal cord but not the hindbrain. If this suggestion is correct, then axon branching in some regions may require PSA, while in other areas axon branching may be independent of PSA.

In the peripheral nervous system, PSA has been investigated in chick motoneurons that innervate the hindlimb. Motoneuron axons exit the anterior lumbo-sacral segment of the spinal cord at about E2.5 (stage 18 of Hamburger and Hamilton, 1951\(^1\)) and traverse the anterior somite (Tosney and Landmesser, 1985). By around E3-E3.5 (stage 21) the first motoneuron growth cones reach the plexus region that lies along the entire anterior-posterior axis of the hindlimb base and is bounded by the myotome, pelvic girdle precursor, endoderm and posterior cardinal vein (Tosney and Landmesser, 1985). At around the same time that axons enter the plexus region they start to express PSA (Tang et al., 1994). Within the plexus region, axons pause and defasciculate, modifying interactions with their neighbours and become reorganised into target-specific bundles (Tosney and Landmesser, 1985). The plexus is, therefore, considered a decision point in the targeting of growing fibres. Under normal conditions, motor axons in this region display complex trajectories with a meandering appearance (the meandering appearance of growing axons presumably results from their "seeking out" appropriate contacts). However, when PSA has been cleaved by endo-N, axons grow along relatively straight trajectories (Tang et al., 1994). This consequence of PSA removal is reversed by

\(^1\) In the present work, wherever a particular stage of development is referred to, this relates to Hamburger and Hamilton, 1951.
simultaneous administration of anti-L1 fab (antigen binding fragment), but not anti-NCAM fab (Tang et al., 1994). L1 has been shown to be important in axon bundling (Landmesser et al., 1988). These findings are consistent with the view that PSA performs a facilitating role in permitting axon defasciculation by attenuating L1-mediated axon-axon adhesion (see Kadmon et al., 1990a; section 1.3).

After progressing from the lumbo-sacral plexus, axons of the sciatic nerve again pause and express PSA as they arrive at their target muscle (Landmesser et al., 1988). Administration of endo-N at the time when axons are elaborating within the iliofibularis muscle results in a reduction in nerve branching (Landmesser et al., 1988; Landmesser, 1992). This suggests that PSA may to be required for fibre defasciculation and/or axon elaboration within target muscle.

In the developing chick visual system, PSA is expressed by the optic tectum, as well as by the axons of retinal ganglion cells as they grow towards and elaborate within the tectum (Schlosshauer et al., 1984) (for a description of the development of the chick visual system see section 2.1.4). When PSA is removed from the optic tectum at around E6-7, when retinal ganglion cell axons start extending in this region (section 2.1.4), the result is a defasciculation of their fibres (Yin et al., 1995). This is the very opposite of what happens in the hindlimb (Landmesser et al., 1988). Yin et al. (1995) note that, while the environment of axons in the hindlimb is PSA-negative, retinal ganglion cell axons, as they enter the optic tectum, encounter a PSA-positive environment. Thus, they argue, in the latter case, PSA cleavage may strengthen axon-environment interactions
more than axon-axon interactions. Thus, in the optic tectum, PSA cleavage may favour axon defasciculation more than it hinders defasciculation. Rutishauser and Landmesser (1996) have also suggested that PSA may protect axons from premature or inappropriate interactions.

At the optic chiasm, however, PSA cleavage seems to have no effect on axons as they start to form the optic tract (Yin et al., 1995). In general, three types of theory have been proposed to account for the specificity of axon guidance: (1) growing axons may be constrained by mechanical factors such as surrounding tissue forming an unpassable barrier, (2) chemical guidance in the form of molecular gradients and (3) electrical factors, such as ions forming a charge gradient. It may be that the first of these is most important where axons are following a course that is already well-defined by the surrounding tissue. Thus, axons at the optic chiasm and in the optic tract may be constrained by surrounding tissue to follow their normal route even when PSA has been removed.

In the rat and mouse, mossy fibre innervation of hippocampal pyramidal cells begins in the early postnatal period and achieves a mature architecture by around P12-P15 (Amaral and Dent, 1981; Gaarskjaer, 1985). PSA is expressed in both the suprapyramidal and infrapyramidal mossy fibre bundles, as well as by some fibres with a dotted appearance crossing the pyramidal cell layer from the latter bundle to the former (Seki and Rutishauser, 1998). Administration of endo-N at P1 has no effect on the infrapyramidal and suprapyramidal mossy fibre bundles themselves (a similar situation to that found in the rat CST (Daston et al., 1996)) but results in an increased number of
fibres following a haphazard path across the CA3 region as assessed at P15 (Seki and Rutishauser, 1998). Similarly, in homozygous NCAM deficient mutant mice, Cremer et al. (1997) observed that: "The axons" in NCAM knockout mutants "invade the pyramidal cell layer, not respecting their normal laminar organisation " (italics added). These findings suggest that PSA in the mammalian hippocampus may be required for the specification of the cyto-architecture. Furthermore, PSA cleavage results in a very large increase in the number of mossy fibre terminal synaptic boutons within the hippocampal CA3 region (Seki and Rutishauser, 1998). During CA3 innervation, there is an initial transient synaptic over-expression followed by a subsequent withdrawal of axons. Seki and Rutishauser (1998) speculate that PSA may facilitate both the selective elimination of over-expressed synaptic contacts as well as enabling axon withdrawal.
**Cell migration.**

Oligodendrocytes and type II (fibrous) astrocytes may arise from a common precursor cell, the oligodendrocyte-type II astrocyte (0-2A) progenitor, that has been frequently studied in culture (see Kiernan and ffrench-Constant, 1993). *In vitro,* 0-2A progenitor cells from rat neurohypophyseal explants do not migrate when PSA has been enzymatically cleaved with endo-N (Wang et al., 1994). This effect is reversible and cells reinitiate migration following cessation of endo-N treatment (Wang et al., 1994). This suggests that, *in vitro,* migration of 0-2A progenitors is PSA-dependent.

Oligodendrocyte precursors of the chick optic nerve originate in the ventricular zone of the third ventricle from around E5 (Ono et al., 1997b). They start to migrate into the optic nerve around E6 and populate the entire optic nerve by around E9 (Ono et al., 1997b). These oligodendrocyte precursors express PSA at their cell surfaces (Ono et al., 1997b). However, migration of oligodendrocyte precursors seems to be unaffected when PSA is cleaved from the optic nerve (Ono et al., 1997b). This suggests that, in the chick optic nerve, migration of oligodendrocyte precursors does not require PSA. These results contrast sharply with the *in vitro* findings of Wang et al. (1994)(discussed above). Ono et al. (1997b), remarked that, *in vitro,* premigratory cells may need to detach from neighbouring precursor cells before they can start migration. PSA cleavage may strengthen cell-cell adhesion thereby hindering cell separation and preventing the start of migration.
By contrast, oligodendrocyte precursors in the optic nerve seem to migrate along axons and may not need to detach from other precursor cells.

During development, luteinizing hormone-releasing hormone (LHRH) producing cells migrate from the olfactory placode to the forebrain in mice (Wray et al., 1989), rats (Daikuku-Ishido et al., 1990) and chicks (Norgren and Brackenbury, 1993). In chicks, LHRH producing cells migrate as normal after endo-N removal of PSA throughout the entire region (Murakami et al., 1998). This suggests that migration of these neurons does not require PSA.

In rodents, the olfactory bulbs receive neurons that are born in the subventricular zone (SVZ) (also referred to as the subependymal zone) beneath the lateral ventricles and migrate rostrally towards the olfactory bulbs (these migrating cells are called the rostral migratory stream). Then, after this period of tangential migration, cells undergo a radial migration to take up their positions within the olfactory bulb. A possible link between PSA and cell migration has been studied in vivo by looking at the distribution of migratory SVZ cells in mutant mice that are deficient in NCAM and, hence, PSA-NCAM (Ono et al., 1994). In homozygous NCAM-deficient mice, fewer precursor olfactory bulb cells migrate rostrally to the olfactory bulb during development, than in wild-type animals (Ono et al., 1994). This same impairment of migration has been reported following endo-N injection into wild-type mice (Ono et al., 1994). This suggests that PSA, rather than other parts of the NCAM molecule, is required for migration of cells in the rostral migratory stream. However, when PSA is cleaved by endo-N in
the olfactory bulb of wild-type animals, radial migration of olfactory bulb precursors that have left the rostral migratory stream is unaffected (Hu et al., 1996). This suggests that tangential migration within the rostral migratory stream is PSA-dependant, but subsequent radial migration within the olfactory bulb does not require PSA.

Hu et al. (1996), further investigated the involvement of PSA in the rostral migratory stream by transplanting cells of the SVZ between wild-type and homozygous NCAM knockout mice. When labelled SVZ cells from NCAM+/+ mice are transplanted into NCAM+/+ mice, large numbers of these cells migrate to the olfactory bulb. This migration was almost completely abolished when PSA was cleaved with endo-N. Almost no cell migration was observed when SVZ cells from NCAM−/− mice were transplanted into NCAM−/− mice, or when cells from NCAM+/+ mice were transplanted into NCAM−/− mice. However, when SVZ cells were transplanted from NCAM−/− mice into NCAM+/+ mice, large numbers of cells migrated to the olfactory bulb (the number of migratory cells was approximately the same as when cells were transplanted between NCAM+/+ and NCAM+/+)(Hu et al., 1996). This somewhat surprising result suggests that cell migration in the rostral migratory stream requires PSA expression in the surrounding environment, but not at the surfaces of migrating cells themselves.

It is possible that the reduced size of the olfactory bulb reported in NCAM-knockout mice (Cremer et al., 1994) may be caused by a reduced migration of olfactory bulb precursor cells. (The rostral migratory stream is considered further below, in the context of the adult.)
2.1.3. Polysialic acid and neural plasticity in the adult.

*The olfactory system.*

In adult rodents, the olfactory bulb continues to receive newly generated neurons from the rostral migratory stream (see section 2.1.2) (Altman, 1969). PSA positive cells have been reported throughout the subependymal layer in both adult rats (Bonfanti and Theodosis, 1994) and adult mice (Rousselot et al., 1995). These PSA-expressing cells have been interpreted as migrating neuroblasts (Bonfanti and Theodosis, 1994; Rousselot et al., 1995). In support of this view, Rousselot et al. (1995), found that most PSA expression within the subependymal zone was closely associated with $[^{3}\text{H}]-\text{thymidine}$ that had been injected intraperitonealy 6 hours before the animals were killed. ($[^{3}\text{H}]-\text{thymidine}$ is incorporated into freshly synthesised DNA and therefore serves as a marker for cells that have recently divided.) It is likely that PSA may be required for the migration of neuroblasts in the rostral migratory stream. In this system, PSA in the adult may, therefore, serve the same function that it is believed to perform during development (Ono et al., 1994).

In adult mammals, olfactory neurons of the olfactory neuroepithelium continually die and are replaced by freshly generated neurons (Graziadei and Monti Graziadei, 1979). Axons of these neurons project, via the olfactory nerve, to the olfactory bulb where they form functional synapses in the glomerular layer. Miragall et al. (1988) reported that, in adult
mice, PSA is expressed by some, but not all, globose basal cells of the olfactory neuro-epithelium. These cells are believed to be the precursors of sensory neurons (Graziadei and Monti Graziadei, 1979). However, Miragall et al. (1988) found that differentiated sensory neurons of the olfactory neuro-epithelium did not express PSA. This suggests that PSA may be expressed by immature sensory neurons and their precursors, but is down-regulated as cells mature. Furthermore, they reported that PSA is expressed by some, but not all, axons within the olfactory nerve. They speculated that, while the axons of freshly generated neurons express PSA, the axons of older neurons had down-regulated PSA. These findings are consistent with the view that PSA is required for the directed axon growth of these sensory neurons.

Within the adult murine olfactory bulb, Miragall et al. (1988) detected weak PSA expression in the nerve fibre layer, but stronger expression in the glomerular and external plexiform layers. In the olfactory bulb of the adult rat, Bonfanti and Theodosis (1994) have reported PSA expression by several cell types throughout all layers of the bulb. Since the adult rodent olfactory bulb continually receives both afferent projections from the neuro-epithelium as well as fresh neurons from the rostral migratory stream it must continually integrate these elements into its circuitry. This requirement for an ongoing integrative capacity implies the retention of a morphoplastic potential throughout the olfactory bulb. PSA expression may, therefore, be required not only by freshly generated cells and projections from the olfactory nerve, but also by other
The hypothalamo-neurohypophysial system.

In the hypothalamo-neurohypophyseal system, neuron-glial relationships and synaptic connections alter during lactation, parturition and prolonged dehydration (Tweedle and Hatton, 1987). In the hypothalamus, PSA is expressed in the supraoptic nuclei (SON) and paraventricular nuclei (PVN), both of which send projections to the neurohypophysis. However, in lactating rats, PSA levels are reduced in the SON and PVN, although expression remains high in the surrounding areas (Soares et al., 2000). Levels of NCAM protein in the SON remain unchanged during lactation (Nothias et al., 1997), suggesting that changes in PSA levels are due to changes in the rate of NCAM polysialylation. Indeed, PSA synthase activity is reduced in the SON and PVN during lactation (Soares et al., 2000). Furthermore, Soares et al. (2000) have reported decreased levels of mRNA for both of the PSA synthase enzymes, PST and STX, in the SON and PVN during lactation. Thus, changes in PSA levels in the SON and PVN seem to be accomplished, at least in part, by changes in the rate of expression of PST and STX.

PSA is expressed in the neural lobe of the hypophysis on the axons and endings of both neurosecretory neurons and their associated pituicytes (specialised glial cells) (Theodosis et al., 1991; Kiss et al., 1993). During periods of enhanced neurosecretion, neurohypophyseal pituicytes undergo extensive morphological changes that bring more endings of
neurosecretory axons into contact with the peri-vascular space (Tweedle and Hatton, 1987). This increased juxtaposition between neurosecretory nerve endings and peri-vascular spaces is likely to assist neurosecretion into the blood stream. Theodosis et al. (1991), suggested that PSA may facilitate this glial morphoplasticity and may, therefore, be a factor in the regulation of neurosecretion. During lactation, PSA levels in the neurohypophysis are reduced (Nothias et al., 1997; Soares et al., 2000). However, unlike the SON and PVN, this is not correlated with a decrease in the levels of PST or STX mRNA (Soares et al., 2000). This suggests that, in the neurohypophysis, changes in PSA levels during lactation, may be achieved by modulating the activity of STX and/or PST at the post-translational levels.
Circadian and seasonal rhythms.

The mammalian suprachiasmatic nuclei (SCN) are believed to house an endogenous circadian rhythm generator. Neurons of these nuclei have also been shown to undergo morphoplastic changes and have been found to express PSA in the rat (Bonfanti et al., 1992), hamster (Glass et al., 1994) and mouse (Shen et al., 1997). Shen et al. (1997) found that homozygous mutant mice that lack NCAM 180 displayed a free running activity/sleep cycle, $\tau$, of 23.5 hours as compared with 23.9 hours in wild type mice of the same strain ($p<0.05$), under conditions of constant darkness (heterozygous mutants had an intermediate $\tau$ of 23.7 h - statistically not significantly different from either of the other 2 groups). All mice entrained normally to a 12h light 12h dark cycle ($\tau$ increased in all groups under conditions of constant light). In trying to home in on the PSA moiety, Shen et al. (1997) used endo-N to specifically cleave this carbohydrate. Using a different strain of mouse with a freerunning $\tau$ of 24.4h, they found that endo-N administration reduced $\tau$ to 24h. These findings suggest that PSA may be involved in the generation and/or maintenance of circadian rhythms but not in their entrainment. It cannot be excluded, however, that PSA may be involved only in SCN output; that is, in the expression of circadian rhythms.

PSA levels also seem to vary seasonally in the higher vocal centres (HVC) of the adult canary (Rousselot and Nottebohm, 1995). In September, song repertoire undergoes significant changes (Kirn et al., 1994). At this time, both PSA levels and neuronal recruitment are high relative to their levels in spring.
when song is more stable (Kirn et al., 1994; Rousselot and Nottebohm, 1995). It is likely that PSA may be required for the modulation of inter-neural circuitry and the integration of freshly generated neurons into that circuitry.

Polysialic acid in other regions of the adult brain.

PSA is expressed the hippocampus of adult mammals (Bonfanti et al., 1992; Seki and Arai, 1993). However, this region will not be considered further in this section, but will be discussed in the context of memory formation in chapter 3.

In 1992, Bonfanti et al., reported mapping the anatomical distribution of PSA throughout the brain and spinal cord of rats aged between 3 and 12 months old. They reported no differences in PSA expression between animals at any of these ages, pointing to a stabilisation of PSA levels by around three months of age. This study confirmed the presence of PSA in several areas such as the olfactory bulb and neurohypophysis, but also revealed that PSA was expressed in numerous other areas. The findings of Bonfanti et al. (1992) showed that, in the adult rat, PSA is confined to discrete regions. However, they also showed that PSA-expressing regions are quite numerous. Furthermore, they observed that PSA is expressed by areas in which adult morphoplasticity has not been reported. They suggested that, if PSA is involved in neural plasticity, then many regions of the mature brain may possess a hitherto unsuspected morphoplasticity.
2.1.4. A partial review of chick central nervous system development.

*Establishment of the brain and eye rudiments.*

Cleavage and early gastrulation occur in the hen oviduct, and the developing egg remains in the body for about 22 hours before being laid. The chick will finally hatch after about 21 days of incubation\(^2\). The neural folds first become visible at around 23 - 26h of incubation (stage 7 of Hamburger and Hamilton, 1951) and meet in the area of the midbrain about 3h later (stage 8). Neural fold fusion in the brain completes at the anterior neuropore around stage 12 (45-49h) and continues posteriorly to near completion at stage 13 (see Bellairs and Osmond, 1998).

At around stage 10, the anterior part of the neural tube, that will develop into the brain, starts to bulge, forming the three primary brain vesicles. The three primary brain divisions are:

1. the prosencephalon (or forebrain) that, by around stages 12-13, starts to divide into the telencephalon (that will become the cerebral hemispheres) and diencephalon;
2. the mesencephalon (or midbrain), that develops to become the large optic lobes, as well as several midbrain nuclei (see later); and
3. the rhombencephalon (or hindbrain) that, by around stage 11, is divisible into the metencephalon (that will develop into

\(^2\) The duration of the incubation period may vary a little depending on factors such as ambient temperature, humidity and strain of chick.
the cerebellum and pons) and the myelencephalon (that will become the medulla oblongata).

The primary optic vesicles have started to form by around stage 9, at the sides of the prosencephalon. The base of the optic vesicles, where they joins the prosencephalon, start to constrict at around stage 10. By stages 13-14, the connection between the optic vesicles and the anterior end of the diencephalon has been reduced to a narrow stalk. At around stage 12, the distal part of the optic vesicle starts to invaginate, becoming juxtaposed to the proximal part of the optic vesicle, forming the optic cup. The optic vesicle is thereby reduced to a narrow strip and a new lumen, that will become the vitreous chamber, is formed. The retina develops from the distal cell layer lining the inside of the vitreous chamber. The lens is not derived from the neural tube, but develops from a thickened region of ectoderm, called the lens placode. It is induced by signals from the optic cup, and is detectable at about stage 12 (see Bellairs and Osmond, 1998).
Overview and overall morphological development.

During most of the first week of embryonic development, the neural tube remains essentially hollow with relatively broad lumina and relatively thin walls in most areas (discussed more fully in the following sections). Primarily during the second embryonic week, both lateral and medial cell migration leads to a widening of the brain and a narrowing of the central lumina. The essential features of the mature brain are present by around 12-13 days (stages 38-39) of incubation. During the third week of embryonic development the most conspicuous advances are the growth of the forebrain hemispheres and development of the cerebellum. The overall structure of the chick brain at various developmental stages is shown for reference in fig. 2.1. The development of the different brain divisions are discussed separately below (for a fuller description of chick brain development see Romanoff, 1960; see also the discussion in section 2.4).
Fig. 2.1. Overall structure of the chick brain at various developmental stages. Dorsal and sagittal sketches of the gross morphology of the chick brain at (a) E5, (b) E8, (c) E10, (d) E13 and (e) E17. Adapted from Pearson, 1972. 1: telencephalon; 2: diencephalon; 3: mesencephalon; 4: cerebellum.
The retina and retino-tectal projections.

In the developing retina, the optic fibre layer (OFL), that consists of retinal ganglion cell (RGC) axons, is discernible by the third day of incubation (E3). The other differentiated retinal layers that are found in the adult are present, but immature, by around E9.

Ganglion cells of the retina are generated from about E2, followed by amacrine cells which become post-mitotic between about E3 and E9 (literature cited in Rogers, 1995). Photoreceptors do not differentiate until between around E10 and E12, and bipolar cells are the last retinal neurons to form between about E11 and E13 (literature cited in Rogers, 1995). Gliogenesis occurs in the inner retina between E12 and E17 (literature cited in Rogers, 1995). Synaptogenesis begins in the retina around E10, and projections from the nucleus isthmo-opticus (IO) (see later) start to arrive around E12 (Clarke et al., 1976).

The first RGC fibres enter the optic stalk around E3, reach the optic chiasm around E4 and arrive at the anterior pole of the optic tectum around E6. Although there is a complete decussation of RGC axons at the optic chiasm in the mature chicken, a transient ipsilateral retino-tectal projection arises between about E6 and E16 (McLoon and Lund, 1982). Between E11 and E18, about 40% of RGC fibres within the optic nerve degenerate (Rager and Rager, 1978), and the first degenerating RGCs have been detected as early as E9 (Rager and Ragar,
The transient ipsilateral retino-tectal projection is probably eliminated by retinal ganglion cell death.

In the optic nerve, cells immunoreactive for myelin basic protein have been reported as early as E12, and some myelin sheath has been detected at E14 (Ono et al., 1999)(the migration of oligodendrocyte precursors into the optic nerve was discussed in section 2.1.2). At E17, Arees (1978) has reported that approximately 1% of axons within the optic nerve are myelinated. This figure remains unchanged up to P3 and then rises to about 5% at P5 (Arees, 1978). At P22, about 50% of optic nerve axons are myelinated, and almost all are myelinated by around 2 months after hatching (Arees, 1978). However, Rager and Rager (1978) reported that about 5-6% of axons in the optic nerve are unmyelinated at 104 days after hatching.

Oligodendrocyte precursors start entering the retina from the optic nerve (see section 2.1.2) at about E10 (Ono et al., 1998). At this stage they are detectable in the OFL of the retina but by E14 are found in their mature location in the inner part of the ganglion cell layer (Nakazawa et al., 1993; Ono et al., 1998). Differentiated oligodendrocytes are detected at E14 (Ono et al., 1998). Myelination of the OFL of the retina begins around E14 and is probably complete by about P7 (Nakazawa et al., 1993).
The telencephalon (forebrain hemispheres).

The telencephalon appears as paired swellings of the neural tube at around 2 days of incubation (stages 12-13 of Hamburger and Hamilton, 1951)(Bellairs and Osmond, 1998). By about E3, the wall of the telencephalon consists of an inner cellular layer (referred to here as the ventricular zone, VZ) and an outer non-cellular layer (the marginal zone, MZ). A cellular mantle zone (MnZ), lying between the VZ and MZ, is starting to develop in the basal zones or the telencephalon at around E3 (Romanoff, 1960). Some thickening of the telencephalic wall begins around E5, although this process does not begin in earnest until about E7 (Romanoff, 1960). The precursors of the olfactory bulbs first appear as ventro-lateral bulges at around E5.

The basal and dorsal regions of the telencephalic neural tube are separated by a small groove called the central sulcus that is apparent by around E3 (Romanoff, 1960). As the telencephalon develops, a lamina, known as the zona limitans, stretches from the central sulcus to the lateral ventricle thereby delimiting the pallial and subpallial regions. The pallium, that arises from the dorsal part of the telencephalic neural tube, comprises the Wulst, the hyperstriatum ventrale (HV), the neostriatum (N) and the ectostriatum (E). The E and parts of the Wulst are the main visual centres of the telencephalon (discussed in section 2.4.3), while the HV and N seem to be largely association areas (Rogers, 1995). The intermediate mediale part of the HV (the IMHV), for example, is involved in several types of learning.
(Kossut and Rose, 1984; Rose 1991; Horn, 1998; Barber et al., 1999; see chapter 3). The subpallium, that develops from the basal telencephalic neural tube, comprises the paleostriatal and archistriatal regions. The lobus parolfactorius (LPO) is a paleostriatal region that is also implicated in memory formation (Kossut and Rose, 1984; see chapter 3). Lesioning studies have also shown that the archistriatum may be involved in the learning and/or expression of a one-trial passive avoidance task (Lowndes and Davies, 1994; see chapter 3) as well as in imprinting (Lowndes et al., 1994). Indeed, it has been suggested that the IMHV, LPO and the archistriatum may form a "memory circuit" (Lowndes and Davies, 1994; an alternative perspective on the location(s) of memory storage is discussed in section 3.1.2).

Most telencephalic neurons become post-mitotic between E5 and E9, although a few granular cells of the olfactory bulb are still being generated at E10 (Tsai et al., 1981). However, almost all olfactory mitral cells as well as most neurons of the E, paleostriatum primitivum (PP), nucleus basalis (Bas) and the archistriatum ventrale have become post-mitotic on or before E5 (Tsai et al., 1981). The majority of telencephalic glial cells are probably generated after about E10 (Tsai et al., 1981). Synapses continue to be formed in the telencephalon until about 1 to 2 weeks post-hatching. However, synapses probably continue to mature until around 8 to 10 weeks post-hatching (see Rostas, 1991).
The diencephalon.

In the wall of the diencephalon, a mantle zone, between the ventricular and marginal zones, is detectable at the beginning of the fourth day of incubation. Towards the end of E5 and during E6, the diencephalic wall undergoes considerable thickening. The primordia of the nucleus rotundus and nucleus ovoidalis are detectable as cell condensations in the dorsal thalamus at E7. By around E12, the mature pattern of the diencephalic nuclei has been established. However, at this time, cell bodies are small, fibres are still growing, some cell migration persists and the nucleus geniculatus lateralis, pars ventralis exceeds its mature size. The development of the chick diencephalon has been described by Kuhlenbeck (1936).

The infundibulum, that will later form the neural lobe of the pituitary gland, is visible as a depression in the inner surface of the diencephalic floor on the fourth day of incubation (Bellairs and Osmond, 1998).

The mesencephalon (midbrain).

The most conspicuous mesencephalic structures in the avian brain are the 2 large optic lobes that develop from the roof of the mesencephalon. Until around E5, the mesencephalon develops as a large, thin-walled single lobe. Between about E5 and E7, this single lobe divides, along the mid-line, into the two optic tecta. Between about E8 and E12, the optic lobes rotate through about 900, bringing the anterior pole into a lateral position and the posterior pole into a medial position.
By the fourth day of incubation, a marginal zone, consisting of neuroblast processes, has developed in the mesencephalic roof. Thickening of the roof of the mesencephalon begins around E5/6. Starting at around E6/7, some cells of the ventricular zone start to migrate through the fibrous marginal zone, moving towards the periphery. The outer fibrous layer therefore sinks deeper into the developing tectum and will eventually form the stratum album centrale (or the deep fibre layer). From around E6 onwards, retinal ganglion cell fibres advance over the tectal surface in a rostro-caudal direction forming the outermost layer of the tectum, the stratum opticum (SO) (Crossland et al., 1975). Advancing fibres reach the central region of the developing SO by about E8 and cover the entire tectal surface by around E13. Penetration of the deeper layers of the optic tectum by retinal ganglion cell fibres within the SO begins in the central region of the tectum around E8 (Rager and von Oeynhausen, 1979). However, ganglion cell fibres continue to branch in this same region of the SO at least until E18 (Thanos and Bonhoeffer, 1987). The differentiated laminar structure of the tectum that is found in the adult, has arisen by around E13 (the development of the optic tectum, as well as its mature structure, has been described by LaVail and Cowan, 1971a,b).

The nucleus isthmo-opticus (IO) is detectable as an eminence in the hind part of the lateral mesocoele by around E8. Neurons of the IO (cells of which send axons to the retina where they connect with amacrine and ganglion cells) are already post-mitotic by about E7 (Clarke et al., 1976). At E13, the IO contains
around 22,000 cells, but this number falls to around 9,500 by the time of hatching (Clarke et al., 1976).

**The metencephalon (cerebellum and pons).**

The cerebellar plate, comprising a neuro-epithelial layer bordering the fourth ventricle and a mantle layer containing neuroblasts, is detectable around the fifth day of incubation. By around E7, the cerebellum is visible as a small rounded pouch (Romanoff, 1960). The cerebellum remains small until about E10 when it starts to grow rapidly and begins to push forwards between the two optic lobes.

Most Purkinje cells arise between E5 and E7, although some are still being generated as late as E13. The proliferation of granule cell precursors in the outer zone of the external granular layer (EGL) persists until around E15. There then follows a massive and rapid inward migration between about E15 and E17, in which cells of the EGL migrate through the Purkinje cell layer (PCL) to take up their final positions in the internal granular layer (IGL). The leading processes of migrating cells associate with Bergmann processes (the fibres of specialised glial cells that have their somata in the PCL), and extend through the developing molecular layer (ML), drawing their cell bodies behind them. Trailing processes of migrating cells form the fibres of the ML. The outer layer of the cerebellum that began as the cellular EGL is thereby transformed into the largely fibrous ML. Eventually, in the IGL, migratory cells detach from Bergmann fibres. At the start of this migration, Purkinje cell arborization, penetrating into the EGL, has already reached its maximum extent. By the time of hatching, the EGL has
disappeared although a few cells remain within the ML and a thin strip of glial cells forms the glia limitans at the cerebellar surface (see Romanoff, 1960; Pearson, 1972).

Cerebellar oligodendrocytes arise from cells in a discrete bilateral focus close to the ventro-medial part of the VZ lining the fourth ventricle (Ono et al., 1997a). From the VZ, oligodendrocyte precursors migrate ventrally and laterally into the pons and then some migrate dorsally into the developing cerebellum (Ono et al., 1997a). Proliferation of oligodendrocyte precursors seems to occur primarily after they have migrated to their final positions (Ono et al., 2001). Differentiated oligodendrocytes are present in the pons as early as E8, and have been detected in the IGL and white matter of the cerebellum at E12 (Ono et al., 1997a).

*The myelencephalon (hindbrain).*

In the chick auditory system, fibres from the cochlear ganglion innervate the nucleus angularis (An) and the nucleus magnocellularis cochlearis (mcc) in the hind-brain. Neurons of the mcc project to the ipsilateral nucleus laminaris (La), via the uncrossed dorsal cochlear tract (CTrU), and to the contralateral La via the crossed dorsal cochlear tract (CTrX)(Parks and Rubel, 1975). Starting at around E7, both the mcc and La migrate, in a medial direction, from their initial lateral location, and only achieve their final position at around E12. Most neurons of the mcc become post-mitotic by around 60h of incubation, while generation of neurons in the nucleus laminaris (La) continues up to around E5 (Rubel et al., 1976). Cell death in the mcc occurs mainly between E11 and E13, with the loss of about 18%
of its cells (Rubel et al., 1976). In the La, cell death begins around E11 but persists until about E15, with the loss of about 84% of its cells (Rubel et al., 1976). The mcc may, therefore, mature a few days earlier than the La.

The earliest stage at which myelin has been detected in the brain, is in the medulla at E11 (Schifferi, 1948). The process of myelination in the brain continues at least up to around E8, and is particularly rapid during the 3 days prior to hatching (Hartman et al., 1979).

2.1.5. Investigations carried out in the present study.

The literature discussed above in sections 2.1.2 and 2.1.3 implicates PSA in morphoplastic processes such as cell migration and axon growth, both during development and in restricted brain regions in the adult. If PSA is involved in chick central nervous system (CNS) development then one would expect PSA to be expressed throughout the CNS during development when cells are proliferating, migrating and extending processes. However, the theory predicts that PSA will be down-regulated by most, but probably not all, CNS structures when they have become mature. Furthermore, since different brain regions become mature at different times (section 2.1.4) one would anticipate PSA down-regulation to occur in different regions at different times depending on when those regions achieve a stable cyto-architecture.

In the present study, immunocytochemistry is used to map the distribution of PSA in the brain, retina and optic nerve of chick embryos throughout the developmental period and in post-hatching chicks of up to 1 week of age. By comparing PSA
expression patterns with what is known of the time-course of morphoplastic events in the chick CNS it should be possible to try to correlate changing PSA expression patterns with those morphogenetic processes.

Multiple immunofluorescence studies are carried out using specific antibodies for myelin, fibrous (type II) astrocytes, neuronal cell bodies and neurofilaments (see methods for details), and immunoreactivities for these markers are compared with PSA expression. Of particular interest is the comparison between PSA expression and the onset of myelination Since the presence of myelin may indicate a stable cyto-architecture while PSA expression suggests that the neural circuitry is still forming, an inverse relationship between polysialylation and myelination might be anticipated (but see results). The other antibodies used in the present study are employed to try to gain some insight into when, during development, different cell types down-regulate PSA. By comparing PSA expression and the expression of cell-type specific markers on the one hand with what is known about the time-course of morphogenetic events, such as cell migration, on the other hand, it may be possible to correlate PSA expression with those events.
2.2. Methods.

2.2.1. Animals.

A total of 44 chicken embryos and 16 post-hatching chicks (Ross Chunky, Maurice Millard Chicks Ltd.) were used in the present study. Fertile eggs were incubated in a communal incubator (Bristol Incubators, model number SH18/6) on an 8/16 hour light/dark cycle at 37.5 - 37.9°C with 50% humidity. Where chicks were to be used post-hatching they were transferred on the eighteenth day of incubation to a hatching incubator maintained on a 12/12 hour light/dark cycle at 37 - 38°C and allowed to hatch. After hatching, chicks were transferred to a communal pen and allowed food and water ad libitum.

Embryos were studied at the following days of incubation: embryonic days 3 (E3), 5 (E5), 9 (E9), 13 (E13), 17 (E17) and 19 (E19), relative to the day of setting. Embryos were staged in accordance with the series of Hamburger and Hamilton (1951). Post-hatching chicks were studied at 1 (P1) and 7 (P7) days post-hatching. The sex of embryos or chicks was not determined.

Whole embryos were used at E3 and E5. Post-hatching chicks and embryos at E9 and older were killed by decapitation and their heads taken for analysis. In some cases, for post-hatching chicks and embryos at E17 and older, whole brains were dissected out by hand and the brains taken for analysis. Brains, embryos or heads were frozen by placing on cover slips resting
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on a metal chuck bathed in liquid nitrogen. Frozen samples were wrapped in aluminium foil and stored at -20°C until use. The work was carried out under project licence number PPL 70/4399, and personal licence number 70/14207.

2.2.2. Antibodies and other reagents.

Primary antibodies.

PSA was identified using a mouse IgM monoclonal antibody (ascites fluid) raised against the polysaccharide capsule of Meningococcus B (Anti-MenB)(Rougon et al., 1986) that recognises PSA units of at least 12 sialic acid residues (a kind gift from G. Rougon)(1/800 dilution for single immunocytochemistry or 1/200 dilution for triple immunofluorescence). Neuronal somata were recognized using a purified IgG₁ mouse anti-neuronal nuclei (anti-neuN) monoclonal antibody (Chemicon International Inc.)(1/75 dilution) that moderately labels most neuronal cell bodies and intensely labels their nuclei (Mullen et al., 1992). Neurofilaments were identified using a purified mouse IgG₁ antibody (anti-NF) that recognizes a 145kDa component of neurofilaments (Oncogene Research Products). Anti-NF was resuspended according to the suppliers guidelines and diluted at 1/100. A rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (anti-GFAP), at a dilution of 1/75, was used to identify fibrous astrocytes (Chemicon International Inc.). Galactocerebroside (GalC)(the major glycolipid found in myelin and expressed specifically by oligodendrocytes (Raff et al., 1979)) was identified using a rabbit polyclonal antibody (anti-GalC) (Biogenesis Ltd.) at a 1/75 dilution.
Secondary antibodies.

For single immunostaining, anti-MenB was detected using a μ-chain specific horseradish peroxidase (HRP) conjugated goat anti-mouse IgM (Sigma Chemical Company)(1/100 dilution). In triple immunofluorescence procedures, anti-MenB was detected using μ-chain specific Rhodamine Red™-X-conjugated affinity purified goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, Inc.) reconstituted according to the suppliers guidelines and diluted 1/100. NeuN and anti-NF were detected using fragment conserved (Fc) specific fluorescein Isothiocyanate (FITC) conjugated to goat anti-mouse IgG (Sigma Chemical Company)(1/50 dilution). Anti-GFAP and anti-GalC were detected using a 7-amino-4-methylcoumarin-3-acetic acid (AMCA) goat anti-rabbit IgG (Vector Laboratories, Inc.)(1/75 dilution). Therefore, in the immunofluorescence studies, anti-MenB labelling was always seen in red, neuronal markers were always observed as green and glial markers were seen as blue.

Other reagents.

The following reagents were obtained from the Sigma Chemical Company: bovine serum albumen (BSA)(purity: minimum 98%); colominic acid; diaminobenzidine (DAB); goat serum; hydrogen peroxide (H2O2); mouse serum; nonidet P-40 (NP-40); poly-l-lysine and Toluidine Blue O. DAKO (R) fluorescent mounting medium (containing 1.5mM NaN3) was purchased from the
Dako Corporation. Disterene plasticiser xylene (DPX) mounting medium was from BDH Chemicals Ltd.

2.2.3. Immunocytochemistry and histology.

Sectioning and fixation procedures.

Frozen 20|im coronal, sagittal or horizontal sections were cut on a Leica CM 1900 cryostat at -16°C and thaw mounted onto BDH Super Premium microscope slides coated in 0.01% poly-l-lysine. Slides were air dried for 30 min at room temperature and frozen at -20°C until use.

Frozen sections were allowed to come to room temperature and were then fixed for 30 minutes in 70% v/v ethanol, washed twice in 50mM tris-buffered saline (TBS) (50mM trizma base, 0.9% NaCl) (pH 7.4) and then rinsed in distilled water. Before staining, sections were drained of excess liquid but not allowed to dry out.

Single immunocytochemistry.

Fixed sections were incubated for 24 hours in a humidified slide box at room temperature with anti-MenB in an incubation buffer (50mM TBS (pH 7.4) containing 1% w/v BSA and 1% v/v goat serum). Sections were washed twice in TBS (pH 7.4), rinsed in distilled water, air dried and incubated for 3 hours at room temperature in a humidified slide box with HRP-conjugated secondary antibody in incubation buffer. Sections were washed and dried as before and the stain visualised by the addition of 0.05% DAB and 0.01% H2O2 (15 min. incubation)
in 50mM TBS (pH 7.4). Sections were washed twice in TBS (pH 7.4) and distilled water then mounted under coverslips using glycerol. In some cases sections from embryos and post-hatching chicks of different ages were incubated at the same time in order to allow comparisons to be made between animals of different ages.

In a few cases, after mounting, cover slips were carefully removed and sections were stained for 1 min. in Gram's stain (Hucker's)(cresyl violet in 2% ethanol). Sections were then rinsed in distilled water and re-mounted as described above.

**Multiple immunofluorescence**

Multiple immunofluorescence was carried out on whole heads at E9, E13, E17, E19, P1 and P7, but not at E3 and E5. These two early stages were not examined because most of the epitopes recognized by most of the primary antibodies used in this study (other than PSA) are not present at these stages (NeuN is expressed by post-mitotic neurons (Mullen et al., 1992), while GFAP and GalC are expressed by differentiated astrocytes and oligodendrocytes respectively).

Fixed sections were incubated for 24 hours in a humidified slide box at room temperature with, in most cases, 4 primary antibodies (either, anti-MenB, anti-NeuN, anti-NF and anti-GFAP; or anti-MenB, anti-NeuN, anti-NF and anti-GalC) in incubation buffer (50mM TBS (pH 7.4) containing 2% w/v BSA and 0.1% NP-40). Anti-GFAP and anti-GalC were applied to neighbouring sections, but never to the same section, since both
were to be visualised using the same secondary antibody. In some primary incubations, either anti-NeuN or anti-NF were omitted from the incubation buffer in order to aid clarity. Slides were washed twice in 50mM TBS (pH 7.4), drained of excess liquid and then incubated in a humidified slide box at room temperature for 2 hours with fluorochrome-conjugated secondary antibodies in incubation buffer. Sections were washed 3 times in 50mM TBS (pH 7.4), drained of excess liquid and mounted under coverslips using Dako fluorescent mounting medium.

**Controls.**

As controls for both single and triple immunocytochemistry, immunolabelling was carried out as described above, but with the following differences:

(1) omission of anti-MenB from the incubation buffer during the primary incubation;

(2) substitution of anti-MenB for mouse serum during the primary incubation; and

(3) incubation with anti-MenB that had been allowed to react with 1mg/ml colominic acid (that consists of oligomers of sialic acid) for 6 hours prior to immunocytochemistry.

In addition, for triple immunofluorescence, in some primary incubations, either 1, 2, 3 or all primary antibodies were omitted from the incubation buffer.


**Histology.**

To ensure accurate identification of neuroanatomical structures, some sections, adjacent to immunolabelled sections, were stained with toluidine blue O. Fixed sections were stained for 20 minutes with 1% toluidine blue O (aqueous). Slides were passed, for 5 minutes, through a series of alcohols (85%, 95% and 100% ethanol) then twice through xylene and mounted under coverslips using DPX.

2.2.4. Analysis and photography of sections.

Slides were viewed, at magnifications ranging from x25 to x1000, on a Zeiss Axiophot light microscope. Sections stained for toluidine blue or single immunocytochemistry were viewed under bright field illumination, with a 12V, 100W halogen filament lamp. For immunofluorescence, fluorescent chromophores were viewed using three filters (for FITC, AMCA or Rhodamine Red) using an HBO 50W mercury lamp.

Based on a visual inspection of sections stained for single immunocytochemistry, the intensity of anti-MenB immunoreactivity was categorised as immunonegative (-), minimal (+/-), weak (+), moderate (++) or intense (+++). This system is only a guide to anti-MenB staining intensity and it does not accommodate differences that fall within a particular category band. When categorizing the intensity of anti-MenB labelling, sections were generally viewed at a magnification of x25. However, this magnification was inadequate when analysing regions such as the retina and cerebellar cortex.
where a magnification of x200 was employed. Also, high magnification was used to check for minimal labelling that was not always apparent at lower magnification.

As a check on the categorization of the intensity of anti-MenB labelling, a few sections were analysed using a Microcomputer Imaging device (MCID) (Imaging Research Inc.). Slides were placed on a Northern Light desktop illuminator (model B 95, Imaging Research Inc.) that transmitted light of a constant intensity through the specimen (ambient lighting conditions were constant throughout). Images were received by an 8 bit MTI CCD72 monochrome camera mounted on a stand above the illuminator (the distance between the camera and the specimen was constant throughout). Images were processed by an NEC Express 5800 computer and presented on a visual display screen. Computer tools were used to draw, freehand with the mouse, the perimeter of target areas and the relative optical density (ROD)\(^3\) within that perimeter was determined automatically by the computer. For each section that was examined, the ROD of an arbitrarily selected region outside of the brain slice was measured and used as a control. This control value was subtracted from each measurement that was taken from that section to give a final ROD for each brain region examined. For each region that was analysed, separate measurements were made from 3 sections. The mean and standard deviation were calculated for each region.

\(^3\) ROD is the log\(_{10}\) of the reciprocal of the gray level transmittance and is calculated automatically by the MCID system.
Colour photographs were taken, under either bright field illumination or dark field illumination, by 35mm cassette automated Axio Mot cameras, integrated into the microscope system. Photographs were taken at magnifications ranging from x 25 to x 1000. For bright field illumination, Fujichrome Sensia II 100 (ISO 100/21°) colour reversal film was used, with light transmitted through appropriate neutral density filters. For dark field illumination, Kodak Ektapress 1600 Gold II (ISO 1600/33°) colour reversal film was employed.

Films were developed as colour slides or black and white negatives and scanned using a Nikon LS-1000 35mm film scanner. Images were captured and processed on a Power Macintosh G3 computer using Adobe Photoshop 5.5. Most slides of sections that had been stained for single immunocytochemistry were scanned in black and white, rather than colour, because black and white images gave a clearer indication of the intensity of anti-MenB labelling. Slides of sections that were to be used for qualitative comparisons were scanned in colour. All images were printed onto Epson photo quality glossy paper using an Epson DX printer.
2.2.5. Terminology.

The terminology of Kuenzel and Masson (1988) is employed wherever applicable.

The term ventricular zone (VZ) is used in the present study to refer to the regions immediately adjacent to the ventricles. This term is used in preference to germinal or ependymal zone since it relates only to anatomical location. In the present study, nothing is thereby implied about the cellular composition of this layer which varies during the course of development.

In the cerebellum, it is unclear precisely when the outermost layer should cease to be termed the external granular layer (EGL) and when the term glia limitans (GIL) becomes more appropriate. In the present study, this distinction is set at the time of hatching. Thus, the outermost layer of the cerebellum is referred to as EGL at E19, but GIL at P1.
2.3. Results.

2.3.1. Specificity of immunostaining and general observations.

Control sections visualised using DAB showed labelling in the lumina and red blood cells of some, but not all, blood vessels. Labelled blood vessels were not observed in sections stained for immunofluorescence. None of the other secondary antibodies used in the present study displayed any non-specific labelling. However, fluorescent particles were often seen scattered across sections.

While most large structures were observed in all replicates, some small structures were only seen in one or two replications or were missed entirely at some time-points. The number of replicates in which particular structures were seen is indicated in the summary tables given at the end of each section. Where structures have been excluded from summary tables, the number of replicates in which the structure was observed is given in the main text. Although only sections visualised with DAB were used for the analysis of anti-MenB staining intensity, the number of replicates quoted refers to combined data collected from sections visualised using DAB and those processed using fluorescence. n=4 unless otherwise stated.
2.3.2. Guide to figures.

The precise location, within the chick brain, of pictures of coronal sections presented in this chapter is given using the system of Kuenzel and Masson (1988). A sagittal view of the chick brain is presented in fig. 2.2. The numbers beneath the drawing refer to the distance, in mm, from a zero point at the level of the bregma. These numbers are given in the figure captions throughout the following sections, and the exact location of coronal planes can be ascertained by reference to fig. 2.2. The numbers given in the figure captions are prefixed with either A or P to indicate whether the section is anterior (A) or posterior (P) to the zero level. The system of Kuenzel and Masson (1988) refers to the brain of 2 week-old chicks which are larger than the brains used in the present study. The distance between unit divisions is therefore less than 1mm in the present study. Furthermore, since the chick brain undergoes structural changes during the period currently being investigated, the relative positions of some structures may be slightly different from their positions in the 2 week-old chick. The co-ordinates given in figure captions should therefore be regarded as approximate.
Fig. 2.2. Sagittal view of the chick brain showing the levels of coronal planes. The numbers above and below the sketch refer to the distance, in mm, from the zero level. Rostral (anterior) is to the right. For abbreviations see p9. After Kuenzel and Masson (1988).
2.3.3. The retina, optic nerve, chiasm and tract.

The retina.

At both E3 and E5, the undifferentiated cellular layer of the retina gave a moderate anti-MenB immunoreaction (fig. 2.3a)(n=3 at both E3 and E5). At E9, all the retinal layers found in the adult are discernible but are still morphologically immature. At E9, both the inner and outer plexiform layers (IPL and OPL), the inner and outer nuclear layers (INL and ONL) and the ganglion cell layer (GCL) were moderately reactive with anti-MenB (fig. 2.3b). At E13, E17 and E19, an intense anti-MenB immunoreaction was observed in the GCL and IPL (fig. 2.3c). At E13, E17 and E19, the OPL was moderately reactive with anti-MenB, the INL gave a weak signal and the ONL was minimally reactive (fig. 2.3c). At both P1 and P7, the GCL and IPL were intensely labelled with anti-MenB (fig. 2.3d). The INL was minimally reactive with anti-MenB at both P1 and P7, but the ONL and OPL were immunonegative at both of these time-points (fig. 2.3d). The optic fibre layer (OFL) was intensely labelled with anti-MenB at all time-points that were investigated (E3-P7)(fig. 2.3a-d). At each time-point that was investigated, no differences were detected in the retinal anti-MenB staining pattern between different regions of the retina. The relative intensities of anti-MenB immunoreactivity in the chick retina are summarized in table 2.1. Fig. 2.3e shows an E5 control section in which the retinal pigment layer was seen but no immunoreactivity was observed.
Fig. 2.3. Anti-MenB immunoreactivity in the retina and a comparison with labelling for the neuronal marker, NeuN. *Continued overleaf*
Fig. 2.3f shows a high magnification view of the GCL at E9 in which anti-MenB immunoreactivity was visualized using DAB and the section was lightly counter stained using cresyl violet. Anti-MenB immunoreactivity was detected around and between cresyl violet-positive cell bodies (arrow in fig. 2.3f) as well as around structures that had the appearance of processes (arrowhead in fig. 2.3f).

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Fig. 2.3. continued. The retina at (a) E5, (b) E9, (c) E13 and (d) P7. The staining pattern observed at E17 and E19 was identical to that detected at E13, and the PI staining pattern was identical to P7. (e) Control section from an E5 embryo. (f) High magnification image showing the GCL at E9 stained for single immunocytochemistry and lightly counter stained with cresyl violet. The arrow indicates anti-MenB immunoreactivity between 2 cresyl violet-positive cell bodies. The arrowhead indicates anti-MenB immunoreactivity around a structure that has the appearance of a process. (g) Anti-MenB positive retinal ganglion cells viewed with fluorescence optics for Rhodamine-Red and (h) the same image as in (g) viewed with optics for FITC to reveal immunofluorescence for NeuN. The same cell is identified in both (g) and (h) by the arrowheads (f, g and h viewed with oil immersion). a: outer nuclear layer; b: outer plexiform layer; c: inner nuclear layer; d: inner plexiform layer; e: ganglion cell layer; f: optic fibre layer; PL: pigment layer; VC: vitreous chamber. Scale bars a-e = 50 µm; f-h = 20 µm.
Immunoreactivities for GFAP or GalC were not detected in the retina at any of the time-points that were studied (data not shown; see discussion). At all time-points from E13 to P7 inclusive, immunofluorescence revealed intense anti-MenB immunolabelling around and between the cell bodies of NeuN positive ganglion cells (fig. 2.3g and h). At high magnification, anti-MenB immunofluorescence was also detected within ganglion cell bodies (fig. 2.3g and h). Intracellular labelling with anti-MenB could not be clearly seen at lower magnification in sections visualised using DAB (see GCL in fig. 2.3d and f). At E13, E17 and E19, anti-MenB immunofluorescence was detected within and around the somata of NeuN positive cells of the INL (data not shown).

**The optic nerve, chiasm and tract.**

The nervus opticus (NO) is only just beginning to form at E3. At E5, E9, E13, E17, E19 and P1, the NO, chiasma opticum (CO) and tractus opticus (TrO) gave an intense anti-MenB immunoreaction (fig. 2.4a-d). At P7, the NO, CO and TrO were only minimally reactive with anti-MenB (fig. 2.4e and f). In most of the NO at P7, anti-MenB labelling could only be seen at high magnification. Furthermore, at P7, anti-MenB labelling was detected beneath some, but not all, parts of the pial surface of the TrO (arrowheads in fig. 2.4e and f). Anti-MenB labelling was not observed beneath the pial surface of the NO or CO at P7 (data not shown).
Fig. 2.4. Anti-MenB immunoreactivity in the nervus opticus and tractus opticus, and a comparison with immunoreactivity for the fibrous astrocyte marker, GFAP (all images are in a sagittal or near sagittal plane). Continued overleaf
At P7, but not at any other time-point that was studied, GFAP positive processes were observed throughout the TrO (fig. 2.4f). GFAP positive processes within the interior of the TrO were non-reactive with anti-MenB (cf fig. 2.4f and g). However, it was impossible to be certain if the anti-MenB labelling beneath the pial surface was carried by GFAP positive processes. The NO was immunonegative for GFAP at all time-points that were investigated (data not shown, see discussion). However, a few GFAP positive processes were detected in the CO at P7, but not at any other time-points that were studied (data not shown; see discussion).

The nucleus opticus basalis (nBOR) was intensely labelled with anti-MenB at E17, E19 and P1, but gave only a weak reaction at P7 (fig. 2.4d and e)(nBOR was not identified earlier than E17).

Fig. 2.4. continued. (a), (b) and (c) The nervus opticus (NO) at E5, E9 and P1 respectively. (d) and (e) The nucleus opticus basalis (nBOR) and tractus opticus (TrO) at P1 and P7 respectively. The dotted line in d marks the base of the diencephalon (dien) and the arrowhead in e indicates anti-MenB immunoreactivity beneath the pial surface of the TrO. (f) and (g) The same view of the TrO observed with optics for (f) Rhodamine-Red (to reveal anti-MenB labelling) and (g) AMCA (to reveal GFAP labelling). The arrowhead in f indicates anti-MenB labelling beneath the pial surface of the TrO. CG: ciliary ganglion; NIII: nervus oculomotorius; Ret: retina. Scale bars: a-e = 500μm; f and g = 50μm.
At E13, E17, E19 and P1, but not at E5 or E9, the NO, CO and TrO contained numerous anti-MenB negative "slits" and holes (fig. 2.5a and b; see also fig. 2.4c). However, at E9, labelling the ON, CO and TrO with anti-NF, revealed the presence of slits and holes in the staining for neurofilaments (fig. 2.5c and e). NF-negative slits were immunoreactive with anti-MenB at E9 (fig. 2.5d and f). Very few anti-NF fibres were detected in the NO or TrO at E13 or later time-points, making a comparison with anti-MenB labelling difficult at these stages (data not shown). (The yellow spots that can be seen in fig. 2.5c-f as well as in some later figures are artifacts.) However, counter-staining of the NO at E13, E17 and P1 with cresyl violet revealed the presence of cell bodies both within anti-MenB negative slits as well as in anti-MenB reactive parts of the NO (fig. 2.5g).

The NO, CO and TrO were immuno-negative for GalC at E9 and E13 (data not shown). At E17, E19 and P1, immunoreactivity for GalC in the NO, CO and TrO was detected in some, but not all, sections in which these structures were seen (fig. 2.6). Anti-MenB negative slits in the NO also appeared as immunonegative slits in the staining for GalC (fig. 2.6).
Fig. 2.5. Anti-MenB immunoreactivity in the nervus opticus at E9 and E13, and a comparison with immunolabelling for neurofilaments (NF) and staining with cresyl violet. Continued overleaf
Fig. 2.6. Immunofluorescence images of the nervus opticus at E19, comparing labelling with anti-GalC and anti-MenB. The same view of the nervus opticus is shown with fluorescence optics for (a) AMCA (showing labelling for GalC) and (b) Rhodamine Red (showing labelling with anti-MenB). The asterisks indicate the same position in both images. Scale bars = 50μm.

Fig. 2.5. Continued. Anti-MenB staining of the nervus opticus (NO) at (a) E9 and (b) E13 showing immunonegative slits at E13 but not at E9. (c) and (d) The same view of the NO at E9 observed with fluorescence optics for (c) FITC (showing NF staining) and (d) Rhodamine Red (showing anti-MenB labelling)(oil immersion). The asterisks mark the same position in both images. (e) and (f) The same view of the tractus opticum (TrO) at E9 observed with fluorescence optics for (e) FITC (showing NF labelling) and (f) Rhodamine Red (showing anti-MenB labelling)(oil immersion). The same position in both images is marked by the arrowheads. (g) The NO at E13 labelled with anti-MenB (red) and counter-stained with cresyl violet (blue)(bright field illumination). Scale bars: a and b = 50μm; c, d, e, f and g = 20μm.
Differences in the intensities of anti-MenB immunoreactivity in the retina, optic nerve, chiasm and tract are summarised in table 2.1.

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**Table 2.1.** Relative intensities of ant-MenB labelling in the chick retina, optic nerve, chiasm and tract. - no labelling; +/− minimal labelling; + weak labelling; ++ moderate labelling; +++ intense labelling; nd: no data; / indicates that the structure is either not present or is undifferentiated. The numbers in parentheses refer to the number of replicates in which the structure was detected. n=4 unless otherwise stated. Abbreviations: ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; OFL: optic fibre layer; NO: nervus opticus; CO: chiasma opticum; TrO: tractus opticum; nBOR: nucleus opticus basalis.
2.3.4. The telencephalon.

At all embryonic stages, anti-MenB immunoreactivity in most parts of the telencephalon appeared homogeneous and it was difficult to distinguish between different regions on the basis of anti-MenB labelling. At P1 and P7, differences in anti-MenB staining intensity often delimited different regions. However, differences in staining intensity were often slight and had to be placed in the same intensity category. Furthermore, in many places, boundaries between different regions were often ill-defined by the anti-MenB staining pattern.

The bulbous olfactorius (BO) is not present at E3 or E5. At all time-points from E9 to P7 inclusive, intense anti-MenB labelling was observed throughout the BO (fig. 2.7). At all time-points from E9 to P7 inclusive, intense anti-MenB immunoreactivity was detected in the nerve fibre layer (NFL) of the BO, as well as in the nervus olfactorius (NI)(fig. 2.7).
Fig. 2.7. Sagittal views showing anti-MenB immunoreactivity in the bulbous olfactorius (BO) at (a) E9 and (b) P7. NFL: nerve fibre layer; NI: nervus olfactorius; VO: ventriculus olfactorius. Scale bars = 400|μm.
At both E3 and E5, intense anti-MenB labelling was detected in the marginal zone (MZ) of the telencephalic neural tube (fig. 2.8a and b). The mantle zone (MnZ), where it is present and was observed, was intensely reactive with anti-MenB at both E3 and E5 (fig. 2.8a). Most of the ventricular zone (VZ) was weakly reactive with anti-MenB at both E3 and E5 (fig. 2.8b). However, at E5, in the region where the MnZ bulges into the telocoele (TC), the VZ displayed an intense anti-MenB immunoreaction (fig. 2.8a).

At E9, the MnZ has expanded to fill most of the ventricular cavities. However, the ventricles are still not completely formed, and telencephalic structures have not taken on a mature configuration. At E9, the VZs displayed weak immunolabelling with anti-MenB (fig. 2.8c and d). At E9, apart from the VZs, the entire telencephalon was intensely labelled with anti-MenB (fig. 2.8c).
**Fig. 2.8.** Anti-MenB immunoreactivity in the telencephalon at E5 and E9. (a) Sagittal view of one hemisphere at E5. (b) The thin roof of the E5 telencephalon shown at higher magnification. (c) Coronal view of the telencephalon at E9. (d) Higher magnification image of the region shown boxed in c. MnZ: mantle zone; MZ: marginal zone; TC: telocoele; VL: ventriculus lateralis; VZ: ventricular zone; Scale bars: a and c = 1mm; b and d = 200μm.
By E13, all of the major divisions of the telencephalon can be distinguished.

The hyperstriatum accessorium (HA), the hyperstriatum intercalatum supremum (HIS) and the hyperstriatum dorsale (HD) were intensely labelled with anti-MenB at all time-points from E13 to P7 inclusive (fig. 2.9). However, at time-points from E19 to P7 inclusive, the HD gave a slightly stronger anti-MenB signal than the HIS (fig. 2.9; see tables 2.2 and 2.3).
Fig. 2.9. Coronal views showing anti-MenB immunoreactivity in the Wulst. (a) Sketch showing the location of images presented in b and c (boxed, level A11.8, see fig. 2.2). (b) and (c) Anti-MenB labelling in the wulst at E19 and P7 respectively. HA: hyperstriatum accesorium; HD: hyperstriatum dorsale; HIS: hyperstriatum intercalatum supremum; HV: hyperstriatum ventrale. Scale bars = 1mm.
All parts of the hyperstriatum ventrale (HV) were intensely reactive with anti-MenB at E13, E17 and E19 (fig. 2.10a, b and d). At P1 and P7, most of the HV was moderately labelled with anti-MenB (fig. 2.10c). However, in almost all sections from P1 and P7 brains, anti-MenB immunoreactivity was slightly stronger in the intermediate medial hyperstriatum ventrale (IMHV) than in adjacent (lateral) parts of the HV (fig. 2.10c and e). At P7, more anterior parts of the HV (the hyperstriatum ventrale pars ventralis (HVv) and the hyperstriatum ventrale pars dorsalis (HVd)) were only weakly labelled with anti-MenB (data not shown).

The hippocampus (Hp) was intensely labelled with anti-MenB at E13, E17 and E19 (fig. 2.10a and b). However, at P1 and P7, while the dorsal part of the Hp was intensely reactive with anti-MenB, the ventral Hp gave only a minimal signal (fig. 2.10c).
Fig. 2.10. Coronal views showing anti-MenB immunoreactivity in the hyperstriatum ventrale (HV) and the hippocampus (Hp). Continued overleaf
At all time-points from E13 to P7 inclusive, in telencephalic regions that were either intensely or moderately labelled with anti-MenB, immunoreactivity was detected around and between the somata of NeuN positive cells as well as within their cell bodies. Anti-MenB labelling was stronger around and between cell bodies than within NeuN positive somata. Fig. 2.11 shows a high magnification image of the IMHV at P7.

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**Fig. 2.10, continued.** (a) Sketch showing the level of images presented in b-e (level A7.6, see fig. 2.2). (b) and (c) Low magnification images showing anti-MenB immunoreactivity at E17 and P1 respectively. (d) and (e) Higher magnification images showing anti-MenB immunoreactivity in the HV at E17 and P7 respectively. N: neostriatum; LH: lamina hyperstriatica. Scale bars: a = 2mm; b and c = 1mm; d and e = 200μm.
Fig. 2.11. Immunofluorescence labelling with anti-MenB and anti-NeuN in the intermediate medial hyperstriatum ventrale (IMHV) at P7. (a) Part of the IMHV viewed with fluorescence optics for FITC to show immunolabelling for NeuN. (b) The same view as in (a) observed with fluorescence optics for Rhodamine Red showing anti-MenB labelling. The arrowhead indicates the same cell body in both images. Scale bars = 20μm.
Most of the neostriatum was intensely labelled with anti-MenB at all time-points from E13 to P7 inclusive (fig. 2.12a-c). However, at P1 and P7, there was only moderate labelling in some medial parts of the N in the region of the field L. However, the field L itself was not clearly distinguished in the present study.

The ectostriatum (E) was intensely labelled with anti-MenB at E13, E17 and E19; weakly reactive at P1 and immunonegative at P7 (fig. 2.12a-f). However, at both E17 and E19, labelling of the E appeared slightly lighter than the intensely reactive N (fig. 2.12b). At P7, some minimal anti-MenB labelling was observed at the lamina medullaris dorsalis (LMD)(fig. 2.12f).

The tractus septomesencephalicus (TSM) was intensely labelled with anti-MenB at E17, E19 and P1, but was largely immunonegative at P7 (fig. 2.12g and h). However, a few anti-MenB reactive fibres were observed within the TSM at P7. The TSM was not identified at E13.
Fig. 2.12. Coronal views showing anti-MenB immunoreactivity in neostriatum (N), ectostriatum (E) and the tractus mesencephalicus (TSM). Continued overleaf
The paleostriatum augmentatum (PA) was intensely reactive with anti-MenB at E13, E17 and E19 (fig. 2.13a and b). At P1, most of the PA was weakly labelled with anti-MenB and at P7 most of the PA was immunonegative (fig. 2.13c and d). However, moderate anti-MenB labelling was observed in some parts of the PA at both P1 and P7 (see fig. 2.13e). The paleostriatum primitivum was intensely labelled with anti-MenB at E13, E17 and E19; gave a minimal reaction at P1 and was immunonegative at P7 (fig. 2.13b-d).

Intense anti-MenB immunolabelling was observed in the lobus parolfactorius (LPO) at all time-points from E13 to P7 inclusive (fig. 2.13b, e and f).

At P1, telencephalic portions of the Fasciculus prosencephali lateralis (FPL) were minimally labelled with anti-MenB and were immunonegative at P7 (fig. 2.13c and d). [The FPL is more thoroughly investigated in its diencephalic portions in section 2.3.5.]

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**Fig. 2.12. Continued.** (a) Sketch showing the level of images presented in b-h (level A9.2, see fig. 2.2). (b) and (c) Low magnification images showing the E and lateral portions of the N. (d), (e) and (f) Part of the E, adjacent to the paleostriatum augmentatum (PA), shown at higher magnification at E17, P1 and P7 respectively. (g) and (h) The TSM at P1 and P7 respectively. HV: hyperstriatum ventrale; LMD: lamina medullaris dorsalis; VL: ventriculus lateralis. Scale bars: a = 2mm; b, c, g and h = 1mm; d - f = 200μm.
Fig. 2.13. Coronal views showing anti-MenB immunoreactivity in paleostriatal regions of the telencephalon. Continued overleaf
The tractus fronto-archistriaticus (FA) was intensely reactive with anti-MenB at P1 and P7, but was not clearly distinguished at embryonic stages (fig. 2.13d).

Intense anti-MenB immunoreactivity was detected throughout the archistriatum (A) at all time-points from E13 to P7 inclusive (fig. 2.14). However, the nucleus taeniae (Tn) was moderately labelled with anti-MenB at E13, gave a weak signal at E17 and E19 and was only minimally reactive at P1 and P7 (fig. 2.14). However, at all time-points from E13 to P7, intense anti-MenB labelling was detected beneath the brain surface at the Tn, in the region of the glia limitans (fig. 2.14).

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**Fig. 2.13. continued.** (a) Sketch showing the paleostriatal regions (shaded) of the telencephalon at level A12.2 (see fig. 2.2). Note that the images shown in b-e are at slightly different coronal levels. (b) The lobus parolfactorius (LPO) and paleostriatum augmentatum (PA) at E17 (level A10.8). (c) The PA and paleostriatum primitivum (PP) at P1 at level A8.6. (d) The PA and PP at P7 at level A8.8. (e) The LPO and PA at P1 at level A10.8. (f) The LPO in its most anterior portions at P7 (level A12.2). (g) Sketch showing the location of the image presented in g. The lamina medullaris dorsalis (LMD) is indicated by crosses. AA: archistriatum anterior; E: ectostriatum; FA: tractus fronto-archistriaticus; FPL: fasciculus prosencepheli lateralis; HV: hyperstriatum ventrale. Scale bars: a and g = 2mm; b - f: 1mm.
Fig. 2.14. Coronal views showing anti-MenB immunoreactivity in archistriatal regions of the telencephalon. (a) Sketch showing the location of images presented in b and c (level A7.6, see fig. 2.2). (b) and (c) Anti-MenB immunoreactivity in the archistriatal regions at E17 and P7 respectively. Ald: archistriatum intermedium, pars dorsalis; Alv: archistriatum intermedium, pars ventralis; Am: archistriatum mediale; LAD: lamina archistriatalis dorsalis; LMD: lamina medullaris dorsalis; N: neostriatum; Tn: nucleus taeniae. Scale bars = 1mm.
At all time-points from E13 to P7 inclusive, immunoreactivity for GFAP was observed around blood vessels and capillaries as well as beneath the brain surface in all parts of the telencephalon including the mid-line. It was impossible to be certain whether perivascular labelling for GFAP occurred in the same cells and processes that were immunoreactive with anti-MenB (data not shown). However, at the brain surface, including the mid-line, in many places, anti-MenB labelling was detected within GFAP positive cell bodies. However, while some GFAP positive cells expressed high levels of anti-MenB immunoreactivity, other GFAP positive cells displayed only low levels of anti-MenB labelling (cf. arrows and arrowheads in fig. 2.15). Furthermore, at the brain surface in the region of the Tn, anti-MenB immunoreactivity did not seem to be confined to GFAP positive cells and processes (see the heavily labelled region marked with a triangle in fig. 2.15). (Using immunofluorescence with anti-MenB and anti-GFAP, the Tn was only identified at E13 and P7.)

Differences in the intensities of anti-MenB immunoreactivity in the telencephalon are summarised in table 2.2.
Fig. 2.15. Co-expression of immunofluorescent labelling with anti-MenB and anti-GFAP at the brain surface in the region of the nucleus taeniae (Tn) at P7. (a) Dark field image of the glia limitans viewed with fluorescence optics for AMCA, showing labelling for GFAP positive cell bodies and processes. (b) The same view as in (a) observed with fluorescence optics for Rhodamine Red, showing anti-MenB labelling within cell bodies that also express GFAP. The same cell, that is heavily labelled with anti-MenB, is indicated by the arrows in both images. A cell that is weakly labelled with anti-MenB is indicated by the arrowheads in both images. The triangle indicates a region of intense anti-MenB labelling where relatively few GFAP positive processes are detected. Both images are viewed with oil immersion. (The intensely labelled area in (b) that is marked with a square is an artifact.) Scale bars = 20μm.
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**Table 2.2.** Relative intensities of anti-MenB labelling in the telencephalon. - no labelling; +/- minimal labelling; + weak labelling; ++ moderate labelling; +++ intense labelling; ++/+ and ++/- indicate that different staining intensities were observed in different parts of the structure. / indicates that the structure is not differentiated. * Although the entire telencephalon was intensely labelled with anti-MenB at E9, and its structures have undergone some differentiation, individual structures could not be distinguished. The numbers in parentheses refer to the number of replicate in which the structure was identified; n = at least 4 unless otherwise stated. Continued overleaf
Gray level analysis of the entire telencephalon gave the following overall ROD values for the whole region:
0.3115 +/- 0.0150 at E9,
0.3545 +/- 0.0141 at E13 and
0.3222 +/- 0.0221 at P1 (mean of 3 sections +/- sd.).

Grey level analyses were carried out on several individual telencephalic regions at E17, P1 and P7. The results of these analyses are presented in table 2.3.

Table 2.2, continued. BO: bulbus olfactorius; HA: hyperstriatum accessorium; HIS: hyperstriatum intercalatum supremum; HD: hyperstriatum dorsale; HV: hyperstriatum ventrale; HVd: hyperstriatum ventrale, pars dorsalis; HVv: hyperstriatum ventrale, pars ventralis; N: neostriatum; E: ectostriatum; PA: paleostriatum augmentatum; PP: paleostriatum primitivum; LPO: lobus parolfactorius; A: archistriatum; Tn: nucleus taeniae; Hp(d): hippocampus (dorsal); HP(v): hippocampus (ventral); TSM: tractus septomesencephalicus.
<table>
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<th>P1</th>
<th>P7</th>
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<td>HA(A11-12)</td>
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<td>0.3181 +/- 0.0190</td>
<td>0.3128 +/- 0.0237</td>
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<td>HIS(A11-12)</td>
<td>0.3102 +/- 0.0155</td>
<td>0.3020 +/- 0.0136</td>
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<td>HD(A11-12)</td>
<td>0.3247 +/- 0.0178</td>
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<td>IMHV(A7.6-7.8)</td>
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<td>0.3198 +/- 0.0231</td>
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<td>HV*(A7.6-7.8)</td>
<td>0.3253 +/- 0.0131</td>
<td>0.2264 +/- 0.0164</td>
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<td>N*(A7.6-7.8)</td>
<td>0.3671 +/- 0.0158</td>
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<tr>
<td>PA(A9.0-9.6)</td>
<td>0.2964 +/- 0.0171</td>
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<td>PP(A9.0-9.6)</td>
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<td>LPO(A10.8-11.2)</td>
<td>0.3618 +/- 0.0136</td>
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<tr>
<td>A(A6.4-6.8)</td>
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<tr>
<td>Tn(A6.4-6.8)</td>
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<td>0.0914 +/- 0.0113</td>
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<td>Hp(d)(A7.6-7.8)</td>
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<tr>
<td>Hp(v)(A7.6-7.8)</td>
<td>0.3481 +/- 0.0203</td>
<td>0.1154 +/- 0.0082</td>
<td>0.0532 +/- 0.0011</td>
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</tbody>
</table>

**Table 2.3.** Relative optical density (ROD) values for selected telencephalic regions. The coronal levels at which the measurements were made are given in parentheses. * Measurements were only taken from medial portions of these structures at the level of the IMHV. Values are the mean of 3 sections +/- sd. For abbreviations see caption to Table 2.2.
2.3.5. The diencephalon.

On the whole, the diencephalon was the most intensely anti-MenB immunolabelled of all brain regions at each stage that was examined.

In the diencephalic roof at both E3 and E5, anti-MenB immunoreactivity was intense in the MZ and VZ, but moderate in the MnZ (fig. 2.17a-b). At both E3 and E5, the floor of the diencephalon, including the infundibulum (Inf), was intensely labelled with anti-MenB (fig. 2.17c). The median eminence (ME) and neurohypophysis (NH) were intensely labelled with anti-MenB at all time-points from E9 to P7 inclusive (fig. 2.17d). With the exceptions of the Inf, ME and NH, discrete diencephalic regions were not be clearly distinguished at time-points earlier than E13. However, intense anti-MenB immunoreactivity was detected throughout the entire diencephalon at E9, E13 and E17.

The nucleus dorsolateralis anterior thalami (DLA), nucleus dorsomedialis anterior (rostralis) thalami (DMA) and the nucleus geniculatus lateralis, pars ventralis (GLv) were intensely labelled with anti-MenB at all time-points from E13 to P7 inclusive (fig. 2.17e and f). The nucleus rotundus (ROT) and nucleus triangularis (T) were intensely labelled with anti-MenB at E9, E13, E17 and E19 (fig. 2.16e). However, at P1, the ROT and T were moderately reactive with anti-MenB, and at P7 both of these nuclei were largely minimally-reactive, although there was some weak staining of the ROT predominantly dorso-laterally (fig. 2.16f).
Fig. 2.16. Anti-MenB immunoreactivity in the diencephalon. 
continued overleaf.
The nucleus ovoidalis (OV) was moderately labelled with anti-MenB at E17, E19, P1 and P7 (fig. 2.16e). The nucleus ansae lenticularis anterior (ALA) was intensely labelled with anti-MenB at E17 and E19, gave a moderate signal at P1 and was immunonegative at P7 (fig. 2.16e and f).

The nucleus pretectalis (PT), nucleus spiriformis lateralis (SpL), nucleus spiriformis medialis (SpM) and the nucleus subpretectalis (SP) were moderately immunoreactive with anti-MenB E17, E19, P1 and P7 (fig. 2.16g). None of these nuclei were identified at earlier stages.

Fig. 2.16 continued: (a) Sagittal view of the diencephalic vesicle at E5. The roof of the diencephalon is shown at higher magnification at E5 in (b), and the floor of the diencephalon at E5 is shown in (c). (d) The median eminence (ME) at E13 (coronal view at level A4.8, see fig. 2.2). (e) Coronal section showing the diencephalon at E17 (level A6.4, see fig. 2.2). (f) Coronal section showing the diencephalon at P7 (level A6.8, see fig. 2.2). (g) Coronal section showing the pre-tectal nuclei at P1 (level A4.8, see fig. 2.2). (g) Coronal sketch showing the location (boxed) of the image shown in g. AL: ansa lenticularis; ALA: nucleus ansae lenticularis anterior; DC: diocoele; DLA: nucleus dorsolateralis anterior thalami; FPL: fasciculus prosencephali medialis; GLv: nucleus geniculatus lateralis, pars ventralis; Inf: infundibulum; MZ: marginal zone; OV: nucleus ovoidalis; PT: nucleus pretectalis; QF: tractus quintofrontalis; ROT: nucleus rotondus; SpL: nucleus spiriformis lateralis; SP: nucleus subpretectalis; T: nucleus triangularis; VZ: ventricular zone. Scale bars: a, d, e, f and g = 400μm; b and c = 50μm; h = 1mm.
In the ROT at E13, E17 and E19, anti-MenB labelling was detected around and between NeuN positive cell bodies, as well as within their cell bodies (fig. 2.17). Anti-MenB labelling around and between cell bodies was stronger than intracellular labelling (fig. 2.17). At P1 and P7, although the intensity of anti-MenB immunoreactivity was much reduced in the ROT, high magnification immunofluorescence continued to reveal anti-MenB labelling within the cell bodies of NeuN positive cells as well as stronger anti-MenB labelling around and between NeuN positive cells (data not shown). That is, the pattern of anti-MenB labelling was the same irrespective of the intensity of labelling [In order to produce high magnification photographs of weakly or minimally immunofluorescent areas, it is necessary to increase the exposure time of the camera. The images thereby produced appear identical to photographs taken of intensely labelled areas. Such images can only be misleading and are therefore excluded from the present work.]

High magnification immunofluorescence revealed the same anti-MenB staining pattern in all diencephalic nuclei including the DLA and GLv.

Although systematic grey level analyses were not carried out on individual diencephalic nuclei, the overall ROD of the entire diencephalon was ascertained at E9, E17, P1 and P7. The following ROD values were obtained:

- 0.3311 +/- 0.0134 at E9,
- 0.3712 +/- 0.0090 at E17,
- 0.3718 +/- 0.0079 at P1 and
- 0.3621 +/- 0.0132 at P7 (mean of 3 sections +/- sd.).
Fig. 2.17. Immunofluorescence images comparing immunolabelling with anti-MenB and anti-NeuN in the nucleus rotundus (ROT). (a) and (b) The same view of the ROT at E13 observed with fluorescence optics for (a) Rhodamine Red (showing anti-MenB labelling) and (b) FITC (showing anti-NeuN labelling)(oil immersion). The asterisks mark the same cell body in both images. Scale bars = 20\(\mu\)m.
Fibre tracts within the diencephalon.

At E13 and E17, the fasciculus prosencephali lateralis (FPL) and the ansa lenticularis (AL) were intensely labelled with anti-MenB (fig. 2.16e and fig. 2.18a). However, at E19 anti-MenB negative strips were observed within the FPL and AL. At P1 and P7, the FPL was largely anti-MenB negative, but contained numerous intensely reactive strips (fig. 2.18b). The number of anti-MenB positive strips in the FPL varied considerably between sections. The FPL was not analysed for anti-NF labelling. In the AL at E13, anti-MenB immunoreactivity was detected both in NF positive areas as well as in NF negative places (fig. 2.18c and d). At P1 and P7, the AL was immunonegative with anti-MenB (see fig. 2.16f). NF labelling in the AL was not investigated at E17 and E19.

The FPL and AL were immuno-positive for GalC at E17, E19, P1 and P7, but not at E13 (fig. 2.18e). However, GalC negative strips were observed crossing the AL and, most noticeably, the FPL at E17, E19, P1 and P7 (fig. 2.18e). GalC negative strips were anti-MenB positive (fig. 2.18e and f). GalC positive areas were either non-reactive with anti-MenB or gave only a minimal signal (fig. 2.18e and f).

In the FPL, a few GFAP positive processes were detected at P1 and P7, but not at earlier time-points (fig. 2.18g). Many GFAP positive processes tended to be orientated along the length of anti-MenB positive strips (two strips run from top left to bottom right in fig. 2.18g and h). However, it was impossible to
Fig. 2.18. Anti-MenB immunoreactivity in the fasciculus prosencepheli lateralis (FPL) and ansa lenticularis (AL) and a comparison with markers for neurofilaments (NF), fibrous astrocytes (GFAP) and myelin (GalC). Continued overleaf
be certain if GFAP positive processes running in anti-MenB positive strips were themselves labelled with anti-MenB. In fig. 2.18(g and h), one GFAP positive process crossing between two intensely anti-MenB reactive strips appears to co-localize with weak anti-MenB labelling (arrowheads in fig. 2.18c and d)(but see discussion). However, many GFAP positive processes were clearly non-reactive with anti-MenB at both P1 and P7 (arrow in fig. 2.18c and d; see also the GFAP positive processes to the left of the arrowheads).

Fig. 2.18. Continued  (a) and (b) Coronal bright field images of the FPL at E17 and P7 respectively (for the location of the FPL see fig. 2.16f). (c) and (d) The same view of the AL at E13 observed with fluorescence optics for (c) FITC (showing anti-NF labelling) and (d) Rhodamine Red (showing anti-MenB labelling). (e) and (f) The same view of the FPL at P7 observed with fluorescence optics for (e) AMCA (showing labelling for GalC) and (f) Rhodamine Red (showing anti-MenB labelling). (g) and (h) The same view of the FPL at P7 observed with fluorescence optics for (g) AMCA (showing anti-GFAP labelling) and (h) Rhodamine Red (showing anti-MenB labelling). The arrowheads in (g) and (h) point to what appears to be a process that is reactive both with anti-MenB and with anti-GFAP (but see discussion). The arrows in (g) and (h) indicate a region that is GFAP positive but anti-MenB negative. All images are in a coronal plane at approximately level A6.8 (see fig. 2.2). Oil immersion was used in c-h. Scale bars: a and b = 200μm; c-h = 20μm.
The commissura anterior (CA) was intensely labelled with anti-MenB at E13, E17, E19 and P1 but was minimally reactive at P7 (fig. 2.19)(n=2 at E13). However, at P7, a few anti-MenB reactive strips were observed within the CA (arrowhead in fig. 2.19c). At E13, anti-MenB immunoreactivity was detected both in parts of the CA that were reactive for NFs, as well as in parts of the CA that were NF negative (fig. 2.19d and e: the asterisk indicates a place that is anti-MenB positive but NF negative). The CA was not examined for immunofluorescence at E17, E19 or P1.

At P7, gaps in the labelling for neurofilaments in the CA were immunonegative with anti-MenB (arrows in fig2.19f and g). Anti-MenB immunoreactivity in the CA at P7 occurred in NF positive places (arrowheads in fig 2.19f and g)(fig.2.19f and g also suggests a tendency for anti-MenB labelling to occur adjacent to NF negative slits). At P7, immunoreactivity for GalC was detected throughout the CA, in both anti-MenB negative as well as anti-menB positive places (the arrowheads in fig2.19f-h indicate a position that is reactive with anti-GalC as well as anti-MenB and anti-NF). No data on the expression of GalC at time-points earlier than P7 were collected.
Fig. 2.19. Anti-MenB immunoreactivity in the commissura anterior (CA), and a comparison with markers for neurofilaments (NF) and myelin (GalC). Continued overleaf
[The sagittal views of the CA shown in fig 2.19f-h appear to reveal a strip of intense anti-MenB immunoreactivity crossing the CA. However, this strip is probably not part of the CA, but a region of surrounding tissue. The bundle of fibres above this anti-MenB positive strip are presumably separating away from the main body of the CA.]

**Fig. 2.19. continued** (a) Position of the CA (shaded) seen in a coronal plane at level A8.2 (see fig. 2.2). Coronal bright images of the CA at (b) E17 and (c) P7. (d) and (e) The same sagittal view of the CA at E13 observed with fluorescence optics for (d) Rhodamine Red (showing labelling with anti-MenB) and (e) FITC (showing labelling for neurofilaments)(oil immersion). The asterisks mark the same position in both images. (f), (g) and (h) The same view of the CA (sagittal plane) at P7 observed with fluorescence optics for (f) FITC (showing labelling for neurofilaments), (g) Rhodamine Red (showing labelling with anti-MenB) and (h) AMCA (showing labelling for GalC)(oil immersion). The arrows in f and g point to a position that is NF negative and anti-MenB negative. The arrowheads in f, g and h indicate a position, adjacent to an NF negative slit, that is reactive with anti-MenB and labelled for NF and GalC. CO: chiasma opticum. Scale bars: a = 2mm; b and c = 100µm; d and e = 20µm; f,g and h = 50µm.
Differences in the intensities of anti-MenB immunoreactivity in the diencephalon are summarised in table 2.4.

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**Table 2.4.** Relative intensities of anti-MenB labelling in the diencephalon. - non-immunoreactive; +/- minimal labelling; + weak labelling; ++ moderate labelling; +++ intense labelling. / indicates that the structure is not differentiated; nd: no data; * indicates that the structure could not be clearly distinguished. The numbers in parentheses refer to the number of replicates in which the structure was identified. n=4-5 unless otherwise stated. DLA: nucleus dorsolateralis anterior thalami; ROT: nucleus rotundus; T: nucleus triangularis; OV: nucleus ovoidalis; GLv: nucleus geniculatus lateralis, pars ventralis; ALA: nucleus ansae lenticularis anterior (rostralis); PT: nucleus pretectalis; SpL: nucleus spiriformis lateralis; SpM: nucleus spiriformis medialis; SP: nucleus subpretectalis.
2.3.6. The mesencephalon (midbrain).

**Tectum opticum and internal tectal nuclei.**

At E3, anti-MenB immunoreactivity in the thin roof of the mesencephalon was moderate in the MZ and minimal in the VZ (fig. 2.20a and b). At E5, the narrow MZ was intensely reactive with anti-MenB, and the broad VZ was moderately labelled (fig. 2.20c and d). [The fibrous MZ of the E5 mesencephalon becomes the stratum album centrale (SAC) of later stages. It is not equivalent to the stratum opticum (SO) since retinal ganglion cell axons have not yet arrived at the tectum. See section 2.1.4.]

The stratum opticum (SO) was intensely labelled with anti-MenB at all time-points from E9 to P7 inclusive (fig. 2.20e-h). Grey level analyses of the SO gave the following ROD values:

- 0.4226 +/- 0.0221 at E9;
- 0.4231 +/- 0.0158 at E13 and
- 0.3601 +/- 0.0362 at P7 (mean of 3 sections +/- sd.).

At E9, the stratum griseum et fibrosum superficiale (SGFS) and the stratum griseum centrale (SGC) are not fully differentiated (LaVail and Cowen, 1971a). The region between the SO and SAC is therefore referred to as SGFS/SGC in fig 2.20. This layer was moderately labelled with anti-MenB at E9 (fig. 2.20e and f). The SGFS and SGC were both intensely labelled with anti-MenB at time-points from E13 to P7 inclusive (fig. 2.20g and h).
Fig. 2.20. Anti-MenB immunoreactivity in the roof of the mesencephalon during its development into the tectum opticum. 

continued overleaf
Grey level analyses were carried out on the SGFS and SGC. To enable a comparison to be made between E9 and later stages, each measurement covered a region that included both the SGFS and the SGC. ROD values for the SGFS/SGC were:

- 0.2445 +/- 0.0228 at E9;
- 0.3649 +/- 0.0118 at E13 and
- 0.3412 +/- 0.0168 at P7 (mean of 3 sections +/- sd.).

The SAC and brachium colliculi superioris (BCS) were intensely labelled with anti-MenB at E9, E13 and E17 (fig. 2.20 d-g; fig. 2.21a). However, the BCS and rostro-ventral parts of the SAC were only minimally reactive with anti-MenB at E19 and were non-reactive at both P1 and P7 (fig. 2.21b-d). However,

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**Fig. 2.20 continued.** (a) and (b) The roof of the mesencephalon at E3 and E5 respectively. (c) Sagittal view of the mesencephalon at E5. (d) Horizontal section of an optic lobe at E9 (rostral is to the top). (e) The tectum opticum at E9 shown at higher magnification. (f) The tectum opticum at E17 (Coronal section). (g) and (h) Coronal views of the tectum opticum at P7 shown at low and high magnification respectively. MC: mesocoele; MZ: marginal zone; VT: ventriculus tecti mesencephali; VZ: ventricular zone; SO: stratum opticum. 1: stratum griseum periventriculare; 2: stratum album centrale; 3: stratum griseum centrale; 4: stratum griseum et fibrosum superficiale; 5: stratum opticum. Scale bars: a and b = 100μm; c, d and g = 1mm; e, f and h = 250μm.
more posteriorly, in its dorso-lateral portions, the SAC displayed weak anti-MenB labelling at P1 and P7 (fig. 2.20g and h).

The ventricular zones gave only a minimal signal with anti-MenB at E9 (fig. 2.20d and e). At E9, between the VZ and SAC, anti-MenB labelling appeared as differentially reactive layers: a moderately labelled layer adjacent to the VZ and a weakly reactive layer just internal to the SAC (fig. 2.20d and e). All layers between the SAC and VT, including the VZ, are collectively referred to as the stratum griseum periventriculare (SGP) (Kuenzel and Masson, 1988). Anti-MenB labelling of the SGP was uniformly intense at all time-points from E13 to P7 inclusive (fig. 2.20f-i; fig. 2.21).

At E13, the nucleus isthmi pars magnocellularis (Imc) was moderately reactive with anti-MenB. Other internal tectal nuclei were not identified at this or earlier time-points. The Imc and nucleus isthmi pars parvocellularis (Ipc) were moderately reactive with anti-MenB at E17 (fig. 2.21b), gave a weak signal at E19 (fig. 2.21c), were minimally reactive at P1 (fig. 2.21d) and were immunonegative at P7 (fig. 2.21e). The nucleus mesencephalicus lateralis pars dorsalis (MLd) was intensely labelled with anti-MenB at E17 and E19 (fig. 2.21b and c), gave a weak signal at P1 (fig. 2.21d) and was immunonegative at P7 (fig. 2.21e)

Tissue medial to the Ipc was intensely labelled with anti-MenB at E17, E19 and P1 (fig. 2.21b-d). At P7, tissue medial to the Ipc was minimally reactive with anti-MenB (fig. 2.21e).
Fig. 2.21. Coronal sections showing anti-MenB labelling in the tectum opticum and isthmic nuclei. (a) Sketch showing the location of images presented in b-e (level A2.8, see fig. 2.2). Anti-MenB labelling is shown at (b) E17, (c) E19, (d) P1 and (e) P7. BCS: brachium colliculi superioris; Cb: cerebellum; Imc: nucleus isthmi, pars magnocellularis; Ipc: nucleus isthmi, pars parvocellularis; MLd: nucleus mesencephalicus lateralis, pars dorsalis; SAC: stratum album centrale; SGP: stratum griseum periventriculare; VT: ventriculus tecti mesencephali. Scale bars: a = 1mm; b-e = 400 μm.
Immunoreactivity for GFAP was detected in the SO at time-points from E13 to P7 inclusive (fig. 2.22). However, because of the intense anti-MenB labelling in the SO it was almost always impossible to be certain if GFAP positive processes were also labelled with anti-MenB. However, there was some indication of such a co-localization at P7 (arrowheads in fig. 2.22, but see discussion).

**Fig. 2.22.** Immunofluorescence images comparing immunoreactivity for the fibrous astrocyte marker, GFAP, with anti-MenB labelling in the stratum opticum (SO) of the tectum opticum at P7. The same view is shown with fluorescence optics for (a) AMCA (to reveal labelling for GFAP) and (b) Rhodamine Red (to reveal labelling with anti-MenB). The arrowheads in both images point to what appears to be the same process (but see discussion)(Both images are viewed with oil immersion.) Scale bars = 20μm.
Immunoreactivity for GalC was detected in some, but not all, parts of the SAC at E17 and E19 (fig. 2.23). Intense anti-MenB labelling was observed in parts of the SAC that were reactive for GalC as well as in places that were GalC negative at both E17 and E19 (fig. 2.23b and c). At P1 and P7, all parts of the SAC that were observed were reactive for GalC. Immunolabelling for GalC was detected in areas that were labelled with anti-MenB as well as in areas that were anti-MenB negative (data not shown).

Immunoreactivity for GFAP was observed in the SAC at E19, P1 and P7 (fig. 2.23d). At E19, anti-MenB immunoreactivity was detected in positions that appeared to be GFAP positive cell bodies (asterisks in fig. 2.23d and e).
Fig. 2.23. Anti-MenB immunofluorescence in the stratum album centrale (SAC) at E19 and a comparison with immunoreactivities for the myelin marker, GalC and the fibrous astrocyte marker, GFAP. (a) Drawing showing the position of images presented in b-e (level A2.0, see fig. 2.2; the boxed regions show the location of images presented in b-e). (b) and (c) show the same view observed with fluorescence optics for (b) AMCA (revealing labelling for GalC) and (c) Rhodamine Red (revealing labelling with anti-MenB). (d) and (e) show the same view of the SAC observed with fluorescence optics for (d) AMCA (revealing labelling for GFAP) and (e) Rhodamine Red (revealing labelling with anti-MenB). The same position, that appears to be a GFAP positive cell body, is marked with asterisks in (d) and (e) (d and e were viewed with oil immersion). Cb: cerebellum; TO: tectum opticum. Scale bars: a = 1mm; b-c = 40μm; d-e = 20μm.
The nucleus isthmo-opticus.

Anti-MenB immunoreactivity in the nucleus isthmo-opticus (IO) was moderate at E17, E19 and P1 and weak P7 (fig 2.24). The IO was not identified at time-points earlier than E17. At E19 and P1, anti-MenB immunoreactivity was detected within NeuN positive cell bodies (fig. 2.24c and d). Strong anti-MenB labelling was observed around the surfaces of NeuN positive cell bodies (fig. 2.24; see discussion). The IO was not analyzed for immunofluorescence at P7.
Fig. 2.24. Anti-MenB immunoreactivity in the nucleus isthmo-opticus (IO) and a comparison with labelling for the neuronal cell marker, NeuN. For the location of the IO see fig. 2.23a. (a) and (b) The IO at P1 and P7 respectively. (c) and (d) The same view of the IO at E19 observed with fluorescence optics for (c) FITC (showing NeuN positive cell bodies) and (d) Rhodamine Red (showing labelling with anti-MenB). (c and d were viewed with oil immersion). The asterisks mark the same positions in c and d. Cb: cerebellum. Scale bars: a and b = 200μm; c and d = 20μm.
Other mesencephalic nuclei and fibre tracts.

At E13, and all later time-points that were investigated, the roots of the nervus oculomotorius (NIII) were largely non-reactive with anti-MenB (fig. 2.25). However, several immunopositive strips were observed apparently within the nerve roots in some, but not all, sections at all time-points from E13 to P7 inclusive (fig. 2.25). However, most anti-MenB positive strips apparently within the NIII were immunonegative for neurofilaments at all time-points from E13 to P7 (asterisks in fig. 2.25c and d). Some of these strips are probably surrounding tissue through which oculomotor fibres are passing. However, at E17 and E19, some NF positive strips within the NIII were also immunoreactive with anti-MenB (arrowheads in fig. 2.25c and d). Strips that were reactive with both anti-MenB and anti-NF were not detected in sections cut at E13, P1 or P7 (see discussion).

In the region of the NIII, counter-staining sections with cresyl violet revealed rows of cell bodies both in anti-MenB negative as well as anti-MenB positive areas (fig. 2.25e and f). At E17, E19, P1 and P7, anti-MenB negative, and NF positive, parts of the NIII were immuno-positive for GalC. (The roots of NIII were non-reactive for GalC at E13.) However, at E17, in some, but not all sections, anti-MenB immunoreactivity was detected in GalC positive parts of the NIII (fig. 2.25g and h).
Fig. 2.25. Anti-MenB immunoreactivity at the level of the nervus oculomotorius (NIII) and a comparison with markers for neurofilaments (NF), myelin (GalC) and cresyl violet staining. Continued overleaf.
In some GalC positive places, anti-MenB immunoreactivity was minimal (arrowheads in fig. 2.25g and h), while occasionally intense anti-MenB labelling co-localized with GalC immunoreactivity (arrows in fig. 2.25g and h).

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**Fig. 2.25. Continued.** (a) Sagittal sketch showing the location of the NIII (shaded) in the chick brain. (b) Bright field image of the NIII labelled with anti-MenB at E13. (c) and (d) Immunofluorescence images showing the same view of NIII at E19 observed with fluorescence optics for (c) FITC (showing NF labelling) and (d) Rhodamine Red (showing anti-MenB labelling). The asterisks in (c) and (d) indicate 2 regions that are NF positive and anti-MenB negative. The arrowheads in (c) and (d) indicate a region that is NF positive and anti-MenB positive. (e) NIII labelled with anti-MenB (red) and cresyl violet (blue) at P1. (f) The same section as shown in (e) but at a higher magnification. (g) and (h) Immunofluorescence images of the NIII at E17 viewed with fluorescence optics for (g) AMCA (showing labelling for GalC) and (h) Rhodamine Red (showing labelling with anti-MenB). The arrows in (g) and (h) indicate a region that is GalC positive and minimally labelled with anti-MenB. The arrowheads in (g) and (h) indicate a region that is GalC positive and intensely labelled with anti-MenB. The dotted lines in (g) delimit the fasciculus longitudinalis medialis (FLM). Rostral is to the right in a and c-f, rostral is to the top in b and to the left in g and h. telen: telencephalon; NIV: nucleus nervi trochlearis; OMv: nucleus nervi oculomotorii, pars ventralis. Scale bars: a = 5mm; b, g and h = 800µm; c, d and f = 200µm; e = 400µm.
Chapter 2

The nucleus nervi oculomotorii pars dorsolateralis (OMdl), the nucleus nervi oculomotorii pars dorsomedialis (OMdm) and the nucleus nervi oculomotorii pars ventralis (OMv) were weakly reactive with anti-MenB at E13 and E17, but were minimally labelled at E19, P1 and P7 (fig. 2.26). However, the nucleus of Edinger-Westphal (EW) was intensely labelled at E17 and E19, moderately reactive at P1 and gave a minimal signal at P7 (shown at P1 in fig. 2.26c). The nucleus nervi trochlearis (nIV) gave a weak anti-MenB immunoreaction at E13 and was minimally labelled at all time-points from E17 to P7 inclusive (shown at E13 in fig. 2.25b).

The fasciculus longitudinalis medialis (FLM) was only minimally reactive with anti-MenB at E13 and non-reactive at all later time-points that were examined. The FLM was non-reactive for GalC at E13. At E17 and E19 some, but not all parts of the FLM were GalC positive (fig. 2.25g).
Fig. 2.26. Coronal sections showing anti-MenB labelling in the nuclei of the nervus oculomotorius at P1. (a) Sketch showing the location of images presented in b and c (level A3.2, see fig. 2.2). (b) and (c) Nuclei of the nervus oculomotorius stained with toluidine blue and anti-MenB respectively. Cb: cerebellum; EW: nucleus of Edinger-Westphal; OMdI: nucleus nervi oculomotorii, pars dorsalis; OMdm: nucleus nervi oculomotorii, pars dorsomedialis; OMv: nucleus nervi oculomotorii, pars ventralis; TO: tectum opticum. Scale bars a = 1mm; b and c = 200μm.
Differences in the intensities of anti-MenB immunoreactivity in the mesencephalon are summarised in table 2.5.

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Table 2.5. Relative intensities anti-MenB immunoreactivity in the mesencephalon. * Owing to differences in staining intensity between different parts of this layer, a meaningful comparison cannot be given. The numbers in parentheses refer to the number of replicates in which the structure was identified. n = 4-5 unless otherwise stated. - non-immunoreactive; + weak labelling; ++ moderate labelling; +++ intense labelling. nd: no data; / indicates that the structure is not present. continued overleaf
2.3.7. The rhombencephalon.

*The cerebellum.*

The cerebellum is not present at E3 or E5 (data on the rhombencephalon at these two stages is shown below). At E9, intense anti-MenB immunoreactivity was detected throughout the cerebellum (fig. 2.27). However, at E9 the cerebellum is still undeveloped and no further detail could be discerned at this time-point.

The nuclei cerebellaris internus (Cbl) as well as fibres within the cerebellum gave an intense anti-MenB immunoreaction at E13 and E17, were weakly labelled at E19, and were minimally reactive at P1 and P7 (fig. 2.27).

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**Table 2.5. continued.** SO: stratum opticum; SGFS: stratum griseum et fibrosum superficiale; SGC: stratum griseum centrale; BCS: brachium colliculi superioris; SAC: stratum album centrale; SGP: stratum griseum periventriculare; Imc: nucleus isthmi pars magnocellularis; Ipc: nucleus isthmi pars parvocellularis; MLD: nucleus mesencephalicus lateralis pars dorsalis; OMdI: nucleus nervi oculomotori, pars dorsolateralis; OMdm: nucleus nervi oculomotorii, pars dorsomedialis; OMv: nucleus nervi oculomotori, pars ventralis; EW: nucleus of Edinger-Westphal; nIV: nucleus nervi trochlearis; IO: nucleus isthmo-opticus.
Fig. 2.27. Anti-MenB immunoreactivity in the cerebellum (Cb). (a) and (b) Sagittal views of the Cb labelled with anti-MenB at E9 and E13 respectively (rostral is to the right in a and b). (c) and (d) Coronal views of the Cb labelled with anti-MenB at E17 and P1 respectively. (e) Coronal sketch showing the location of images presented in c and d (level A0.4, see fig. 2.2). Cbl: nucleus cerebellaris internus; FLM: fasciculus longitudinalis medialis; FU: fasciculus uncinatus. Scale bars: a-d = 400μm; e = 2mm.
At E13, the external granular layer (EGL) was intensely labelled with anti-MenB (fig. 2.28a-b). By E17, with the virtual completion on inward cell migration, the cellular EGL has been largely replaced by the cell-sparse molecular layer (ML). At E17, the ML was intensely labelled with anti-MenB (fig. 2.28c). However, the remaining EGL displayed an intense anti-MenB signal at E17 (fig. 2.28c). At E19, anti-MenB immunoreactivity was moderate in the ML (fig. 2.28d). At P1 and P7, minimal anti-MenB labelling was detected in the ML, but intense labelling was observed beneath the pial surface in the region of the glia limitans (fig. 2.28e). The surface of the cerebellum was immunonegative for GFAP at all time-points that were investigated (data not shown, see discussion).

The Purkinje cell layer (PCL) was intensely reactive with anti-MenB at E13; weakly labelled at E17; minimally reactive at E19 and immuno-negative at P1 and P7 (fig. 2.28a-e). The internal granular layer (IGL) was intensely reactive with anti-MenB at E13 and E17; moderately labelled at E19 and minimally reactive at P1 and P7 (fig. 2.28a-e). Minimal anti-MenB labelling in the ML and IGL at P1 and particularly at P7 was often, though not always, so slight that it was only detectable using high magnification immunofluorescence.
Fig. 2.28. Anti-MenB immunoreactivity in cerebellar folia and a comparison with the myelin marker, GalC. Continued overleaf.
White matter (WM) within the cerebellar folia was intensely reactive with anti-MenB at E13 and E17; gave a moderate signal at E19 and was minimally reactive at P1 and P7 (fig. 2.28a-e). Immunofluorescence for GalC was detected in some, but not all, parts of the cerebellar WM at E17, E19 and P1 (fig. 2.28f). At each of these 3 time-points (E17, E19 and P1), there was no difference in the intensity of anti-MenB immunoreactivity between areas of the cerebellar WM that were GalC positive and areas that were negative for GalC (fig. 2.28f and g). At P7, all parts of the WM that were observed were immuno-positive for GalC.

At any given time-point, the same anti-MenB staining pattern was observed in all ten cerebellar folia (see fig. 2.27).

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**Fig. 2.28. Continued.** (a) Sagittal sketch of the cerebellum (Cb) showing the location (boxed) of images presented in b-g. Bright field images showing anti-MenB labelling at (b) E13, (c) E17, (d) E19 and (e) P7. Immunoreactivity at P1 (not shown) was intermediate between E19 and P7 (see fig. 2.28). (e) and (f) The same view of cerebellar white matter (WM) at E17 observed with fluorescence optics for (e) AMCA (showing labelling for GalC) and (f) Rhodamine Red (showing labelling with anti-MenB). The dotted line in e delimits an area of WM that is GalC negative. EGL: external granular layer; GIL: glia limitans; IGL: internal granular layer; ML: molecular layer; PCL: Purkinje cell layer. Scale bars: a = 1mm; b-g = 50μm.
Differences in the intensities of anti-MenB immunoreactivity in the cerebellum are summarised in table 2.6.

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**Table 2.6.** Relative intensities of anti-MenB labelling in the cerebellum. +/- minimal labelling or non-immunoreactive; + weak labelling; ++ moderate labelling; +++ intense labelling; nd no data; / indicates that the structure is not present; ? indicates that the identity of the structure is uncertain. The numbers in parentheses refer to the number of replicates in which the structure was identified; n=4-6 unless otherwise stated. EGL: external granular layer; GIL: glia limitans; ML: molecular layer; PCL: Purkinje cell layer; IGL: internal granular layer; WM: white matter; Cbl: nucleus cerebellaris internus.
The pons and myelencephalon.

At both E3 and E5 the floor of the rhombencephalon is particularly thick relative to other areas of the neural tube (Fig. 2.29). In the floor of the rhombencephalon at both E3 and E5, anti-MenB immunoreactivity was intense in the MZ and MnZ (fig. 2.29). In the VZ, anti-MenB immunoreactivity was moderate at both E3 and E5 (fig. 2.29)(n=3 at both E3 and E5).
Fig. 2.29. Sagittal views showing anti-MenB immunoreactivity in the rhombencephalon. (a) The entire rhombencephalon at low magnification at E3. (b) and (c) The floor of the rhombencephalon at higher magnification at E3 and E5 respectively. Note the different magnifications in b and c. Rostral is to the right in b and c. Is: isthmus; MC: mesocoele; MZ: marginal zone; MnZ: mantle zone; RC: rhombocoele; SC: spinal cord; VZ: ventricular zone. Scale bars: a = 500μm; b and c = 50μm.
At E17 and E19, the nucleus motorius nervi trigemini (MnV) and the nucleus sensorius principalis nervi trigemini (nPrV) were both moderately labelled with anti-MenB (fig. 2.30a and b). Both of these nuclei were minimally labelled at P1 (fig. 2.30c). At P7, most of the pons was anti-MenB negative (fig. 2.30d). The roots of the nervus trigeminus (NV) were anti-MenB negative at time-points from E17 to P7 inclusive (fig. 2.30). None of the above structures were identified at time-points earlier than E17.

The fasciculus uncinatus (FU) was moderately reactive at E17 (see fig. 2.30b) and gave only a minimal reaction at P1 (fig. 2.30c). However, at P7, some sections showed intense anti-MenB labelling of the FU (fig. 2.30d). However, fibres of the FU as they ascend into the cerebellum were non-reactive with anti-MenB at P7 (see fig. 2.27d).

Throughout the rhombencephalon, the fasciculus longitudinalis medialis (FLM) gave a minimal anti-MenB signal at E13 and was non-reactive at time-points from E17 to P7 inclusive (see figs. 2.30-2.32).
Fig. 2.30. Anti-MenB immunoreactivity at the level of the nervus trigeminus. (a) Coronal sketch showing the location of images presented in (b) - (d)(level AO.6, see fig. 2.2; the boxed region in (a) indicates the approximate position of images shown in (b) - (d)). Anti-MenB immunoreactivity is shown at (b) E17, (c) P1 and (d) P7. Cb: cerebellum; FLM: fasciculus longitudinalis medialis; FU: fasciculus uncinatus; nPrV: nucleus sensorius principalis nervi trigemini; NV: nervus trigeminus; TO: tectum opticum. The crosses mark the nucleus motorius nervi trigemini. Scale bars: a = 1mm; b-d = 400μm.
At E13, the nucleus angularis (An), nucleus magnocellularis (mcc) and nucleus laminaris (La) were intensely labelled with anti-MenB (fig. 2.31a-c). However, at E13, the region surrounding the mcc and La was only minimally reactive with anti-MenB (fig. 2.31b and c). This region surrounding the mcc and La was immunoreactive with GalC at E13 (fig. 2.31c and d).

The An and La were weakly labelled with anti-MenB at E17, and were non-reactive at E19, P1 and P7 (fig. 2.31e-h). The mcc was non-reactive with anti-MenB at E17, E19, P1 and P7 (fig. 2.31e-h).

The crossed dorsal cochleal tract (CTrX) was immunonegative with anti-MenB at E17 (the CTrX is marked with an asterisk in fig. 2.31e).

While much tissue at around the level of the eighth cranial nerve was intensely immunoreactive with anti-MenB at E17 (fig. 2.31e), at both E19 and P1, much of this tissue at this level gave only a minimal signal (fig. 2.31g). At P7, apart from two, not clearly definable bilateral regions of anti-MenB immunoreactivity (marked with asterisks in fig. 2.31h), the nucleus vestibularis medialis (VeM) and the nucleus nervi glossopharyngei (nIX) were the only clearly defined anti-MenB positive structures at this level (fig. 2.31h).
Fig. 2.31. Anti-MenB immunoreactivity at the level of the eighth cranial nerve (Level P1.0, see fig. 2.2). Continued overleaf.
Anti-MenB immunoreactivity was intense throughout most of the medulla at E17 and E19 (fig. 2.32a). At P1 and P7, most of the medulla was non-reactive with anti-MenB, although some labelling was detected in ventral its parts (fig. 2.32b). The nucleus nervi glossopharyngei et nucleus motorius dorsalis nervi vagi (nIX-X) was intensely reactive with anti-MenB at all time-points from E17 to P7 inclusive. The glia limitans was non-reactive with anti-MenB at P1 and P7 (fig. 2.32b).

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**Fig. 2.31. continued.** (a) Coronal sketch showing the location (boxed) of images presented in b, e, g and h. (b) Coronal view at E13. (c) Sagittal view of the nucleus magnocellularis (mcc) and nucleus laminaris (La) at E13. (d) Immunofluorescence image of a sagittal section neighbouring c viewed with fluorescence optics for AMCA (showing labelling for GalC). The dotted line in d delimits the La. (e) Coronal view at E17. The asterisk indicates the crossed dorsal cochleal tract (CTrX). (f) The same section as in e shown at higher magnification. (g) and (h) Coronal images at P1 and P7 respectively. The asterisks in h indicate two regions of ill defined anti-MenB labelling. An: nucleus angularis; Cb: cerebellum; FLM: fasciculus longitudinalis medialis; nIX: nucleus nervi glossopharyngei; VeM: nucleus vestibularis medialis. Scale bars: a = 1mm; b-h = 400 μm.
Fig. 2.32. Anti-MenB immunoreactivity at the level of the ninth and tenth cranial nerves at (a) E17 and (b) P7 (approximately at level P2.8, see fig. 2.2). Cb: cerebellum; FLM: fasciculus lonitudinalis medialis; nIX-X: nucleus nervi glossopharyngei et motorius dorsalis nervi vagi. Scale bars = 400μm.
Relative differences in anti-MenB immunoreactivity in the pons and myelencephalon are summarised in table 2.7.

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**Table 2.7.** Relative intensities of anti-MenB labelling in the pons and myelencephalon. - non-immunoreactive; +/- minimal labelling; + weak labelling; ++ moderate labelling; +++ intense labelling; nd: no data; / indicates that the structure is not differentiated. Numbers in parentheses refer to the number of replicates in which the structure was identified; n=4 unless otherwise stated. nPrV: nucleus sensorius principalis nervi trigemini; MnV: nucleus motorius nervi trigemini; An: nucleus angularis; mcc: nucleus magnocellularis; La: nucleus laminaris; VeM: nucleus vestibularis medialis; nIX-X: nucleus nervi glossopharyngei et nucleus motorius dorsalis nervi vagi.
2.4. Discussion.

2.4.1. Specificity and characteristics of immunostaining and general remarks.

The absence of any signal, other than in some blood vessels, in sections incubated in the absence of anti-MenB, indicates that the secondary antibodies, directed against anti-MenB, are not recognizing other epitopes. In immunofluorescence experiments, the absence of any reaction in sections incubated without primary antibodies indicates that all the secondary antibodies are only recognizing their appropriate primary antibodies. The absence of a signal in sections incubated with anti-MenB previously reacted with colominic acid suggests that the antiserum is not contaminated with other factors that react both with molecules in the tissue and with the secondary antibodies. The anti-MenB immunoreaction observed in the present study would, therefore, seem to reflect the presence of PSA. Furthermore, the anti-MenB antibody employed in the present study has been widely used by other workers, none of whom reported non-specific binding (for example, Bonfanti et al., 1992; Rousselot and Nottebohm, 1995). PSA associated with the Na$^+$ channel (Zuber et al., 1992) does not seem to be recognized by the anti-MenB antibody used in the present study (personal communication from G. Rougon cited in Bonfanti et al., 1992). The anti-MenB immunoreactivity detected in the present study therefore seems to be due to polysialylated NCAM.
Chapter 2

The anti-MenB antibody used in the present investigation seems to recognise PSA chains of about 12 sialic acid residues (Rougon et al., 1986; Finne et al., 1987; Hayrinen et al., 1995). With such an unusually high degree of polymerization, it is possible that anti-MenB only recognizes PSA when it is in a particular conformation. Thus, in the present study, the presence of PSA might not be revealed if its degree of polymerization is too low and/or if it is in the wrong conformation. Therefore, the absence of an anti-MenB immunoreaction does not necessarily mean an absence of PSA. Also, an absence of an anti-MenB immunoreaction may reflect PSA levels having fallen below a detectable threshold or that epitopes have been rendered inaccessible to antibodies. Nonetheless, with due reservations, in the following discussion, an anti-MenB immunoreaction will be considered to reflect the presence of PSA. The absence of an anti-MenB immunoreaction will be considered to reflect an absence of PSA.

Because PSA is expressed at the membrane surface, it is often impossible, at the light microscope level, to be certain if an observed anti-MenB immunoreaction is associated with the cell surface or with surrounding processes (see, for example, Skoff, 1990). This problem has been clearly shown in the adult mouse cerebellum, where GFAP positive processes of Bergman glia unsheathe Purkinje cell bodies (see fig. 2 in Wilkin and Levi, 1986). In this case, although immunoreactivity for GFAP appears around the cell surfaces of Purkinje cells, GFAP is clearly not expressed at the surfaces of Purkinje cell bodies themselves (immunoreactivity for GFAP was not detected in Purkinje cell layer in the present study; the cerebellum is
discussed later). In the present study, while strong anti-MenB labelling was consistently detected around NeuN positive cell bodies (eg. fig. 2.11), only in the IO did anti-MenB form clear rings around NeuN positive cells (fig. 2.24c and d). However, the above considerations show that, even in the case of the IO, it is not certain that PSA is expressed at the surface of these cells. Furthermore, one should not conclude that neurons in areas other than the IO do not carry PSA at their cell surfaces. The difference between the IO and other areas may be due to the unusual structure of the IO that consists of a convoluted layer of cells (the isthmo-optic system is considered in section 2.4.3; for review see Clarke 1992). A clear discrimination between immunolabelling of the cell surface membrane and neuropil, as well as between neuronal and glial processes, generally requires immuno-electron microscopy (see Skoff 1990).

In the present study, in many parts of the chick brain, anti-MenB immunolabelling was observed within the cell bodies of NeuN positive cells. In most cases, it is likely that anti-MenB is recognizing PSA that has been newly synthesized by the neuron (PSA that has been internalized prior to degradation may also be recognized). However, if cells are able to direct PSA expression to specific membrane regions (discussed below in the case of retinal ganglion cells) then it remains uncertain whether freshly synthesized PSA is destined for the surface of the cell body or only its processes. The detection of PSA within the neuronal somata is therefore not conclusive evidence of PSA expression at the surface of the cell body.
Neuronal and glial processes may, in places, run adjacent to one another. Thus, at the light microscope level, even an apparent co-localization of immunoreactivities for GFAP and PSA is not conclusive evidence that GFAP positive processes express PSA themselves. In the FPL at P7, for example, one GFAP positive fibre appeared to be weakly reactive with anti-MenB (fig. 2.18g and h). However, many GFAP positive processes were clearly anti-MenB negative in the FPL. One must therefore consider the possibility that the one GFAP process that appeared to carry PSA may have been PSA negative itself but was running adjacent to a PSA positive process. This question requires clarification at the electron microscope level.

The present study has used only a small number of antibodies as markers of particular cell types. In principle, a fuller picture could be achieved by employing a larger number of probes. For example, antibodies against vimentin could be used to detect radial glia and tanycytes (Alvarez-Buylla et al., 1987, 1988). Antibodies against carbonic anhydrase II and against glutamine synthetase could be employed to recognize protoplasmic astrocytes (Linser, 1985). However, practical constraints mean that such studies could only be conducted on limited regions of the brain. More detailed studies should, in general, also be complemented with investigations at the electron microscope level.
2.4.2. Polysialylation and myelination of the fibre tracts and nerves.

The co-localization of immunoreactivities for GalC and PSA frequently observed in fibre bundles (such as the optic nerve and cerebellar white matter (figs 2.6 and 2.28)) in the present study suggests that the presence of myelin did not render PSA epitopes inaccessible to the anti-MenB antibody. However, this observation does not exclude the possibility that access of anti-MenB to PSA may be inhibited as the amount of myelin increases. For example, much myelination of the optic nerve occurs during the first week post-hatching (Arees, 1978), a period when the present study has shown that anti-MenB immunoreactivity almost disappears from the optic nerve (fig. 2.4). As the amount of myelin in the optic nerve increases, PSA may be increasingly rendered inaccessible to anti-MenB. However, this concern seems unwarranted. At E13, the roots of the oculomotor nerve were largely unlabelled with anti-MenB and were also non-reactive for GalC. This suggests that, in this region, PSA is down-regulated before the onset of myelination. Consequently, the absence of an anti-MenB immunoreaction cannot be attributed to the presence of myelin, at least in this case. Furthermore, in the SAC of the optic tectum (fig. 2.23) and in the cerebellar white matter (fig. 2.28) the intensity of anti-MenB labelling was the same in both GalC positive and GalC negative places. Taken together, these observations suggest that the presence of myelin did not interfere with the detection of PSA in the present study.
In the present study, immunoreactivity for GalC was never detected in the optic fibre layer (OFL) of the retina, even as late as P7. In conflict with the present study, other investigations have shown this layer to be myelinated even before hatching (Nakazawa et al., 1993; Ono et al., 1998; see section 2.1). Furthermore, Ono et al. (1998) detected immunoreactivity with the 04 antibody, that recognizes several epitopes including GalC (Bansal et al., 1989), as early as E14. Since GalC is expressed at the membrane surface, it would seem unlikely that, in the present study, anti-GalC would have failed to penetrate tissue to reach its epitope. It cannot be excluded that, in the chick retina, the 04 antibody used by Ono et al (1998) was recognizing epitopes other than GalC (Sommer and Schachner, 1981; Bansal et al., 1989). Furthermore, it is possible that myelin in the avian retina is, in some way, unusual. In most mammals, oligodendrocyte precursors do not cross the lamina cribrosa to enter the retina and the retina never becomes myelinated. Since the OFL of the avian retina does become myelinated, one might wonder whether some specialization of myelin may be required in order to better facilitate the passage of light through the retina to the photoreceptors. Nonetheless, it would certainly seem surprising if myelin in the chick OFL does not contain GalC. The consistent failure of the present study to detect GalC in the OFL of the chick retina, therefore remains difficult to explain.
In the present study, GalC immunoreactivity was first detected at the level of the eighth cranial nerve at E13 (fig. 2.31) (the auditory system is discussed below). No other GalC expression was observed in any other central nervous structures at that time. In the hind-brain, the presence of myelin has been reported as early as E11 (Schifferi, 1948). However, it was not the aim of the present study to produce a spatio-temporal map of myelin distribution, and it is possible that GalC expression has been missed in some areas at this and other time-points.

Some molecules that occur in myelin (possibly including myelin associated glycoprotein (MAG)) seem to be able to inhibit axon growth (Walsh and Doherty, 1996). On the basis of such findings, it might be suggested that myelination begins only after the establishment of mature neural connections. If PSA is down-regulated following the establishment of mature connections, then an inverse relation between polysialylation and myelination may be anticipated. The present study points to a down-regulation of PSA from most fibre bundles primarily during the third embryonic and first post-hatching weeks. Although the process of myelination begins and progresses mainly during this same period (Schifferi, 1948; Hartman et al., 1979; present study), the current investigation does not suggest that there is any direct relationship between myelination and PSA down-regulation. The results presented in the current study suggest that, in some fibre tracts and nerve roots, such as the FLM and roots of the third cranial nerve (fig. 2.25) (discussed below), PSA is largely down-regulated before
the onset of myelination. However, in other nerves and fibre tracts, such as the optic nerve (fig. 2.7), SAC of the optic tectum (fig. 2.23) and cerebellar white matter (fig. 2.28), immunoreactivity for GalC was detected at time-points when PSA levels were high, suggesting that myelination begins before the onset of PSA down-regulation. Furthermore, in fibres in the region of the primary auditory nuclei (discussed below) myelination and PSA down-regulation appear to proceed approximately simultaneously (fig. 2.31). Thus, the present results, not only show no inverse relationship between PSA and myelin, but also suggest that polysialylation and myelination are regulated independently. It may be worth adding that, using electron microscopy, Bartsch et al. (1990) have reported that PSA is expressed by both myelinated and unmyelinated fibres in the optic nerve of the 16 day-old mouse.

Immunoreactivity for GalC indicates the presence of mature oligodendrocytes. In regions such as the cerebellar white matter where PSA expression persisted as myelin was being laid down, it was not always clear from the present results whether PSA was carried by myelinating oligodendrocytes or only by axons. This question is worth investigating (possibly in the cerebellum) by looking at PSA expression in white matter at the electron microscope level. However, in fibre tracts where PSA was down-regulated before the onset of myelination (such as the FLM and NIII, see fig. 2.25) it is clear that oligodendrocytes are PSA negative as they start to lay down myelin.
However, in the optic nerve, some of the cells detected within PSA negative slits may be oligodendrocytes (fig. 2.5). If this suggestion is correct, then it implies that oligodendrocytes in the optic nerve down-regulate PSA before they start to form myelin (the optic nerve is considered below).
2.4.3. Polysialic acid in the visual system.

The retina, retino-tectal projections and optic tectum.

The results presented in the current investigation point to some degree of PSA down-regulation by the inner and outer nuclear layers of the retina between E9 and E13 (fig. 2.3). Using immunoblotting with anti-NCAM, Schlosshauer et al. (1984) reported PSA down-regulation by all layers of the chick retina between E5 and E10. However, the present study actually shows an increase in PSA levels in the IPL and GCL between E9 and E13. It is difficult to see how methodological differences can account for the discrepancy between the present study and the study of Schlosshauer et al. (1984). It is therefore difficult to see how these conflicting results can be sensibly reconciled.

If PSA is involved in neural plasticity, then the decrease in PSA levels detected in the present study in the outer retinal layers (ONL, OPL and INL) between E9 and P1 may correlate with the establishment of a stable cyto-architecture in those layers. The retention, and indeed augmentation, of PSA in the IPL and GCL may be required if the modulation of inter-neural connections continues during, and beyond, the first week post-hatching. A relatively prolonged period of neural plasticity in these layers may be related to the amount of time required for retinal ganglion cell (RGC) axons to form specific connections within the optic tectum (discussed below). The modulation of tectal connections might, in turn, require further modulation of connections within the retina.
In the present study, high levels of PSA expression were evident in the OFL of the retina from E3 to P7 (fig. 2.3). The SO of the optic tectum, that consists largely of axons of retinal ganglion cells (RGCs), was intensely labelled with anti-MenB at all time-points from E9 to P7 (fig. 2.20). However, the NO, CO and TrO, that carry these same RGC axons towards the brain, expressed only minimal amounts of PSA at P7 (fig. 2.4). In the SO at P7, although brain surface astrocytes may express PSA (fig. 2.22), the intensity of anti-MenB immunoreactivity in this layer was so high that it is almost certain that RGC axons express PSA when they are within the SO. Similarly, in the retinal OFL at P7, although labelling for neither GFAP nor GalC were detected in the present study, the high intensity of anti-MenB labelling suggests that PSA is being expressed by RGC axons within this layer. This suggests that, by P7, RGC axons express PSA at their membrane surfaces when they are within the retina and when they are in the optic tectum, but not when they are in the NO, CO or TrO. This differential PSA expression by different parts of the same axon might arise in a number of ways. Firstly, retinal ganglion cells may possess mechanisms that are able to direct the PSA-NCAM molecule to different regions of the membrane. Secondly, PSA-NCAM may be inserted into the axon membrane throughout its length but PSA may subsequently be cleaved by sialidases that are present in the optic nerve, chiasm and tract but absent in the retina and optic tectum. Thirdly, PSA-NCAM might only be inserted into the cell membrane in regions where specific environmental signals are present. Fourthly, PSA-NCAM may be inhibited from being inserted into the axon membrane by specific
environmental signals that are present in the optic nerve, chiasm and tract but absent in the retina and tectum. These third and fourth suggestions are not mutually exclusive and a combination of permissive and inhibitory signals may be required. In these cases, the minimal anti-MenB reactivity detected within the NO, CO and TrO may be attributable to PSA-NCAM within RGC axons that is being transported to the optic tectum. Fifthly, PSA-NCAM may be synthesised locally in the optic tectum and transferred to RGC fibres, rather than being produced in the retinal ganglion cells themselves and subsequently transported along their axons. In this fifth scenario, PSA may be synthesized by RGCs and expressed at the axon surface within the retinal OFL, but may be inhibited from being transported along axons beyond the retina. The question of how particular membrane molecules are targeted to particular regions of the cell membrane is of considerable importance. The data presented in the current study would seem to suggest that PSA-NCAM in the chick visual system may be a useful model in which to investigate this question. However, an electron microscope study to confirm PSA expression by RGC axons in the retina and optic tectum, as well as the absence of PSA in the optic nerve, is an essential starting point for such a line of enquiry.

The PSA detected in the present study in NF negative parts of the optic nerve and tract at E9 is probably carried by glial precursors (fig. 2.5). By E9, the presence of PSA positive oligodendrocyte precursors has been reported at the chiasmal end of the optic nerve (Ono et al., 1997b). In the present study, at E13, E17 and P1 some, but possibly not all, cells in the optic
nerve were PSA negative (fig. 2.5g). It is likely that these cells are glial precursors that have finished migrating and down-regulated PSA (it is unclear from the present results whether these cells are precursors of oligodendrocytes or astrocytes or both).

In the TrO, although PSA was detected beneath the pial surface up to P7, GFAP positive processes within the tract were clearly PSA negative (fig. 2.4f and g). Furthermore, GFAP positive cell bodies were not observed beneath the pial surface of the TrO (fig. 2.4f and g). It therefore remains uncertain which elements of the TrO are expressing PSA. The most likely explanation would seem to be that glial cells beneath the pial surface of the TrO express PSA but have not yet started to express GFAP.

In the present study, GFAP positive processes were not detected in the optic nerve earlier than P7. Even at this stage, only a tiny amount of GFAP was detected at the chiasmal end, but not the retinal end, of the optic nerve. The present data therefore tend to suggest that GFAP first appears in the optic tract and starts to appear later at the chiasmal end of the optic nerve, and later still at the retinal end of the optic nerve. However, Ono et al. (1999) have reported the presence of GFAP positive processes at the retinal end of the optic nerve at around E14, with large numbers of GFAP positive processes detected throughout the optic nerve at E18. The data obtained in the present study therefore conflict with the results of Ono et al. (1999) not only in the time-course of the first appearance of GFAP but also in the direction of that appearance (ie. from the brain end to the retinal end of the optic nerve rather than vice versa). Since GFAP is an intracellular molecule, it may be
undetected by immunocytochemical methods if the cell membrane is not sufficiently permeabilized. However, the detection of GFAP in the optic tract in the present study suggests that this is unlikely to have been a problem in the current investigation. It cannot be excluded that differences between the results obtained in the present study and those of Ono et al. (1999) may arise from differences in the strain of chick. Nonetheless, the absence of immunoreactivity for GFAP in the optic nerve in the present study must be questioned.

PSA is expressed by astrocytes in the optic nerve of adult mice (Bartsch et al., 1990). Whether PSA is expressed by astrocytes in the optic nerve of the adult chicken is unknown. The present study has shown that GFAP positive astrocytes in the optic tract are PSA negative at P7. Furthermore, the almost complete loss of PSA from the chick optic nerve detected in the present study by P7, indicates that glial cells in the optic nerve are PSA negative by this stage. Thus, if PSA is expressed in the optic nerve of the adult chicken, that would indicate a de novo upregulation rather than a retention of PSA. This question is worth investigating by carrying out immunocytochemical studies at the light and electron microscope levels in the adult chicken.

The present study points to an increase in PSA levels in the SGFS and SGC of the optic tectum between E9 and E13 (fig. 2.20). Since RCG axons start to penetrate these layers from about E8 onwards (Rager and von Oeynhausen, 1979) this increase in PSA levels may be associated with the growing, branching and arborization of RGC axons within the SGFS. Enhanced PSA expression may also be required by tectal cells
as they establish synapses and other relationships with ingrowing RGC fibres.

In the present study, high levels of PSA expression were detected in the SO, SGFC and SGC as late as one week post-hatching (fig. 2.20). Thanos and Bonhoeffer (1987) have reported that RGC axons are still branching in the central region of the SO of the optic tectum between E16 and E18. Since RGC axons first invade and arborize within the SGFS in this central region, it is likely that branching within the SO continues in other areas later than E18. Furthermore, since the generation of retinal ganglion cells continues up to E12 (Kahn, 1973), some of their axons may still be arriving at the posterior SO close to the time of hatching. Arborization of these axons within the SGFS would therefore be expected to continue for some time after hatching. Furthermore, it is possible that tectal connectivity patterns may be modified by visual experience. This plasticity may require PSA. The present results are consistent with the view that neural connectivity within the optic tectum continues to develop during the first week after hatching and that PSA is involved in this process.
Central projections of the tecto-fugal system.

Birds possess two visual systems: the tecto-fugal and thalamo-fugal pathways that are considered to be equivalent to the mammalian extra-geniculate and geniculo-striate systems, respectively (Deng and Rogers, 1998a). (The thalamo-fugal system is discussed below and the retino-tectal projections, as well as the tectum itself, that comprise the first part of the tecto-fugal system, were considered above.) From the optic tectum, cells of the SGC, send projections, both directly and via the pre-tectal nuclei, to both the contralateral and ipsilateral ROT and T (Ehrlich and Mark, 1984; Deng and Rogers, 1998b). From these nuclei, fibres ascend to the ipsilateral ectostriatum (E) of the telencephalon. The present investigation shows that PSA was lost from the E between E17 and P7 (fig. 2.12) and was largely down-regulated from the ROT and T between E19 and P7 (fig. 2.16). PSA expression in the pre-tectal nuclei remained moderate from E17 to P7 (fig. 2.16). It is immediately noticeable that the "direction" of PSA down-regulation in the tecto-fugal system is from the higher to the lower centres, with the SO, SGFC and SGC (discussed above) still intensely labelled at P7, almost a week after PSA has disappeared from the E. Intuitively, one would expect PSA down-regulation to occur in the opposite direction, with "fine-tuning" of neural connections occurring in higher centres, associated with integration with other inputs, after a stable morphology has been established more peripherally. On the basis of the data obtained in the present study, the proposed
function of PSA in neural plasticity suggests that a stable cyto-architecture may be established in the E as early as E17 (PSA down-regulation occurring subsequent to this). That is, at a time when retino-tectal axons are still growing and tectal connections are still being formed (Thanos and Bonhoeffer, 1987; discussed above). The present study therefore suggests that the establishment of mature connectivity patterns within some regions of the visual system (such as the E) are independent of the establishment of mature connections within other areas (such as the optic tectum).
The thalamo-fugal system.

In the thalamo-fugal system, retinal ganglion cell axons synapse in the DLA. From the DLA, fibres ascend via the FPL (the lateral forebrain bundle) to the ipsilateral visual Wulst and, via the supraoptic decussation (SOD) and FPL, to the contralateral visual Wulst (Boxer and Stanford, 1985). In contrast to the tecto-fugal system, there was no indication of PSA down-regulation by diencephalic nuclei of the thalamo-fugal visual system or by the Wulst (figs. 2.9 and 2.16). If PSA is involved in neural plasticity, the detection of PSA in the DLA and HA as late as P7 suggests that the modulation of intercellular relationships is still continuing in these regions one week after hatching.

In the male chick, between about P5 and P12, there is a structural asymmetry in the thalamo-fugal system, with the left DLA (that receives projections from the right eye) having a stronger connection with the forebrain than the right DLA (that receives projections from the left eye) (Adret and Rogers, 1989). At P21, this asymmetry is no longer present. This implies either a loss of some connections from the left diencephalon to the visual Wulst and/or a growth of connections from the right diencephalon to the visual Wulst. If PSA is required for the modification of thalamo-fugal interconnections then PSA expression in this system may be expected to persist for 2 to 3 weeks after hatching. The data presented in the present study are consistent with this suggestion. Furthermore, some of the PSA positive strips
detected in the FPL as late as P7, may be fibres of the thalamo-fugal system that are either still growing or are degenerating.
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The isthmo-optic system.

Cell death in the nucleus isthmo-opticus (IO) occurs mainly between about E13 and E18 (Clark et al., 1976; for review see Clarke, 1992). The results obtained in the present study show moderate levels of PSA expression in the IO up to P1, with reductions occurring between P1 and P7 (fig. 2.24). Cells of the IO probably rely for neurotrophic support, mainly on brain derived neurotrophic factor (BDNF) in the contralateral retina (von Bartheld and Johnson, 2001). However, it is likely that local and afferent sources of neurotrophic factors may also be important (see, for example, von Bartheld, 1996; Herzog and von Bartheld, 1998.) If PSA facilitates access by neurotrophins to cell surface receptors (Yang et al., 1995), then its expression by cells of the IO during the period of cell death may enhance neurotrophic support for cells that carry it (Vutskits et al., 2001). However, the present study shows that PSA was expressed in the IO after the cell death period, suggesting that PSA may be required for further modulation of inter-neural connections. Numerous studies have investigated cell death and its regulation in the IO (Clarke, 1992). The IO would therefore seem to be a good system in which to investigate a possible role for PSA in the regulation of cell death.
2.4.4. Polysialic acid in the auditory system.

The present study shows that PSA is down-regulated by the mcc and La a few days after the end of the cell death period (PSA down-regulation occurring between E13 and E17 in the mcc, and between E13 and E19 in the La)(fig. 2.31). That the La still expresses some PSA at E17, while the mcc is immuno-negative at this time, may be related to the earlier cessation of cell death in the latter nucleus (Rubel et al., 1976; see section 2.1.4). As suggested above in the case of the IO, PSA may facilitate access by neurotrophins, such as BDNF, to their cell surface receptors (see Yang et al., 1995; Muller et al., 2000; Vutskits et al., 2001). The results presented in the current study are consistent with this view. However, while the IO retains PSA for some time after the end of the cell death period, no such prolonged period of PSA expression was detected in the mcc and La. A possible interpretation of this difference, is that while the mcc and La have established a stable cyto-architecture by the end of the cell death period, further re-modelling continues in the IO.

In the present study, at E13, fibres in the region of the mcc and La already showed signs of PSA down-regulation (fig. 2.31a-c). These fibres include the CTrU that connects the mcc and ipsilateral La (Parks and Rubel, 1975). By E17, both the CTrU and the CTrX, that connects the contralateral mcc and La, were PSA negative. This suggests that connections between the brain stem auditory nuclei may have become stabilized before E17 and, possibly, as early as E13.
The An and La send ipsilateral and contralateral projections to the MLd in the midbrain, a region that is considered to be homologous to the mammalian inferior colliculus (Conlee and Parks, 1986). The present study has shown that PSA is lost from the MLd between about E19 and P1 (fig. 2.21). This is about 6 days after PSA down-regulation in the mcc, and about 4 days after PSA down-regulation in the La and An. The main auditory region in the diencephalon is the OV, and in telencephalon the main auditory area is the field L of the neostriatum. In the present study, both of these areas continued to show moderate PSA immunoreactivity at P7. Thus, in the auditory system, PSA seems to be down-regulated in the following sequence: (1), in the mcc, a primary hind-brain auditory nucleus; (2), in the An, also a primary auditory nucleus, and the La that receives direct projections from the mcc and An; (3), in the MLd in the mid-brain. In the auditory regions of the diencephalon and telencephalon PSA expression persists for at least a week after it has been lost from the MLd. That the An retains PSA for a little longer than the mcc may be because the An, unlike the mcc, sends projections to the midbrain (Conlee and Parks, 1986). The An may, therefore, need to synthesize PSA while its fibres are starting to make connections in the MLd. As remarked above in the case of the visual system, one would expect PSA expression to persist longer in higher centres where neural circuitry may be expected to undergo a longer period of modification. The present study shows that, in the auditory system, unlike the visual system, the PSA expression pattern is broadly in keeping with this suggestion.
2.4.4. The telencephalon.

The PSA down-regulation detected during the period examined in the present study in the Hp, Tn, E and much of the paleostriatal complex (excluding the LPO, discussed below) suggests that a stable cyto-architecture may have been established in these areas, certainly by P7. Conversely, the high levels of PSA expression maintained in the Wulst, N, LPO and A up to P7 suggests that the neural circuitry may still be undergoing modifications in these areas.

Throughout the period examined in the present study, while PSA levels decreased in much of the HV, there was some indication that slightly higher levels of PSA expression were maintained in the IMHV (fig. 2.10; table 2.3). The IMHV is an area known to be important in passive avoidance learning (Kossut and Rose, 1984; Patterson et al., 1990; Rose 1991; Chapter 3), sickness-conditioned learning (Barber et al., 1999) and imprinting (McCabe et al., 1981; 1982). In the rat, PSA is retained in the hippocampus into adulthood (Bonfanti et al., 1992; Seki and Arai, 1993), where it may be important in the late stages of memory formation (Doyle et al., 1992a; see chapter 3). The retention of high levels of expression at P7 in the chick IMHV may be related to the neural plasticity that occurs during memory formation. However, the present study has shown that PSA is still widely expressed in the telencephalon at P7, and further down-regulation throughout this region may be anticipated before a mature expression
pattern is established. One would certainly be interested to see if PSA is expressed in the IMHV of the adult chicken.

Both the LPO, that is also implicated in passive avoidance learning (Kossut and Rose, 1984; Gilbert et al., 1991; Rose, 1991), and the archistriatum, that has been implicated in both passive avoidance learning (Lowndes and Davies, 1994) and imprinting (Lowndes et al., 1994) expressed high levels of PSA throughout the period examined in the present study (figs. 2.13 and 2.14). Some of the PSA expressed in the LPO and archistriatum may function in memory formation. As with the IMHV, one would be interested to know if the LPO and archistriatum express PSA in the adult chicken. However, in the IMHV, LPO and archistriatum, it would seem likely that only a small proportion of the PSA present in these areas is concerned with memory-related plasticity. A possible role for PSA and its synthesis during passive avoidance learning is investigated in chapter 3.
In the glia limitans, in the telencephalon, co-localization of immunoreactivities with anti-GFAP and anti-MenB within the somata of brain surface cells supplied convincing evidence that brain surface astrocytes synthesize PSA (fig. 2.15). However, the considerations raised in section 2.4.1 in relation to the co-localization of labelling with anti-NeuN and anti-MenB apply to GFAP positive cell bodies. PSA synthesis by a particular astrocyte does not imply that all the processes of that cell carry PSA or that PSA is expressed at the surface of the cell body. Indeed, at the brain surface of the olfactory bulb in 60 day-old mice, Miragall et al. (1988) have reported PSA expression in areas of glia-glia and axon-glia contact, but not in areas where glia contact the basement membrane. Also, although PSA is expressed by astrocytes in the optic nerve of 16 day old mice (Bartsch et al., 1990), it seems to be absent between the basal lamina and astrocyte endfeet (the visual system is discussed below). Whether PSA is restricted to particular regions of the astrocyte membrane in the glia limitans of the chick telencephalon is unclear from the present results.

In the present study, although PSA was down-regulated from the Tn, immunoreactivity for PSA was seen to persist beneath the pial surface. At the brain surface in the region of the Tn from E13 to P7, although the present study has shown that PSA is synthesized by GFAP positive cells, PSA is not confined to brain surface astrocytes and their processes (see the triangle in fig. 2.15). It seems likely that neuronal processes in the region of the glia limitans, at least in this area, also continue to express PSA. Addressing this question will require further work at the electron microscope level.
In the brain of the adult canary, Rousselot and Nottebohm (1995) have reported the presence of PSA at all brain surfaces. Whether PSA is expressed at the brain surface of the adult chicken is unknown. This question is worth investigating by extending the current study to look at PSA expression in the adult chicken.
2.4.6. The diencephalon.

The only diencephalic nuclei that displayed significant PSA down-regulation were the ROT, T (discussed in section 2.4.3) and ALA. PSA down-regulation by the latter nucleus is probably associated with PSA down-regulation by the AL (see below).

The PSA detected in NF negative parts of the CA and AL at E13 is presumably carried by glia or their precursors (fig. 2.18c and d and fig. 2.19d and e) (glial migration and differentiation in these fibre tracts has not, to the best of my knowledge, been investigated, the degree of differentiation undergone by these cells at E13 is therefore uncertain). By P7, the NF negative parts of the CA, that presumably contains glial cell bodies, were PSA negative (fig. 2.19f and g). Immunofluorescence data at time-points between E13 and P7 were not collected. In the FPL at P7, most, possibly all, GFAP positive processes were PSA negative (see fig. 2.18g and h; see also the considerations raised in section 2.4.1). Thus, although the present results point to a down-regulation of PSA by glia within diencephalic fibre tracts between E13 and P7, it remains uncertain when precisely, during this time period, PSA is down-regulated. While the collection of additional data to clarify this matter would be welcome, this question would be more fruitfully addressed as part of more detailed studies that go beyond the scope of the present work. However, because of the inaccessibility of central fibre tracts and the lack of other studies on their glial elements, other regions, such as the optic nerve, are likely to be more useful systems to investigate.
The disappearance of most of the PSA from the CA, AL and FPL between P1 and P7 is certainly attributable to PSA down-regulation by axons. However, the detection of small amounts of PSA in NF positive parts of the CA at P7 indicates that a few axons within the CA express PSA at this stage (fig. 2.19f and g). Similarly in the FPL, the PSA positive strips detected at P1 and P7 are probably PSA expressing axons (fig. 2.18b). It is possible that a small proportion of axons within these fibre tracts are still growing at P7 and that this growth requires PSA. Thus, while most fibres have established stable connections and down-regulated PSA, a few growing fibres stand out as PSA positive. The source and target of these fibres is uncertain from the present data, although some fibres in the FPL may form connections of the thalamo-fugal visual system (discussed in section 2.4.3). However, a question mark should be raised regarding the above suggestion. In the CA at P7, co-localization of anti-MenB and NF with GalC suggests that PSA positive axons may also be myelinated (arrowheads in fig. 2.19g and h). As remarked in section 2.4.2, myelin seems to contain molecules that are inhibitory to axon growth (see Walsh and Doherty, 1996). If this is the case, then it suggests that PSA carried by myelinated axons may serve a function unrelated to axon growth. What such a function might be is unclear.
2.4.7. The mesencephalon.

The TO and IO have already been discussed in connection with the visual system (section 2.4.3) and need not be considered further here.

In the present study, at E13 and at all later time-points that were investigated, the roots of the oculomotor nerve were largely PSA negative but contained several immunoreactive strips (fig. 2.25). While many of these strips may be surrounding tissue through which oculomotor fibres are passing, at E17 and E19 the co-localization of labelling with anti-NF and anti-MenB suggests that PSA is carried by some nerve fibres at least at these time-points (fig. 2.25c and d). That fibres reactive with both anti-NF and anti-MenB were not detected at other time-points indicates either that such fibres are absent at other time-points or that they were missed in the present study. On the basis of the present results, one should not conclude that PSA positive fibres within the oculomotor nerve are only present at E17 and E19 (one would certainly suspect that they are present at E13, see below). This question might be clarified as part of a more detailed study that should include the use of tracer dyes in conjunction with immunocytochemistry to identify the source and target of PSA positive fibres.
The present study suggests that PSA has been down-regulated by the majority of fibres within the oculomotor nerve before E13, but that it continues to be expressed by some fibres certainly as late as E19. Most fibres within the oculomotor nerve are somatic motor axons that arise from the OMdI, OMdm and OMv, and supply the extra-ocular eye muscles (Bubien-Waluszewska, 1981). Based on the results obtained in the present study, it seems likely that most, possibly all, of these axons down-regulate PSA before E13. The oculomotor nerve also contains parasympathetic fibres that arise from the EW and synapse in the ciliary ganglion (CG). Motor neurons of the CG send axons that innervate the ciliary and iris muscles, as well as blood vessels in the eye. Compared with the somatic motor axons, parasympathetic projections therefore encounter an additional junction point in the CG. One might therefore expect that these parasympathetic projections may require more time to achieve a mature configuration. In the present study, the EW displayed strong immunoreactivity for PSA as late as P1, more than 4 days after PSA was down-regulated in the other oculomotor nuclei. It is therefore possible that some of the PSA positive fibres observed within the roots of the oculomotor nerve at E17 and E19 is expressed by axons of EW neurons. Some proprioceptive fibres from the eye muscles also travel in the oculomotor nerve and probably terminate in the nucleus mesencephalicus nervi trigemini (nVM) (Bubien-Waluszewska, 1981). The integration of proprioceptive information may require a prolonged period of re-modelling of the neural circuitry for some time after hatching. This plasticity may require PSA. In line with this suggestion, one may
speculate that some of the PSA detected in the roots of the oculomotor nerve is expressed by afferents to the nMV and is concerned with proprioception.

The above suggestions are not mutually exclusive, and the PSA expression detected in the roots of the oculomotor nerve may be explained by a combination of the factors discussed above. Furthermore, it is possible that PSA is carried by different fibres at different time-points. Indeed, *de novo* upregulation of PSA by some oculomotor fibres, including somatic motor axons, cannot be excluded on the basis of the present data.

The cresyl violet-stained cells detected in PSA negative parts of the oculomotor nerve roots as early as E13 are probably glia within the nerve itself (fig. 2.25e and f). This suggestion implies that glia within the oculomotor nerve roots have already down-regulated PSA by E13 (this same conclusion was drawn in relation to glia in the optic nerve in section 2.4.3). The cresyl violet-stained cells in PSA positive strips may be cells in the surrounding tissue. However, it cannot be excluded that some of these cells are PSA positive glia within the oculomotor nerve that might, possibly, still be migrating.
2.4.8. The cerebellum.

The present study has shown that PSA is very rapidly down-regulated by the chick cerebellum during the few days prior to hatching (figs. 2.27; 2.28).

At E13, the Purkinje cell layer, including the somata of the Purkinje cells themselves, was very intensely stained for PSA (fig. 2.28b). The detection of PSA within Purkinje cell bodies suggests that these cells are actively synthesizing PSA at that stage. By E17 this layer gave a moderate reaction, and by E19 appeared as an almost non-reactive strip between the immuno-positive molecular and granular layers (fig. 2.28c and d). At E17, PSA was not observed within Purkinje cell bodies suggesting that these cells stop synthesizing PSA between E13 and E17. The PSA detected apparently at the Purkinje cell surfaces at E17 will have been synthesized by Purkinje cells prior to E17 and/or may be carried by Bergman glia (Wilkin and Levi, 1986; see also section 2.4.1) By about E13, Purkinje cell arborization within the EGL is maximal (see Romanoff, 1960). Thus, the present study suggests that Purkinje cells stop synthesizing PSA after they have established connections with the processes of pre-migratory and migratory cells within the EGL/ML. The present study also suggests that PSA down-regulation by Purkinje cells begins at the same time as granule cell precursors are migrating through the Purkinje cell layer (see section 2.1.4).

The PSA detected in the EGL at E13 is probably carried, for the most part, by granule cell precursors prior to their inward migration (fig. 2.28b). The continued expression of PSA in the
Chapter 2

ML at E17 suggests that cells that have migrated from this region retain PSA on their processes (within the ML) for some time after inward migration. Synaptic contacts may still be forming in the ML at E17 and this process may require PSA. The present results also imply that granule cell precursors also express PSA during their migration from the EGL to the IGL. This cell migration may require PSA (Wang et al., 1994; see sections 2.1.2). However, several studies have suggested that cells do not need to express PSA in order to migrate successfully (Hu et al., 1996; Ono et al., 1997b; Murakami et al., 1998). The question of whether cerebellar cells require PSA for successful migration should be tested by looking at the consequences of PSA cleavage by endo-N in the cerebellum.

Once having migrated into the IGL, granule cells elaborate processes within that layer. In the IGL, the present study shows that PSA is down-regulated relatively soon after the arrival of inwardly migrating cells. This suggests that stable intercellular connections may be established relatively quickly. Given the rapid development of the chick cerebellum, this conclusion is not surprising.
PSA down-regulation in the mouse cerebellum shows a similar correlation with developmental events as reported here in the chick (Hekmat et al., 1990). In the mouse, granule cell migration occurs between about P4 and P15, with PSA being gradually down-regulated between about P8 and P21, and becoming completely undetectable by P30 (Hekmat et al., 1990). This more protracted down-regulation presumably reflects the slower development of the murine cerebellum. One notable difference between the findings of Hekmat et al. (1990) and those reported here is that, in the former study, PSA down-regulation in the Purkinje cell layer occurred simultaneously with down-regulation in the other layers.

Although PSA expression persisted in the glia limitans up to P7 (fig. 2.28), the absence of GFAP labelling makes it impossible to be certain if PSA is carried by surface astrocytes (the glia limitans is discussed in the case of the telencephalon in section 2.4.5). However, it certainly seems likely that the brain surface reaction is attributable to PSA expression by surface astrocytes. Whether PSA is carried by neuronal processes at the cerebellar surface as late as P7 is unclear from the present data.
2.4.9. The pons and medulla oblongata.

The present study has shown that, by P7, PSA has been down-regulated throughout most of the pons and medulla (figs. 2.30; 2.31; 2.32). Much of this down-regulation is certainly associated with a loss of PSA by nerve fibres. In the medulla, most of the PSA down-regulation detected by P1 is certainly attributable to a loss of PSA from fibres that are descending into or ascending from the spinal cord.

PSA was strongly expressed in the VeM up to P7 (fig. 2.31). It is possible that "fine-tuning" of the vestibular system may require on-going behaviour and experience in the post-hatching period. PSA in the VeM might, therefore, be correlated with morphoplastic events that are associated with vestibular learning and maturation. Furthermore, the VeM receives inputs from cerebellar Purkinje cells (Wold, 1981). PSA expression in the VeM may, therefore, be associated with the maturation of output systems from the cerebellum and/or their integration with vestibular inputs.

The reasons for the retention of PSA by the nIX-X as late as P7, as well as the PSA up-regulation by the FU between P1 and P7 are unclear.

Although PSA expression by surface astrocytes persisted in many areas (discussed in the preceding sections), this was not the case in the medulla oblongata (see fig. 2.32). In the medulla at P7, GFAP positive cells of the glia limitans were PSA negative. The reason for this difference between the medulla and more rostral structures is unclear. Speculation is difficult
since the function of PSA expression by brain surface astrocytes is itself uncertain.

2.4.10. Summary and conclusions.

The present study shows that, in general, in the neural tube during the first week of embryological development, PSA levels tended to be moderate in the ventricular zones and higher in the mantle and marginal zones (the roof of the diencephalon seems to be exceptional in that the VZ was intensely reactive with anti-MenB at E3 and E5, fig. 2.16a-b). While the VZ consists largely of proliferating cells, the MnZ contains many post-mitotic neuroblasts and the MZ is comprised mainly of neuroblast processes. The present study therefore suggests that cells increase PSA expression as they differentiate into process bearing neuroblasts. It is possible that PSA expression at the process surface facilitates the movement and growth of neuroblast processes. From their very genesis, these processes grow in very specific directions (Windle and Austin, 1936). If PSA facilitates access of extra-cellular molecules to their surface receptors (Yang et al., 1996; Vutskits et al., 2001), then PSA may enable growing processes to respond to extra-cellular signals and grow in appropriate directions.

The present study suggests that, in the brain as a whole, PSA levels increase between around E5 and E9 and remain relatively high until around E13. In keeping with this finding, Sevigny et al. (1998) have reported that, in the embryonic chick brain, PSA synthase activity rises sharply between E5 and E8 and then remains relatively constant until E12. During
the third embryonic week and the first week post-hatching, the present study shows that PSA was down-regulated in many brain regions. Sevigny et al. (1998) have reported that, in the embryonic chick brain, PSA synthase activity declines by around 50% between E12 and E15, then declines by about another 50% between E15 and E18 (i.e. a fall of about 75% between E12 and E18). While the results obtained in the current investigation would certainly predict decreases in PSA synthase activity between E12 and E18, the magnitude of the decrease detected by Sevigny et al. (1998) is surprisingly large. However, during the period from E13 to the time of hatching, the present study has shown widespread PSA down-regulation by fibre tracts. The overall amount of PSA in PSA positive fibre bundles may be extremely high when compared with brain nuclei because of the proportionately larger amount of cell membrane present in the fibre bundles. The loss of PSA from fibre tracts detected in the present study may, therefore, explain much of the reduction in PSA synthase activity detected by Sevigny et al. (1998).

In many discrete brain regions where PSA down-regulation was detected in the present study (e.g., the Imc and Ipc), the decrease in PSA levels occurred evenly and simultaneously across the entire region. Furthermore, in all intensely labelled nuclei, cell-sized anti-MenB negative gaps were never observed with high magnification immunofluorescence. This implies that, prior to PSA down-regulation, PSA is synthesized by all cell types within that nucleus. These observations further imply that PSA is simultaneously down-regulation by all cell types, and all of their processes, within most nuclei where PSA down-
regulation was observed. This suggests that in most brain nuclei, PSA expression may be regulated at the regional level. However, no evidence for such regional regulation was found in the fibre tracts (discussed in the preceding sections). Indeed, the detection of PSA positive fibres within largely PSA negative fibre bundles was a common finding of the present study. This suggests that PSA expression is not regulated at the regional level in nerve fibres tracts.
CHAPTER THREE

AN INVESTIGATION INTO A POSSIBLE ROLE FOR POLYSIALIC ACID AND SIALYLTANSFERASE ACTIVITY FOLLOWING PASSIVE AVOIDANCE TRAINING IN THE DAY OLD CHICK.

3.1. Introduction.

3.1.1. Background.

The one trial passive avoidance task was introduced by Cherkin in 1969 and takes advantage of the fact that day old chicks peck spontaneously at small conspicuous objects in their field of view. If a bead coated in an unpleasant tasting substance, such as methylanthranilate (M), is offered the chick pecks once, shakes its head in a characteristic "disgust" response, and avoids a similar but dry bead when offered subsequently. The spontaneity and comparative simplicity of the behaviour, as well as the ready acquisition of the avoidance response, makes it an appealing model for the study of the biochemical, temporal and morphological processes associated with learning. Chicks that have pecked a bitter bead and subsequently avoid a dry bead show a number of biochemical, physiological and morphological differences from control chicks that have pecked
a water coated bead, or birds that have pecked a bitter bead but have been rendered amnesic (for a recent review see Rose 2000).

3.1.2. Localisation of specific brain regions.

Perhaps the most basic question that may be asked about the biological basis of memory is where, within the brain, it happens. Two-deoxyglucose (2-DG) autoradiography identified the IMHV, the PA and the LPO, as areas of potential interest following passive avoidance training (Kossut and Rose, 1984). To maximise the chances of finding areas of potential interest, Kossut and Rose gave 2-DG before training. It was not, therefore, possible to discriminate between the concomitants and correlates of learning. This distinction was made in a subsequent study (Rose and Csillag, 1985) which confirmed the likely importance of the IMHV and LPO but eliminated the PA. Presumably the PA was involved in the behaviour and/or experience of pecking and/or tasting the bitter bead, but not in subsequent memory processing. Interestingly, in the adult canary, it is the LPO, HV and parts of the HA that are the main recipients of neurons freshly generated in the ventricular zones (Alvarez-Buylla and Nottebohm, 1988). An immediate comparison, therefore, presents itself with the mammalian hippocampus, an area known to be important in both spatial (Becker et al., 1996; Murphy et al., 1996) and avoidance learning (Doyle et al., 1992a), and also a region that displays ongoing neurogenesis into adulthood (Kaplan and Hinds, 1977).

If an area is necessary for memory formation, consolidation and/or subsequent expression then lesions to that area should
be amnestic depending on when, in relation to training, the lesion is made (Rose, 1981). If chicks are given bilateral or left IMHV lesions on the day of hatching and are trained the following day they show the disgust response and immediate avoidance but are amnesic when tested 3 or more hours later (Patterson et al., 1990). Pre training right IMHV lesions are without effect. If bilateral or left IMHV lesions are made an hour or more after training the bird shows avoidance on test 24 hours later (Patterson et al., 1990). These experiments point to the initial importance of the left IMHV but further show that the memory trace seems to migrate from that area and that the IMHV is not needed for the expression of avoidance (see Rose 1991, 1995).

The LPO would seem a likely site for this relocated engram. Indeed, bilateral LPO lesions, but not unilateral lesions to either side, made 1 hour after training are amnestic (Gilbert et al., 1991). Pre-training LPO lesions, however, are without effect (Gilbert et al., 1991). One interpretation of this is that the memory trace moves from the IMHV to the LPO. With the LPO removed the trace either remains trapped in the IMHV or migrates elsewhere (Rose, 1991). This memory flow seems to be from the left IMHV to right IMHV and thence to the LPO, since if pre-training LPO lesions are made followed by post-training right IMHV lesions the chick is amnesic (Gilbert et al., 1991). Left IMHV post-training lesions are without effect if the LPO has been ablated pre-training (Gilbert et al., 1991). The trace thus seems to flow from left IMHV to right IMHV but, with the LPO removed, cannot become established elsewhere. This conclusion is supported by the result that pre-training
right IMHV lesions followed by post-training LPO lesions, which are otherwise amnestic, do not produce amnesia (Gilbert et al., 1991). This suggests that, with its normal route blocked, the memory trace simply remains in the left IMHV.

While these results point to the dynamic fluidity of developing memory, the brain is still more plastic. If the right IMHV is lesioned pre-training, a post-training left IMHV lesion is not amnestic (Gilbert et al., 1991). In this case the memory cannot simply have remained in the left IMHV, it must have moved elsewhere. Interestingly, bilateral pre-training LPO lesions followed by post-training bilateral IMHV lesions result in amnesia (Gilbert et al., 1991). With the LPO removed the trace cannot escape from the right IMHV. Plasticity has its limits. Conceivably, the developing memory is more flexible when in the left IMHV than when it reaches the right IMHV.

This flow of memory seems to be more than a straightforward engram relocation. If chicks are trained on a bitter yellow bead and subsequently offered, sequentially, both a yellow and a blue bead they peck only the latter. However, if post-training bilateral IMHV lesions are made the chick avoids both (Patterson and Rose, 1992). Thus, cues relating to colour seem to have been lost while those relating to form are still available. What might be regarded as a single memory is not a unitary thing. It has many aspects: size, shape, colour and so on. While cues relating to colour may remain in the left IMHV, those relating to form migrate to the LPO. Different learning experiences may therefore leave a memory trace in the same area where they share common features (eg., they both possess colour), whereas some experiences may possess aspects that
have no parallels in another experience and may leave traces in additional areas.

The results of these lesioning experiments thus point, on the one hand, to elements of both localised and distributed storage and, on the other hand, to elements of both fluidity in the developing memory as well as the existence of stable loci. They also back up the results of the 2-DG autoradiography experiments (Kossut and Rose, 1984; Rose and Csillag, 1985) in pointing to the IMHV and LPO as sites of engram development following passive avoidance training.
3.1.3. Models of memory development.

Memory formation is not an instantaneous event but a biological progression - a cascade of biochemical interactions. Furthermore, this progression is not a purely smooth continuous process, but seems to be divisible into discrete temporal components (Gibbs and Ng, 1976; 1977; 1979). This conclusion rests on the finding that blockage of particular molecules or processes causes amnesia at certain times, though not at others, while different agents are amnestic at other time points (see section 3.1.4). Early, short term processes are believed to depend on increased transmitter release, receptor up-regulation and release of retrograde messengers resulting in enhanced synaptic efficacy. Later, longer term processes require protein synthesis and extensive structural remodelling (see Rose 1991, 1995). Gibbs and Ng (1976) proposed 3 stages: short term memory (STM), lasting for about 10 minutes after training; intermediate term memory (ITM), available from about 20 to 50 minutes post-training; and long term memory (LTM), available from 60 minutes to at least 24 hours post-training. Subsequently, they further divided ITM into a phase A and phase B (Gibbs and Ng, 1984). There does, however, seem to be some flexibility in the duration of these stages, with ITM persisting to around 70 minutes post-training in chicks trained in isolation (Gibbs and Ng, 1979).

If memory "passes through" distinct phases, or, better, it takes on different forms, the question arises as to the nature of the relationship between these phases. To a first glance, the simplest view would seem to be a sequential, linear progress in
which memory is represented in an early phase then transferred from there to the next phase (one process at a time) (see fig. 3.1a). An alternative proposal is a parallel processing model (more than one process at a time) (fig. 3.1b). In this type of model the distinct phases arise with a greater independence. Representation in a later phase is not necessarily dependent on passage through an earlier one. In this case, however, early processes, other than STM, that ultimately give rise to later phases must be envisaged: a sort of nascent-LTM.

(a) Linear processing.

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STM  ITM(A)  ITM(B)  LTM
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(b) Parallel processing.

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STM ITM(A) ITM(B) LTM
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**Fig. 3.1.** Schematic representation of 2 different perspectives on memory processing. See text for details.
3.1.4. Biological processes.

While no attempt will be made here to fit the biochemistry of passive avoidance learning to the schemes presented in the last section, it should become clear that the types of processes described do fall into different categories. Terms such as STM and LTM or shorter term processes and longer term processes will be used loosely depending only on when, in relation to training, particular events occur. Furthermore, it should be noted that this review is not intended to be exhaustive.

**Synaptic transmission.**

It has long been thought that memory formation may depend on activity-dependent changes in synaptic efficacy such that the synapse is strengthened if both pre and post synaptic cells are simultaneously active (Hebb, 1949). If this is the case the existence of some mechanism able to recognise this simultaneous activity is implied. Much attention has focused on the N-methyl-D-aspartate (NMDA) glutamate receptor. Opening of its intrinsic Ca$^{2+}$ channel requires both L-glutamate binding and relief of Mg$^{2+}$ blockage brought about by depolarisation of the post synaptic cell beyond a sufficient threshold. Its activation therefore depends on just the sort of pre and post synaptic activity that is required, enabling it to act as a molecular coincidence detector. Administration of the selective antagonist MK-801, 1 hour or 20 min before, or 5 min after, training is amnestic on test at 3 hours, suggesting that the NMDA receptor is involved as a necessary component in an early memory phase (Burchuladze and Rose, 1992). Glycine
binding to an allosteric site on the NMDA receptor also seems to be necessary (Steele and Stewart, 1993).

The most abundant L-glutamate receptor is that selectively activated by the agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Although the AMPA receptor has been implicated in producing the post synaptic depolarisation required to relieve the NMDA Mg$^{2+}$ blockage, it seems to be involved in the later stages of memory development, its maintenance and/or recall (Steele and Stewart, 1995), but not in the early stages (Burchuladze and Rose, 1992). Intracerebral injection of the selective AMPA antagonists CNQX, DNQX or NBQX are not amnestic on test at 3 hours if administered 5 min post training, but do result in amnesia on test at 6.5 hours if given at 4.5 or 5.5 hours post training (Steele and Stewart, 1995; for a review of the involvement of glutamate in passive avoidance learning see Ng et al., 1997).

Neurotransmitter involvement in memory development following passive avoidance training is not confined to glutamatergic systems. Increases in the level of forebrain muscarinic acetylcholine (ACh) receptors have been detected using quinuclidinylbenzilate (QNB) binding (Rose et al., 1980). This increase is not significant at 10 min, is maximal at around 30 min and had returned to control levels by 3 hours (Rose et al., 1980). Administration, 15 min before training, of the protein synthesis inhibitor cycloheximide or the Na$^{+}$/K$^{+}$ ATPase inhibitor ouabain abolishes the increase (Rose et al., 1980). While ouabain is amnestic on test at 30 min, there is no significant reduction in the percentage avoidance of chicks
injected with cycloheximide compared with saline injected controls. This suggests that at 30 min, memory is being sustained by processes that do not require ACh muscarinic receptors. Ligand binding has also detected decreases in nicotinic ACh receptors, while administration of the nicotinic antagonist scopolamine has been shown to be amnestic (Holscher, 1995).

GABA transmission has also been implicated in the early stages of passive avoidance learning (Clements and Bourne, 1996), and Crowe et al. (1991) have found enhanced forebrain noradrenaline levels associated with ITM(B) as well as amnesia at 30 min post training, arising from blockage of β receptors.

**Phosphorylation.**

Incubation of chick forebrain post synaptic density (PSD) and synaptic plasma membrane (SPM) proteins with (\(^{32}\)P)-ATP results in lower in vitro phosphorylation of the pre-synaptic 52 kDa protein, B-50 in M trained compared with W trained or untrained chicks (Ali et al., 1988). This may be regarded as suggesting that there are fewer sites available for phosphorylation in the M trained birds, which is taken to imply that potential sites are already occupied. That is, there is an increased *in vivo* phosphorylation following M training. The effect, first detectable around 10 min, increases with time up to about 30 min, and subsequently declines to control levels by around 6 hours (Ali et al., 1988).

B-50 is a protein kinase C (PKC) substrate. This enzyme exists in a number of isoforms. While the brain specific γ form is largely membrane bound, the α and β forms are distributed
between membrane and cytosol. It has been suggested that regulation of B-50 phosphorylation is achieved by changes in the ratio PKC(cytosolic): PKC(membrane) (Akers et al., 1986). Consistent with the hypothesis that training leads to a translocation of PKC α and/or β to the membrane, an increase in the membrane-bound, Triton-extractable form has been detected in the left IMHV of M trained chicks 30 min after training (Burchuladze et al., 1990). The phenomenon is lateralized - but the asymmetry is interesting. The proportion of membrane bound PKC is higher in the right than the left IMHV of W controls: training abolishes the asymmetry. The impression is that of the ratio in the left IMHV being in a primed, depressed state. An involvement of PKC seems to be a necessary component of memory processing since administration of the inhibitors H7 or mellitin is amnestic (Burchuladze et al., 1990).

The function of B-50 phosphorylation by PKC is unknown. However, it has been implicated in synaptic transmission, membrane conductivity, Ca$^{2+}$ influx and/or activation of the phosphatidyl inositol system presumably affecting intracellular Ca$^{2+}$ stores.

**Immediate early genes.**

Passive avoidance learning may require immediate early gene (IEG) expression. It has been suggested (Rose, 1995) that their expression, occurring around 30 min after training, is a pivotal point in the transition between relatively short term synaptic events and protein synthesis-dependent LTM.
Large increases in c-fos mRNA have been found in both the left and right IMHV and LPO 30 min. after passive avoidance training using northern blotting and in situ hybridisation (Anokhin et al., 1991). Exposure to 1 hour of activity in a rich visual environment leads to significantly enhanced levels of forebrain c-fos mRNA, especially in the IMHV, medial forebrain and cerebellum. Increases were not found in birds that had accommodated to the enriched environment for 2 days, suggesting that the novelty of the experience is most important for c-fos expression. However, these genes are very sensitive to induction by a variety of sensory stimuli, including pecking at a bitter bead. Some caution is therefore warranted.

In a subsequent experiment involving an appetitive task (Anokhin et al., 1991) 3 groups of chicks were exposed to a "pebble floor" that required them to discriminate between grains of food and inedible pebbles (a fourth group remained undisturbed in their pens throughout, acting as quiet controls). The experiment was conducted over 2 days. On day 1 the first 2 groups were exposed to a floor with no food. On day 2 food was included on the floor for group 2 but not group 1. Group 3 had food on both days. Thus, groups 1 and 3 had the same experience on day 2 as day 1, while group 2 chicks were exposed to a novel situation on day 2. The number of pecks at pebbles and/or food were recorded over an 8 min period on day 2, after which c-jun levels were estimated by northern blotting. Expression of c-jun was greater in all 3 groups than in quiet controls, but the largest difference was in group 2, that had been exposed to a novel situation on the second day, even though more pecks were recorded from group 3. This suggests
that, while sensory stimulation and behavioural interaction affect c-jun expression, some of the differences found are specifically attributable to learning.

**Protein synthesis and cell adhesion molecules.**

If long term memory formation involves synaptic remodelling, a requirement for protein synthesis in general, and CAM synthesis in particular, is suggested. Intracerebral injections of the protein synthesis inhibitor, anisomycin, are amnestic on test at 24 hours if administered between 30 min before and 1.5 hours after training, or at 4.5 hours post training. Perhaps the simplest explanation for this is that the ongoing flow of proteins to the membrane is interrupted and synaptic connections can only remain in their original format. However, anisomycin injections given between 1.5 and 4.5 hours, or at later time points, are not amnestic (Freeman et al., 1995). The inhibition is, therefore, more specific than just interruption of the flow. This suggests that memory formation following passive avoidance training involves at least 2 separate waves of protein synthesis.

The involvement of particular CAMs has been investigated by studying the amnestic affects of administering specific antibodies or recombinantly expressed fragments (Scholey et al., 1995). The logic of this is that, if LTM formation requires de-adhesion and subsequent re-adhesion in a modified connection pattern, there may be an intervening period during which CAM extracellular domains become temporarily exposed. Antibodies binding exposed epitopes at this time might be
expected to prevent subsequent re-adhesion and may, therefore, be amnestic (Rose, 1995).

Polyclonal or monoclonal anti-NCAM antibodies are amnestic on test at 24 hours if given around 5.5 hours post-training. Anti-L1 antibodies are also amnestic if given at this time point but also when administered immediately prior to training (Scholey et al., 1995). Administration of recombinantly expressed L1 fragments containing the Ig I-VI domains are amnestic if given around the time of training but not at 5.5 hours, while fragments containing the FN-III domains produce amnesia if administered at 5.5 hours but not at earlier times (Scholey et al., 1995). It has been proposed (Rose, 1995) that these differences may reflect fragments containing FN-III domains interfering with transduction mechanisms, a likely component of STM, while the Ig-domain-containing fragments affect the adhesive interactions implicated in LTM formation.

A requirement for protein synthesis and CAM function suggests a requirement for their glycosylation. Fucose is incorporated, almost exclusively, into glycoproteins rather than being metabolised via other routes. It is, therefore, well suited as a marker in glycoprotein studies. Passive avoidance training results in increased \(^{3}\text{H}\)-fucose incorporation into chick forebrain SPM glycoproteins lasting up to at least 24 hours post training (Burgoyne and Rose, 1980; Sukumar et al., 1980). This enhanced incorporation is abolished if chicks are rendered amnesic by a mild electric shock just after training, implying that increased fucosylation is a necessary component of memory formation (Rose and Harding, 1984). A large proportion of fucosylglycoproteins are located at the synaptic
membrane. SDS-PAGE showed that a pre synaptic molecule of 50 kDa and post synaptic components of 28, 33, 41, 62-80, 100-120 and 150-180 kDa are affected by training (Bullock et al., 1992). Learning-related changes in the incorporation of (3H)-fucose and (14C)-fucose at 6 and 24 hours are complex, showing both increases and decreases. It would seem, then, that enhanced fucosylation is more than just a consequence of increased protein processing.

If memory formation is fucosylation-dependent then blocking this process should lead to amnesia. 2-deoxygalactose (2-dgal) competes with galactose for incorporation into the pre terminal site of the growing carbohydrate chain but, because of its structure, prevents terminal fucosylation. (3H)-2-dgal becomes incorporated into the same glycoproteins (and only those glycoproteins) that become labelled after (14C)-fucose injection (Bullock et al., 1990). Its action therefore seems to be quite specific. If chicks are injected immediately before or up to 1 hour after passive avoidance training they are amnesic on test at 24 hours. 2-dgal is not amnestic if the injection is delayed to between about 2 and 5.5 hours but becomes so again if administered between about 5.5 and 8 hours. It is not amnestic at later time points (Scholey et al, 1993). This points to two waves of fucosylation: a result that accords with the two waves of protein synthesis reviewed above (Freeman et al., 1995).

Fucosylation requires initial fucose activation by phosphorylation and subsequent insertion of the activated sugar into the carbohydrate chain. These reactions are catalysed by fucokinase (FK) and fucosyltransferase (FT) respectively. Training has no effect on FT activity but changes
in FK have been detected (Lossner and Rose, 1983). Although the effect is only significant in the right forebrain base (a region that includes the LPO), changes in specific areas within the larger areas analysed may have been diluted by the inclusion of tissue not specifically involved in memory. It is noticeable that activity in the right forebrain base of control chicks is lower than in other areas, and that training raises activity to a level comparable with other areas of control birds (cf. PKC and morphological parameters where training also seems to abolish an asymmetry (Burchuladze et al., 1990; Patel and Stewart, 1988)).

**Morphological changes associated with passive avoidance training.**

Increases have been detected in spine density in large multipolar projection neurons of the IMHV 25 hours after training (Patel and Stewart, 1988); the effect being larger in the left than the right hemisphere. Interestingly, comparison of control birds showed an asymmetry, with spine density being higher in the right than the left hemisphere. Training abolished the asymmetry (Patel and Stewart, 1988).

Morphological changes are not confined to the number of spines. Significant increases in spine head diameter and significant decreases in mean spine stem length are reported in the left IMHV at 25 hours post-training (Patel and Stewart, 1988). Training appears to alter the relationship between mean length of the post-synaptic thickening in the left and right IMHV and increases in the number of vesicles per pre-synaptic button in the left IMHV at 24 hours post-training (Stewart et
al., 1984). These findings suggest some intriguing hemispheric effects. However, these investigations were only carried out at about 1 day after training. It would be interesting to see a time course for changes in these morphological parameters, since it cannot be excluded that, in different regions, the same processes occur at different times: hemispheric differences at a given time point may, therefore, be a reflection of the memory trace having reached different stages of its development in different regions (section 3.1.2; Rose, 1991).
3.1.5. Studies on a possible involvement in Polysialic acid during Memory formation.

Given the proposed role for PSA in neural plasticity, and its presence in the mammalian hippocampus (Seki and Arai, 1993), an area known to be important in learning, it is a reasonable hypothesis that hippocampal PSA may be involved in learning. Doyle et al. (1992a) looked for an association between learning and polysialylation using adult rats in a step down avoidance paradigm. Following injection of \(^{3}H\)-N-acetyl-mannosamine, a sialic acid precursor, increased incorporation of radioactivity was detected in the hippocampus at 12 and 24 hours following training. This increased incorporation seems to be the product of polysialylation of NCAM 180. NCAM levels themselves remained unchanged throughout. This result suggested a role for enhanced NCAM polysialylation in a late consolidation phase of memory formation.

Immunocytochemistry demonstrated that, at 12 hours after step-down avoidance training, increases in PSA expression were restricted to a distinct population of granule cells at the border of the hilus and granule cell layer (Fox et al., 1995). This is the same cell population already shown from other studies to express PSA in the adult rat (Seki and Arai, 1993). Enhanced PSA levels have also been found in the same cell population following training in a Morris water tank, a spatial learning paradigm (Murphy et al., 1996), indicating that the changes are not task-specific.
In 1996, Bekker et al. found that injections of endo-N into the rat hippocampus (that removed hippocampal PSA expression for a period of 4 days) resulted in reduced spatial learning and/or recall on the second, but not the first, day of training in a Morris water tank. This finding is important in pointing to polysialylation as a necessary component of memory processing, and not merely a relatively non-specific consequence of the training procedure. Furthermore, Becker et al. (1996) showed that administration of endo-N does not seem to impair visual discrimination or motor behaviour since endo-N treated rats find a platform in the Morris tank as quickly as controls when the platform is visible.

Increases in dendritic spine density in the hippocampus have been detected from 6 to 24 hours following passive avoidance training in the rat, with levels subsequently declining to control values by 72 hours (O'Malley et al., 1999). These changes are consistent with the selective stabilization model (Changeaux and Danchin, 1976) that suggests a stable cytoarchitecture may be established by an initial over-expression of synaptic connections followed by a subsequent selective elimination phase. If this type of model is applicable to memory formation (Doyle et al., 1992b), increases in PSA levels seem to be occurring towards the end of the period when synaptic overexpression is maximal and immediately prior to the period of synaptic selection. This suggests a role for PSA, not in the initial synaptic overexpression but, rather, in preparing/priming synapses for a subsequent selection. This view bears a parallel with the suggestion made by Seki and Rutishauser (1998) that PSA may be required for the
withdrawal of overexpressed connections in the developing hippocampus.

Although the mammalian hippocampus is an area of known, ongoing neurogenesis (Kaplan and Hinds, 1977), the generation of new neurons does not seem to be necessary for learning to occur; at least at the time when memory is developing (Fox et al., 1995). Increased PSA levels are not restricted to newly generated neurons since the number of PSA positive cells at the hilus/dentate border increases by around 30% by 24 hours post-training, whereas there is no increase in the number of cells incorporating bromo-deoxyuridine (BrdU) (Fox et al., 1995).
3.1.6. Investigations conducted in the present study.

Section 3.1.5 described a number of studies that investigated a possible role for PSA in learning in adult rats. The literature reports no comparable studies in the chick. The increased dendritic spine density detected at 24 hours post-training in both the chick (Patel and Stewart, 1988) and the rat (O'Malley et al., 1999) suggests that the timecourse of passive avoidance learning may be the similar in both species. The detection of enhanced PSA levels at 12-24 hours following step down avoidance training in the rat (Doyle et al., 1992a) therefore suggests a large time window in which to investigate PSA expression in the chick.

In principle, there are a number of ways of investigating a possible role for PSA in memory formation following passive avoidance training in the chick: [1] direct detection of differences in PSA levels between trained and untrained birds; [2] increases in the incorporation of labelled PSA precursors; [3] identification of changes in the activities of enzymes associated with its synthesis or degradation; [4] interventive measures that remove PSA or prevent its up-regulation, such as the administration of endo-N, should result in amnesia, if administered at an appropriate time or, conversely, [5] if enhanced PSA levels can be detected following passive avoidance training then rendering trained chicks amnesic by, for example, a subconvulsive shock should reduce PSA expression to control levels. The first and third of these approaches are adopted in the present study. In the first
experiment, immunocytochemistry is used to look at PSA levels in the IMHV and LPO and then, in a second set of experiments, sialyltransferase activity is assayed at several timepoints following passive avoidance training.

3.2.1. Animals and passive avoidance training.

Animals were treated as described in section 2.2.1. Day-old chicks were transferred, in pairs, to aluminium pens (20 x 25 x 20cm) illuminated overhead by red, 25W light bulbs. Chick starter crumb was sprinkled on the floor of each pen and the birds left to acclimatise for one hour. Chicks received 3 pre-training 10 second presentations of a 2.5mm diameter white bead at 5 min. intervals. Chicks that failed to peck on at least 2 out of 3 trials were excluded from subsequent treatments (87% of chicks satisfied this criterion). Chicks were trained, 10 min. after pre-training, by a 10 second presentation of a 4mm chrome bead coated in either methylanthanilinate (M) or water (W) or with a dry bead (D). Birds left undisturbed throughout served as quiet controls. Five minutes before testing, chicks were presented with the white bead as a check on pecking and discrimination. Chicks that failed to peck were excluded. Chicks were tested at an appropriate time with a 10 second presentation of a dry 4mm chrome bead. Only those that responded correctly (that is, avoid if trained on M, peck if trained on W or a dry bead) were taken for analysis (80% of chicks gave the correct response).
3.2.2. Chemicals.

Cytidine 5'-monophospho-N-acetyl[4,5,6,7,8,9-14C]neuraminic acid (CMP-14C NeuNAc) (specific activity 294mCi/mmol) was obtained from Amersham International. Methylantranil ate (M), Coomassie brilliant blue G250, phosphotungstic acid (PTA) and fetuin were obtained from The Sigma Chemical Company. Complete™ (a protease inhibitor cocktail) was from Boehringer Mannheim, SOLVABLE™ was from Du Pont and EMULSIFIER-SAFE™ was from Packard. Antibodies and other reagents were as described in section 2.2.2.

3.2.3. Effects of passive avoidance training on anti-MenB immunoreactivity

Chicks were trained as described in section 3.2.1 using either a dry bead (n=6) or a bead dipped in methylanthranil ate (n=6) and tested for recall at 12 hours post-training. Chicks were killed by decapitation, whole forebrains removed by hand and frozen by placing on cover slips resting on a metal chuck.

4 Fetuin, the main glycoprotein in fetal calf serum, is an α-globulin with an Mr of 48 kDa. It carries 6 carbohydrate groups: 3 O-linked to ser/thr and 3 N-linked to asparagine accounting for about 20% and 80% of bound carbohydrate respectively (Spiro, 1960). Green et al. (1988) identified 23 di- and tri-antennary N-linked oligosaccharides containing from 1 to 4 α2-3 and α2-6 linked NeuNAc residues. Therefore, when used as an exogenous acceptor in ST assays, fetuin probably accepts NeuNAc residues attached via α2-8 linkages rather than via additional α2-3 and/or α2-6 linkages. Furthermore, in vitro ST assays have shown that fetuin can become polysialylated (see, for example, Yoshida, 1995b).
bathed in liquid nitrogen. Frozen samples were wrapped in aluminium foil and stored at -20°C until use.

Frozen coronal brain sections were cut as described in section 2.2.4, at the levels of the LPO (between levels A11.6 and A10.8 of Kuenzel and Masson (1988)) and IMHV (between levels A7.8 and A7.4 of Kuenzel and Masson (1988))(for precise location of these coronal planes see fig. 2.2 in section 2.3.2). Two sections were cut from each brain at each level (i.e. 4 sections per brain). Additional sections were cut for use as controls. A small cut was made in the left hemisphere of each section to allow subsequent identification of the left and right sides of the brain. Single immunocytochemistry was performed as described in section 2.2.3 using anti-MenB as the primary antibody and anti-mouse IgM-HRP conjugate as the secondary antibody. For control sections, anti-MenB was omitted from the incubation buffer during the primary incubation.

Sections were analysed using a Microcomputer Imaging device (MCID) (Imaging Research Inc.) as described in section 2.2.4. Slides were placed on a desktop illuminator that transmitted light of a constant intensity through the specimen (ambient lighting conditions were constant throughout) and images were received by a monochrome camera maintained at a constant distance above the specimen. Computer tools were used to draw, freehand with the mouse, the perimeter of target areas (left and right IMHV and left and right LPO) and the relative optical density (ROD) within that perimeter was determined automatically by the computer. To serve as a standard, a line was drawn around the right hand half of each section and the ROD determined as before (the left hand side of each section
was not used because of the cut made in this region to enable left and right sides of the brain to be distinguished). The ROD of the right hand side of 4 control sections, that had been incubated without anti-MenB in their primary incubations, was also measured and the mean of these 4 values was determined and used in subsequent calculations. Standardised relative optical density (SROD) was calculated by using the following equation:

$$SROD = \frac{[\text{ROD(target area)} - \text{ROD(control)}]}{[\text{ROD(half section)} - \text{ROD(control)}]}$$

For each brain, each target area was analysed in two sections, SROD was calculated for each target region and the mean of 2 SRODs was calculated. Statistical analysis was carried out using a Mann-Whitney U test.
3.2.4. Determination of protein content.

Protein content of homogenised samples was determined using a modification of the Bradford method (1976). Samples at appropriate dilutions were added in duplicate to a 96 well microtitration plate (10μl/well). Wells were cleared by addition of 30μl/well 10mM NaOH and revealed with 250μl/well Bradford dye (50ml 95% ethanol; 100ml Coomassie brilliant blue G250; 100ml H₃PO₄). Plates were read on a spectrophotometer at 595nm. BSA at concentrations of 50, 100, 150, 200, 250, 300, 400, 500, 600 and 700 μg protein/ml (10μl/well) served as protein standards from which calibration curves were constructed. Protein content of samples was determined from the linear portion of the graph (fig 3.2).

![BSA standards graph](image-url)

**Fig. 3.2.** Calibration curve plotted from BSA standards of known protein content. Absorbance of diluted samples is read from the graph as μg protein/ml homogenate and converted to mg/ml original homogenate.
3.2.5. Sialyltransferase assays.

Sialyltransferase assays were carried out, using a modification of the method of Breen and Regan (1986), in 50mM Tris-HCl (pH 7.0) containing 10mM MgCl₂, 3mg/ml Triton X-100, Complete™ (one tablet per 50ml solution), 1mg fetuin as exogenous acceptor and the enzyme homogenate preparation in a final volume of 420 µl. Reactions were initiated by the addition of 50nCi CMP-({sup}14{sub}C)-NeuNAc (170 pmol) and incubated for 45 min. at 37°C. Reactions were terminated by the addition of 420µl of 12% trichloroacetic acid (TCA)/ 1% phosphotungstic acid (PTA) and precipitated on ice for 30 minutes. Proteins were gathered by centrifugation at 12000g and 4°C for 3 min., washed twice in 420µl 12% TCA/1% PTA and solubilized overnight at 50°C in 1ml SOLVABLE™. Controls were incubated for the same time in the absence of CMP-({sup}14{sub}C)-NeuNAc, after which time the label was added and the reaction immediately terminated. Samples were transferred to scintillation vials, 8ml EMULSIFIER-SAFE™ added, left overnight and counts estimated on a Beckman LS 1707 scintillation counter.

3.2.6. Characteristics of Sialyltransferase activity.

Four one-day old Ross Chunky chicks were killed by decapitation, whole forebrains removed and frozen in 10% w/v 50mM tris-HCl (pH 7.0) containing Complete™ (one tablet per 50ml solution). Samples were stored at -20°C until use. Samples were thawed on ice and homogenized using a teflon-glass homogenizer for 10 up/10 down strokes and protein content was determined using the Bradford (1976) method as
described in section 3.2.4 (homogenate dilutions of 1/25 and 1/50 were used).

Incorporation of NeuNAc with respect to time was determined by carrying out sialyltransferase assays, as described in section 3.2.5, for 10, 25, 45 and 90 minutes with 0.5mg protein as the enzyme source. Results were expressed as pmol NeuNAc incorporated/mg protein. NeuNAc incorporation with respect to enzyme concentration was investigated by conducting sialyltransferase assays (section 3.2.5) using 0.1, 0.2, 0.5 and 1mg protein in 45 min incubations (pH 7.0). Results were expressed as pmol NeuNAc incorporated/hour. pH optimum was determined by conducting assays at pH 6.5, 7.0 and 7.5 using 0.5mg protein in 45 min incubations. Results were expressed as pmol NeuNAc incorporated/mg protein/hour. To confirm that fetuin was serving as the acceptor substrate for transferred NeuNAc, sialyltransferase assays were carried out as described in section 3.2.5. using 1mg protein in 45 min incubations at pH 7.0. Assays were carried out either with 1mg fetuin present in the reaction mixture or in the absence of fetuin. Results were expressed as pmol NeuNAc incorporated/mg protein/hour. All assays were performed in triplicate with 3 immediately-terminated controls serving as a measure of non-specific binding. Results were plotted as the mean of 3 assays minus the mean of 3 controls, for each assay.
3.2.7. Sialyltransferase activity following passive avoidance training.

Chicks were trained as described in section 3.2.2, and tested for recall at either 6, 12 or 24 hours post-training in 3 separate experiments. Four groups of chicks were used in each experiment: methylanthanilate (M) trained, water (W) trained, birds trained on a dry (D) bead and quiet controls that remained undisturbed throughout (n=7 in all cases).

After testing, chicks were killed by decapitation at the time-points given above and the left and right IMHVs and left and right LPOs dissected out by hand with the aid of a brain mould (for the location of these areas see fig. 3.3). Tissues were frozen in 0.5ml 50mM Tris-HCl (pH 7.0) containing Complete™, and stored at -20°C until use.

Only the left LPOs were used for subsequent biochemical procedures. Frozen samples from other areas remained in storage at -20°C to be used depending on the data obtained from the left LPOs.
Fig. 3.3. Schematic drawings of coronal slices of day-old chick brains prepared using a brain mould. The position and angle of cuts is shown in the sagittal view (stereotaxic coordinates after Youngren and Phillips, 1978). The first slice represented by face A (taken at level a) was used for dissecting the LPO, and the second slice, represented by face B (taken at level b) was used for dissecting the IMHV. For abbreviations see p9.
Samples were thawed on ice and polytroned on a Kinematica CH 6005 for 10 seconds. Protein content was determined using the Bradford (1976) method (homogenate diluted to 1/10 and 1/20) as described in section 3.2.4. Sialyltransferase assays were carried out in duplicate as described in section 3.2.5 using 190 μl homogenate in 45 min incubations. The mean of 4 immediately terminated reactions, serving as a measure of non-specific binding, was subtracted from each mean value. Samples were coded before carrying out assays so that the experimenter was blind as to which samples came from which birds. Activity was expressed as pmol NeuNAc incorporated/mg protein/hour. Statistical analyses were carried out using a one way analysis of variance (ANOVA).
3.3. Results.

3.3.1. Relative optical density analysis of anti-MenB immunoreactivity following passive avoidance training

SROD was higher in every LPO than in any IMHV, with no overlap in the data (fig. 3.4). At 12 hours post-training there were no differences in SROD between M-trained birds and controls in any of the regions investigated (fig. 3.4) (Mann Whitney tests: Left IMHV: U= 17; Right IMHV: U= 18; Left LPO: U= 13; Right LPO: 13.5. n=6 in all cases, p> 0.05 in all cases).

Fig. 3.4. Standardized relative optical density (SROD) of anti-MenB immunoreactivity in (a) the left IMHV, (b) right IMHV, (c) left LPO and (d) right LPO at 12 hours post-training. (Methylanthranilate-trained birds: closed bars; birds trained on a dry bead: open bars. n=6 in all cases, error bars represent SEM).
3.3.2. Characteristics of sialyltransferase activity.

NeuNAc incorporation was linear with respect to time up to 45 minutes (fig. 3.5) and with respect to protein at least up to 1mg, the highest concentration used (fig. 3.6). Incorporation was essentially the same at pH 6.5 and 7.0 and was much lower at pH 7.5 (fig. 3.7). All subsequent assays were carried out for 45 min at pH 7 with protein concentration within the linear range. Only minimal NeuNAc incorporation was detected when fetuin was excluded from the assay mixture (fig. 3.8).

NeuNAc incorporation: pmol/mg protein.

![Graph](Image)

Fig. 3.5. Sialyltransferase activity in chick whole forebrain homogenate. Extent of reaction measured as NeuNAc incorporation with respect to time (n=3, error bars represent the standard deviations).
Fig. 3.6. NeuNAc incorporation with respect to protein concentration (n=3, error bars represent the standard deviation).

Fig. 3.7. pH dependence of ST activity (n=3, error bars represent the standard deviation).
NeuNAc incorporation: pmol/mg protein/hour

Fig. 3.8. NeuNAc incorporation as a measure of sialyltransferase activity in chick whole forebrain homogenate: (A) with fetuin present in the reaction mixture and (B) without fetuin. (n=3, error bars represent the standard deviation).
3.3.3. Sialyltransferase activity following passive avoidance training.

There were no differences in ST activity in the left LPO between any of the groups of chicks at any of the time-points examined. Typically activity was around 7 pmol NeuNAc transferred/mg protein/hour at 6 hours post-training; 6.5 pmol NeuNAc transferred/mg protein/hour at 12 hours post-training and 6 pmol NeuNAc transferred/mg protein/hour at 24 hours post-training. These results are summarised in figs. 3.9 - 3.11. Results of statistical analyses are given in the relevant figure captions.
Experiment 1: Sialyltransferase activity in the left LPO at 6 hours post-training.

NeuNAc incorporation: pmol/mg protein/hour.

Fig. 3.9. ST activity in the left LPO at 6 hours post-training. M: methylantranilate trained; W: water trained; D: chicks trained on a dry bead; Q: quiet controls (n=7 in all cases, error bars represent SEM). F3,24 = 0.18336; p>0.25.
Experiment 2: Sialyltransferase activity in the left LPO 12 hours post-training.

NeuNAc incorporation: pmol/mg protein/hour.

Fig. 3.10. ST activity in the left LPO 12 hours post training. (n=7 in all cases +/- SEM) For abbreviations see caption to fig. 3.9. F3,24 = 0.5808; p>0.25.
Experiment 3: Sialyltransferase activity in the left LPO at 24 hours post-training.

NeuNAc incorporation: pmol/mg protein/hour.

Fig. 3.11. ST activity in the left LPO at 24 hours post training. (n=7 in all cases +/- SEM). For abbreviations see caption to fig. 3.9. F_{3,24} = 0.7996; p>0.25.
3.4. Discussion.

3.4.1. Anti-MenB immunoreactivity following passive avoidance training.

The failure of this study to detect any differences in the intensity of anti-MenB immunoreactivity between trained and control birds at 12 hours following passive avoidance training may be explained in a number of ways. Firstly, it is possible that any enhancement in PSA expression does not occur at 12 hours post-training and this study has looked at an inappropriate time-point. Secondly, passive avoidance training in the chick might not lead to increased PSA expression at all in the IMHV or LPO. However, any differences in PSA levels between trained and control birds may be undetectable using this methodology because of the high background levels of anti-MenB immunoreactivity in the day-old chick forebrain (see chapter 2). Immunocytochemistry has successfully detected enhanced hippocampal PSA expression following passive avoidance training in the adult rat (Fox et al., 1995). However, background levels of PSA are much higher in the forebrain of the day-old chick than in the hippocampus of the adult rat. Indeed, in the study of Fox et al. (1995), increases in the number of PSA positive cells could be detected post-training because many cells were PSA negative in control animals. Such an approach could not be used in this study because no PSA negative neurons can be unequivocally detected in the IMHV or LPO of the day-old chick (chapter 2). Because of this high signal-to-noise ratio discussed above, immunocytochemistry is probably not a useful approach for
addressing the question of whether PSA levels are enhanced following passive avoidance training in the chick. For this reason, immunocytochemistry was not used further to look at other time-points (alternative approaches to the investigation of PSA involvement in passive avoidance learning in the chick are considered in section 3.4.4).

3.4.2. Characteristics of sialyltransferase activity.

The linearity of ST activity with respect to time up to 45 minutes observed in this study (fig. 3.5) is the same as described in cerebral P2 fractions from 12 day old Wistar rats assaying transfer of NeuNAc to asialofetuin and employing essentially the same method as used here (Breen and Regan, 1986). However, while α2-8 STs have been shown to exhibit activity towards fetuin, transfer to asialoglycoproteins has not been reported with cloned enzymes (see, for example, Yoshida et al., 1995). On the other hand, α2-3 and/or α2-6 STs transfer NeuNAc to asialofetuin but not to fetuin (Kojima et al., 1996a,b). Therefore, transfer to fetuin implies α2-8 ST activity while transfer to asialofetuin, as assayed by Breen and Regan (1986) implicates the involvement of α2-3 and/or α2-6 STs. Linearity up to 3 hours has been obtained for ST activity towards NCAM from stage 33 embryonic chick brain, with about 70% of transferred NeuNAc in the form of PSA (Oka et al., 1995). However, while Oka et al. (1995) may have looked at the same enzyme as that assayed here (discussed below), the different assay methods make comparison difficult.
While the study of Breen and Regan (1986) found linearity up to 2mg protein, in the present study assays were only carried out up to 1mg protein (fig. 3.6). Oka et al. (1995), assaying PSA synthase activity towards purified NCAM, found linearity up to 60μg NCAM in a final volume of 50μl. However, higher concentrations of NCAM were not investigated and differences in the protocol employed by Oka et al. (1995) and in the present study make meaningful comparisons difficult.

ST activity was essentially the same at pH 6.5 and pH 7, but lower at pH 7.5 (fig. 3.7). This is comparable to an optimum of pH 6.5 reported for ST activity with respect to asialofetuin in forebrain P2 fractions from Wistar rats (Breen and Regan, 1986). However, an optimum of pH 7 - 7.5, with much lower incorporation at pH 6.5, is reported in adult rat brain golgi fractions (Breen and Regan, 1988a). An optimum of pH 6.0, although with significant activity at pH 5.5 and 6.5, is reported for PSA synthase activity from embryonic chick brain (Oka et al., 1995). However, in the present study activity was not assayed below pH 6.5.

The sialyltransferase activity characterised in this study includes both the endogenous and exogenous reactions, since NeuNAc incorporation into all proteins, not just fetuin, was assayed. However, when fetuin was excluded from the assay mixture, incorporation of NeuNAc into reaction products was minimal (fig. 3.8), suggesting that fetuin is serving as the main acceptor substrate for transferred NeuNAc.

It is not clear whether PSA synthesis requires the activity of a single polymerase enzyme (Eckhardt et al., 1995), or if an
initiase enzyme is required to attach the first \( \alpha_{2,8} \) NeuNAc residue onto an already attached \( \alpha_{2,3} \) linked NeuNAc. If the latter is the case, then the assays carried out here are only assaying the initiase and not the polymerase activity. [Since about 21 nmol fetuin is present and only about 3-4 pmol NeuNAc are transferred, there is insufficient acceptor substrate for the polymerase if it is unable to act directly upon fetuin lacking \( \alpha_{2,8} \) linked NeuNAc (this is not normally present on fetuin).] Thus, although the method used in the present study is assaying \( \alpha_{2-8} \) ST activity, it is not entirely certain that it is assaying polyST activity. In principle, this uncertainty can be addressed in two ways. Firstly, the assay protocol can be extended to include digestion of the reaction products by endo-N. The extent of polysialylation can then be estimated by assessing radioactivity in the fraction released by enzymatic digestion to give a measure of polyST activity (see Oka et al., 1995). However, since the assay protocol used in this study failed to reveal any differences between trained and control birds, the additional investment in an extended protocol is difficult to justify. Nonetheless, this methodology could be employed, with a limited number of assays, purely to confirm the presence of PSA. Secondly, ST assays could be conducted with unlabelled CMP-NeuNAc followed by electrophoresing the reaction products and immunoblotting with anti-MenB to confirm the presence of PSA-fetuin. However, this was not done in the present investigation because it would not reveal what proportion of the transferred NeuNAc was in the form of PSA. It would, therefore, say nothing about the level of polyST activity following passive avoidance training.
3.4.3. Sialyltransferase activity following passive avoidance training.

The time points investigated in this study were chosen to cover a range when changes in ST activity might be expected to occur, preceding anticipated increases in PSA levels (see section 3.1.5). However, if increases in PSA levels occur in the chick following training, then increased ST activity might be anticipated between 6 and 12 hours post-training. That is, the time points chosen in this study may have assayed ST activity before any possible increases occur and then again after they had returned to basal levels. Such a short lived increase in ST activity would, however, seem unlikely. Furthermore, the cost involved in investigating ST activity at other time points (such as 9 hours post-training) is difficult to justify on the basis of the data already obtained.

Since the data presented in this study derive from 3 separate experiments, a direct comparison between the data obtained at different time-points cannot be made. It is, however, noticeable that, for chicks in all 4 conditions, mean ST activity is highest at 6 hours post-training and lowest at 24 hours post-training, with values at 12 hours being intermediate. Since training was carried out at about 24 hours post-hatching, chicks in the third experiment were, on average, 18 hours older than chicks in the first experiment at the time of testing. The lower levels of ST activity at the later time-points may, therefore, be attributed to a developmental decrease in ST activity.
3.4.4. Alternative approaches to the study of polysialylation following passive avoidance training in the chick.

As noted in section 3.1.6, there are, in principle, several approaches to investigating possible changes in PSA expression following passive avoidance training. For example, radiolabelled mannoseamine, a sialic acid precursor, can be injected, at various time-points following passive avoidance training, and its incorporation into proteins estimated. This approach has already been successfully employed following step-down avoidance training in the rat (Doyle et al., 1992a). Alternatively, enzyme linked immunosorbent assays (ELISAs), utilising anti-MenB, possibly in conjunction with anti-NCAM (Dubois et al., 1995), may be employed to give a direct estimation of PSA-NCAM levels. However, because of the large amount of PSA present in the chick forebrain (see chapter 2), neither of these approaches would seem to offer a potentially fruitful line of enquiry.

An alternative approach to the study of polysialylation following passive avoidance training is the use of interventive methods. Firstly, a possible amnestic affect of PSA removal can be investigated by injecting endo-N. One objection to this approach is that, given the high levels of PSA in the chick forebrain, administration of endo-N may have many consequences unrelated to memory formation (such as interfering with synaptic maturation, for example). However, injections of endo-N can be made directly into the IMHV (see, for example, Scholey et al., 1993), and by optimising the administration protocol it may be possible to restrict PSA
removal to this region. Nonetheless, PSA cleavage within the IMHV may inhibit other processes that might, in turn, affect memory formation and/or behavioural expression of the passive avoidance task.

To investigate any possible amnestic affect of administering anti-MenB, as has been done using anti-NCAM (Scholey et al., 1993), would seem a dubious way to proceed. If this large IgM becomes attached to PSA at the cell surface, this would be expected to de-stabilise intercellular contacts. Since this is precisely the function proposed for PSA itself, administration of ant-MenB might be considered more likely to augment, rather than block, PSA function. On the other hand, a possible amnestic affect of administering anti-MenB should not be discounted if PSA functions in some other, perhaps more complex, manner.

The two known polyST enzymes, PST and STX (see section 1.4.1), may subserve different functions (see, for example, Eckhardt et al., 2000). In mice, PST seems to be required for the maintenance of LTP (Eckhardt et al., 2000) (whether STX is involved in LTP has not, to the best of my knowledge, been investigated). If polysialylation is required for learning the passive avoidance task, then one may speculate that PST, rather than STX, is involved in the process (STX may be required for other developmental processes not directly related to memory formation). If this is the case, then memory formation may involve relatively large changes in PST levels. (If only changes in PST and/or STX activity occur following passive avoidance training, then it may be impossible to distinguish between the activities of the 2 enzymes.)
principle, PST levels can be investigated using ELISAs with anti-PST antibodies following passive avoidance training. Such an approach may, therefore, offer a means of circumventing the problem of high background levels of PSA and ST activity. Alternatively, the possible amnestic affects of ST antisense can be investigated. However, to date, neither of the known polyST enzymes (PST and STX) have been cloned from the chick. Thus, although both PST and STX display very high sequence homology between mammalian species, the production of appropriate antisense probes may have to await the cloning of these enzymes from the chick. Possible prevention of PSA synthesis by using ST antisense also presents the same problems as discussed above in relation to PSA removal by using endo-N. Nonetheless, antisense technology would, in principle, seem to offer an alternative approach to the study of possible PSA involvement in passive avoidance learning.
The principal aim of the first part of the present work was to produce a spatio-temporal map of the anatomical distribution of PSA in the developing chick brain and visual system. Emphasis was placed on the gross anatomical level and a broad overview of changing PSA expression patterns was established. However, in the present work, chicks were only studied up to one week after hatching. PSA expression patterns certainly continue to change after this stage and extending the present investigation to include older animals would produce some worthwhile data and would, in a sense, "complete" the present work.

In the present investigation, the use of immunofluorescence and histological dyes helped to clarify some aspects of PSA expression (these have already been discussed in chapter 2 and do not require recapitulation here). However, more questions were raised than were answered (particularly in relation to PSA expression by white matter glia, see section 2.4). Given the broad anatomical level at which the present work has focused, it is inevitable that questions arise. Indeed, on the whole, it is a positive outcome of the present work that further lines of enquiry are suggested.
The second component of this thesis investigated PSA levels and ST activity following passive avoidance training. The failure of the present study to detect differences in PSA levels and ST activity between chicks trained on the passive avoidance task and control birds, underscores the value of using adult animals to study the function of PSA. Since PSA levels are much reduced in the adult, relative to the young animal (Bonfanti et al., 1992), changes in PSA levels may be more readily detected in adult animals. Because of the high levels of PSA in the day-old chick brain, no firm conclusions can be drawn from the present study regarding a possible role for PSA or ST activity during memory formation in this animal. Thus, the question of whether passive avoidance training leads in increased PSA levels or enhanced PSA synthase activity in the chick IMHV and/or LPO, remains open.
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