STUDIES ON NEURONAL NETWORK ACTIVITY OF OLFACTORY BULB, SPINAL CORD AND FRONTAL CORTEX GROWN ON MICROELECTRODE ARRAYS *IN VITRO*: THE ROLE OF GAP JUNCTIONS IN NETWORK INTEGRATION

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Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy

December 2005

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ACKNOWLEDGEMENTS

I would firstly like to thank my supervisors at the University of Nottingham, Dr Terry Parker and Dr Rob Mason, and my industrial supervisor Dr Richard Heal at QinetiQ for all their help and guidance throughout my PhD. I would also like to thank Prof Guenter Gross at the University of North Texas for letting me to work at his lab and everyone else at CNNS for great times we had.

Many thanks to Dr Lisa Storer for day to day help and encouragement in the good old E84. And to Karen and Jane, it wouldn't have been the same without you. I would also like to thank Nima Abbassi-Ghadi, 'my' medical student, for all his help.

I would also like to thank my friends and family. Special thanks to Peter for his love and for staying awake all those long nights listening to me complaining about the hard life of a PhD student. And finally, a big thank you to my mum, Maija, and dad, Heikki for always being there. I've learnt a lot from you and have kept my dad's words in my mind throughout the PhD. "Suuri oppi hulluttaa". I couldn't agree more.

PUBLICATIONS

UNPUBLISHED ABSTRACTS

Roschier, U.M.A. & Gross, G.W. (2003): The effect of gap junction blockers on the spontaneous network dynamics of mouse frontal cortex and spinal cord cultures. The 3rd International Conference on Substrate-Integrated Microelectrodes, Denton, TX, USA.

Roschier, U.M.A., Heal, R., Mason, R., Parker, T.L. & Gross, G.W. (2003) The effect of gap junction blockers on the spontaneous network activity of mouse frontal cortex and spinal cord cultures. Society for Neurosciences meeting, New Orleans, LA, USA.

ABSTRACT

This project focused on understanding the mechanisms involved in CNS integration. The anatomy and physiology of mammalian olfactory system was investigated in order to develop an organotypic *in vitro* sensory system to increase our understanding of sensory processing at a neural network level. The olfactory network cultures grown on multielectrode arrays (MEAs) were found to only rarely exhibit electrical activity and it was decided this was an unsuitable preparation for the purposes of this study. The spinal cord was chosen as a secondary sensory system, initially in co-culture with dorsal root ganglia and then alone, with special interest in gap junction function.

Gap junctions have received increasing attention as contributors to pattern generation in neuronal ensembles, including the generation or modification of highly coordinated, intense bursting states. The main result section of this study explored the effects of four gap junction blockers (carbenoxolone (CBX), halothane, 1-octanol and oleamide) on the spontaneous activity of mouse and rat frontal cortex and spinal cord cultures grown on microelectrode arrays (MEAs). It was our hypothesis that the characteristic coordinated bursting seen in most frontal cortex and in some higher density spinal cord cultures would be influenced via gap junction communication.

The four compounds tested generated interesting, and in one case paradoxical effects. Frontal cortex cultures were all inhibited in a dose-dependent manner, which included total cessation of activity by halothane, CBX, 1-octanol, or

oleamide (at concentrations 250μM, 100μM, 20μM, 20μM, respectively). All cultures showed spontaneous recovery at lower concentrations and reversibility after culture medium changes at higher concentrations. In addition, measurements of network burst rates and coefficients of variation of burst period indicate that burst coordination among channels was reduced by these compounds. These responses were generally mirrored in the spinal cord, except for CBX, which produced a paradoxical transient intense increase in network spike and burst production.

The results of this study show the effect of the gap junction blockers to be not only tissue specific, but also to differ from species to species. It is still unclear whether these differences seen really are through the blockade of gap junctions, or due to the secondary effects of the blockers used. Further studies showed that strychnine (1μM) prevented this transient activity increase in spinal cord networks, implying that CBX may temporarily block glycine inhibition. Blocking intracellular calcium mobility with thapsigargin (up to 5μM) did not affect the effects of gap junction blockers used.

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BIC	bicuculline methobromide
BME	Eagles' basal medium
CBX	carbenoxolone
CX	connexin
DAB	3,3'-diaminobenzidine
DIV	days *in vitro*
DMEM	Dulbecco's minimum essential medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DSP	digital signal processor
FBS	foetal bovine serum
FCM	full culture medium
FCS	foetal calf serum
GABA	γ-aminobutyric acid
GPBS	glucose-phosphate buffered saline
GBSS	Gey's balanced salt solution
GFAP	glial fibrillary acidic protein
HBSS	Hanks balanced salt solution
HMDS	hexamethyldisilazane
ITO	indium-tin oxide
KPBS	potassium phosphate buffered saline

L-AP4	L-2-amino-phosphonobutyrate
MAP	multichannel acquisition processor
MEA	multielectrode array
MEM	Eagle's minimum essential medium
NBM	neurobasal medium
NCS	new born calf serum
NGS	normal goat serum
NHS	normal horse serum
NMDA	N-methyl-D-aspartate
NRS	normal rabbit serum
PAS	periodic acid Schiff's staining
PBS	phosphate buffered saline
PDL	poly-D-lysine
PHA-L	*Phaseolus vulgaris* Leucoagglutinin
P-ORN	poly-L-ornithine
SIG	signal input board
(S)UPW	(sterile) ultra-pure water
TBS	tris buffered saline
TTX	tetrodotoxin

CHAPTER 1

GENERAL INTRODUCTION

1.1. INTRODUCTION

In this study the anatomy and physiology of two mammalian sensory systems were investigated in order to increase our understanding of sensory processing at a neural network level. Originally the study focused primarily on the olfactory system (i.e. olfactory epithelium and olfactory bulbs) with secondary attention paid to the dorsal root ganglia and the spinal cord. After an experimental evaluation of the olfactory system and discussions with other research groups working in the area, notably Prof. G.W. Gross (CNNS, University of North Texas, USA) and Dr E.D. Herzog (Department of Biology, Washington University, USA), it was decided the olfactory system was an unsuitable preparation for the purposes of this study. In particular olfactory network cultures on multielectrode arrays (MEAs) were found to only rarely exhibit electrical activity. Therefore the dorsal root ganglia and the spinal cord became the main focus of the remaining study.

The spinal cord cultures were used to investigate of the effects of gap junction blockers on the spontaneous network dynamics in spinal cultures. This was

extended to include the frontal cortex for comparative purposes as the spinal cord and frontal cortex cell cultures expressed different electrical behaviours.

1.2. CELL MEMBRANE BIOLOGY

It is important to understand the basic anatomical, physical and chemical properties of the cell environment contributing to the biology of ion transport across the cell membrane in order to understand electrophysiological experimentation. Cells have a plasma membrane consisting of phospholipids, separating the cytoplasm from the extracellular space. The membrane forming phospholipids contain lipophilic and hydrophilic residues spontaneously arranged so that the lipophilic compounds face each other (Fig. 1.1.). This means that in the cell membrane there are bilayers of phospholipids, forming an effective barrier to charged molecules, in which membrane proteins, such as receptors and ion channels, are set in.

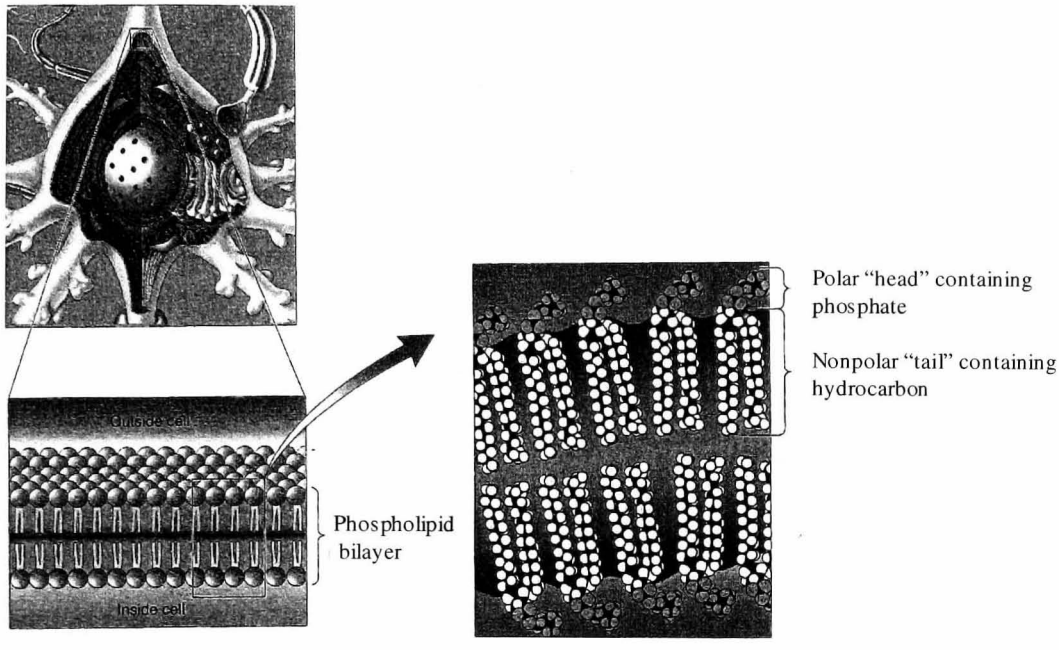

Figure 1.1. The phospholipid bilayer of a neuronal membrane forms a barrier to water-soluble ions (From Bear *et al.*, 2001). The hydrophilic heads face the intra- and extracellular environments and the hydrophobic tails face each other as shown in the cross section through the membrane.

The extra- and intracellular fluids are watery salt solutions and conduct ions very effectively. Therefore the cell membrane acts as an insulation layer between the two solutions. The ion channels, transporters and pumps in the cell membrane break down the insulator properties of the cell membrane. The osmolarity of both extracellular and intracellular contents is controlled tightly to protect the cell membrane. An overview of the contents present in the cell environment is given in Table 1.1.

Ion	Intracellular range (mM)	Extracellular range (mM)
Na^+	5-20	130-160
K^+	130-160	4-8
Ca^{2+}	50-1000nM	1.2-4
Mg^{2+}	10-20	1-5
Cl^-	1-60	100-140
HCO_3^-	1-3	20-30

Table 1.1. Intracellular and extracellular distribution of the main ions (Molleman, 2003).

The concentration differences between the extra- and intracellular fluids cause concentration gradients for each of the ions across the cell membrane inducing diffusion from the higher to the lower concentration. For example, if the cell is only permeable to potassium, the potassium ions diffuse out of the cell causing the inside of the cell to become negatively charged. The diffusion continues until the negative charge inside the cell is large enough to keep the potassium ions inside the cell. Therefore the concentration differences, for example the intracellular high potassium and low sodium concentrations are maintained actively by the cell, mainly by Na^+/K^+ pumps. On the other hand, for example calcium ions are pumped out of the cells by Ca^{2+}/Na^+ exchangers. The activity and the distribution of ions on either side of the membrane causes the charges on either side of the cell membrane to be imbalanced; the inside of the cell at rest is negatively charged in relation to the outside. This difference causes an electrical potential over the cell membrane (Molleman, 2003). When the

membrane is at rest, the difference of the potential inside and outside the cell is approximately -65mV. During the action potential (Fig.1.2.) the membrane potential becomes briefly positive.

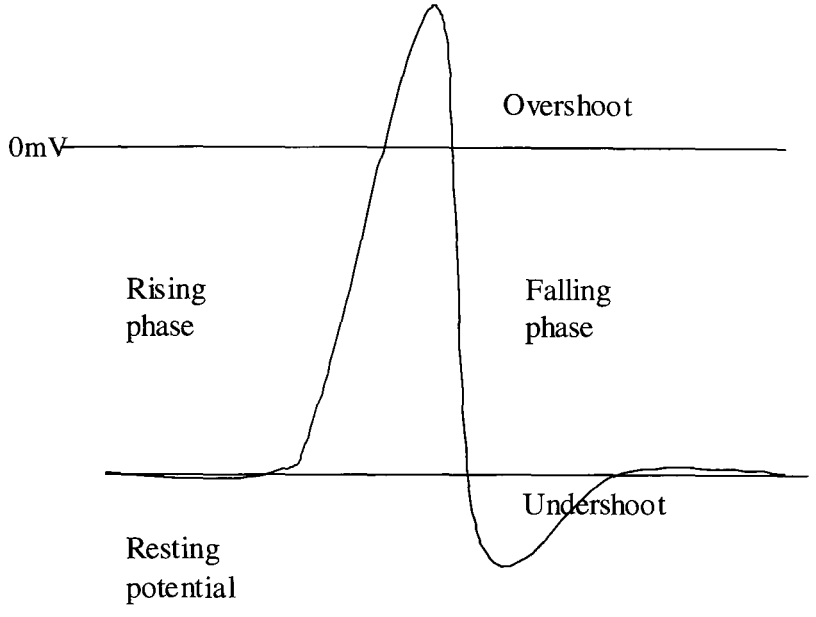

Figure 1.2. An overview of different phases of an action potential.

The rising phase of the action potential is characterised by a rapid depolarisation of the cell membrane. The change in the membrane continues until the membrane potential reaches a peak value of about 40mV. The positive phase of the action potential is called an overshoot and during this phase the inside of the cell is positively charged compared to the outside. The overshoot is followed by a falling phase, a rapid repolarisation causing the membrane to become more negative than the original resting potential. This phase is called an undershoot. The undershoot is followed by a gradual increase in membrane potential until the resting potential is restored. The entire action potential lasts about two milliseconds (Bear *et al.*, 2001).

1.3. SPINAL CORD AND DORSAL ROOT GANGLIA

The role of the spinal cord in the central nervous system is to conduct sensory information from the peripheral nervous system to the brain, to conduct motor information from the brain to skeletal, cardiac and smooth muscles and glands, and to serve as a minor reflex centre responsible for some simple reflexes like the withdrawal reflex. The nerve impulses reaching the spinal cord through sensory neurones are transmitted to the brain. Signals arising in the motor areas of the brain travel back down the cord and leave in the motor neurones. 31 pairs of spinal nerves, containing both, sensory and motor axons, arise along the spinal cord. All the sensory axons pass into the dorsal root ganglia, where their cell bodies are located and then on into the spinal cord itself within the spinal column, whereas all the motor axons pass into the ventral roots before uniting with the sensory axons to form the mixed nerves (Fig. 1.3.). The dorsal root ganglia, groups of nerve cell bodies located outside of the central nervous system, contain the nerve cell bodies of unipolar neurones that carry afferent information from sensory receptors in the periphery to the central nervous system for processing.

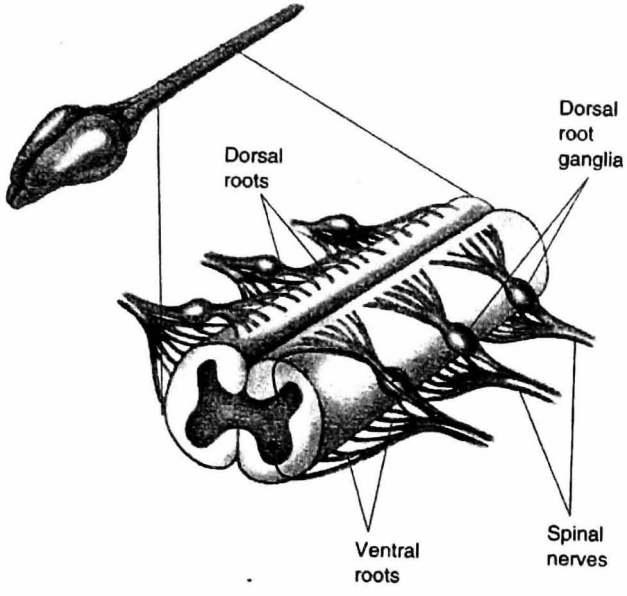

Figure 1.3. The spinal cord (From Bear *et al.*, 2001). The spinal cord runs inside the vertebral column with axons entering and exiting via the dorsal and ventral roots respectively. The rooms form the spinal nerves.

The motoneurones of the spinal cord are activated by the glutamatergic inputs from the primary afferents, interneurones and descending inputs. The transmission is mediated by both NMDA (N-methyl-D-aspartate) and non-NMDA glutamate receptors (e.g. Ziskind-Conhaim, 1990). The earliest synaptic potentials from the motoneurones have been recorded at the embryonic day 16, showing slow rise rates and long durations. These potentials can be blocked by NMDA receptor antagonists. The slow excitatory postsynaptic potentials with fast rates of rise are mediated by AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors and appear at least a day later (Ziskind-Conhaim, 1990), emphasising the importance of NMDA receptors for spinal network development. In case of an early postnatal blockade of the NMDA receptors, the developmental decrease of gap junctional coupling between the

motoneurones during the first two weeks of the postnatal development (see 5.1.1.) is prevented (Mentis *et al.*, 2002). Several different types of connexion proteins are expressed by the developing motoneurones. The connexin expression is regulated during the postnatal development (Chang *et al.*, 1999).

The NMDA-mediated synaptic activity has a critical role in the postnatal refinement of spinal synaptic circuits: The period of plasticity of the spinal cord circuitry can be prolonged by neonatal blockade of NMDA receptors (Maier *et al.*, 1995). The increase in glutamatergic synaptic activity during the normal development is associated with the maturation of the locomotory function of the spinal cord. It is possible that this leads to the decrease of the gap junctional coupling of the motoneurones providing an activity-dependent mechanism for the modification of motor control due to the loss of motoneurone coupling reducing the extent of synchronised motoneurone activity and allowing for independent recruitment of motoneurones (Mentis *et al.*, 2002).

The dorsal horn is the primary area for receiving somatosensory input. It is laminar structure, each of the laminas receiving specific types of input. It contains high concentrations of several types of receptors congregating in lamina II, an area receiving most of fine, presumably nociceptive, somatosensory input. Many of these receptors reorganise themselves in response to various stimuli or pathological situations. Neuroactive ligands such as peptides and excitatory or inhibitory amino acids are also organized in a laminar fashion (Coggeshall & Carlton, 1997).

1.4. FRONTAL CORTEX

Frontal cortex, the cortex of the frontal lobe, is the portion of the brain involved with reasoning, planning, abstract thought and other complex cognitive functions in addition to motor function. The frontal lobe is divided from the parietal lobe by the central culcus and the cortex can be divided into three areas; the primary motor cortex responsible for the motor functions, the premotor cortex anterior to the primary motor cortex including Broca's and supplementary motor areas and, the rest of the frontal cortex, the prefrontal cortex.

The primary motor cortex projects to and from several brain areas, including olfactory cortex and hypothalamus and also sends additional projections to areas such as the hippocampus. It also has massive descending projections to spinal cord.

The physiological role of the frontal cortex is to participate in different aspects of the active adaptation to the environment, i.e. skeletal and ocular movement, reasoning and the spoken language. Also the visceral actions and emotional behaviour are, to some extend, controlled by areas of the frontal cortex (Fuster, 2000). The cognitive functions of the frontal cortex consist of the activation and processing within and between the networks of neurones (e.g. Fuster, 1997). The neuronal networks of the frontal cortex are highly organised, specific and defined by their connectivity. The lowest level of the cortical hierarchy is the

primary motor cortex, representing and executing elementary muscular movements. The premotor cortex on the other had has a higher role as a substrate for more complex, goal and trajectory defined movements. Finally, the highest in the cortical hierarchy is the prefrontal cortex, representing the broad schemas of action in skeletal and speech domains (Fuster, 2000).

In general, the cortex processes the sense data and formulates appropriate motor responses. The importance of the cortex is represented by the large size of the cortical areas – 80% of the brain volume in modern man is occupied by the neocortex and its connections (Passingham, 1982).

The cortex is formed of a laminated sheet of cells, varying in thickness from 0.8mm in mouse visual cortex to 3mm in some areas of the human motor cortex. However, the number of neurones in a column extending from the cortical surface to the white matter is practically the same in all species and all cortical areas (e.g. Rockel *et al.*, 1980), due to the larger cell-to-cell distances in the thicker cortices. In terms of the laminar organisation, three different types of cortex can be identified: granular, agranular and homotypical. The primary visual cortex is an example of the granular cortex. The small cell bodies are, typically to primary sensory areas, densely packed in the middle layer of the cortex. The motor cortex on the other hand lacks this distinct small cell layer and is therefore called agranular. The third cortex type, homotypical cortex, contains varying populations of granule cells. It includes most of the association cortical areas and is the least understood area of the cortex (Douglas & Martin, 1990).

All three types of cortical areas contain the same two basic cell types, those with spiny dendrites (the stellate and pyramidal cells) and those whose dendrites are smooth (the smooth cells). The two cell types differ not only morphologically, but also structurally and functionally. For example, the spiny neurones are excitatory, whereas the smooth neurones are thought to be inhibitory. About 90% of all cortical neurones fall into these two groups but the proportions of the different types vary between the cortical layers (Douglas & Martin, 1990). Layer 1 is relatively cell free containing none of the spiny cell types. On the other hand, about 75% of all the neurones in the layer 4 of the granular cortex are stellate neurones. Overall the stellate neurones form around 10% of the neuronal populations in the granular cortex. In the remaining layers, the pyramidal cells form 70-80% of the neuronal population (Powell, 1981) and the proportion of the smooth cells is constant at about 20% in layers 2-6 (Gabbott & Smogyi, 1986). The other main neuronal elements found in cortex are axons providing inputs to the cortex. The axons projecting to the cortex arise from several sources, including the thalamus (Douglas & Martin, 1990).

The spiny and smooth cells form different types of synaptic connections. The spiny neurones (and the thalamic afferents) form asymmetrical synapses that display pronounced electron-dense areas under the postsynaptic membrane, but not under the presynaptic membrane. The smooth cells, on the other hand, form synapses with more symmetrical pre- and postsynaptic electron densities (Colonnier, 1968). Both types of synapses are found on all cortical neurones, but vary on their distribution: asymmetrical synapses are mainly found on

dendritic spines and shafts, whereas the symmetrical synapses are concentrated around the soma and proximal dendrites (e.g. Beaulieu & Colonnier, 1985).

There are four different types of receptors for excitatory amino-acids in the cortex: NMDA, quisqualate, kainate and L-AP4 (L-2-amino-phosphonobutyrate). Due to the lack of specific receptor antagonists, these receptors can be grouped in two functional classes: the NMDA receptor and non-NMDA receptors. The important agonists for the NMDA receptor are NMDA, aspartate and L-glutamate and for non-NMDA receptor agonists include kainate, quisqualate and L-glutamate. The receptors are antagonised by non-specific excitatory amino acid antagonists, such as kynurenate (Douglas & Martin, 1990).

1.5. PRIMARY NEURONAL CELL CULTURE

This experimental study was carried out using primary neuronal cell cultures due to the lack of cell lines available, that would have exhibited the full range of neuronal cell characteristics required, e.g. spontaneous generation of electrical activity and synapse formation. The neuronal primary cells form synapses and become spontaneously electrically active following the separation from the donor animal. They do not, however, continue to divide.

Primary culture systems have been widely used for studying the neuronal functions in many regions of the central nervous system. These cultures are a well established method for studying the electrical activity and the mechanisms causing this activity in an intact brain circuitry. There are two commonly used primary neuronal cell culture types – organotypic slice cultures and dissociated cell cultures. The electrical activity in these cultures has been shown to be very similar to that *in vivo* within single neurone, at single synapse and within the complex network circuitry (Cotman *et al.,* 1994).

1.5.1. Organotypic Slice Cultures

The brain slice preparation for neurochemical studies was first used by McIlwain *et al.* (1951). The goal of this method is to prepare a slice of tissue having those neurones, glia and synapses that are important for the experiment in a viable condition. The tissue should remain organotypically organised, but at the same time be accessible for experimental manipulation. The tissue should contain neurones displaying cellular differentiation and, at the same time, permit studies of the development and characteristics of axonal connectivities within and between different areas of the brain. The results of this method, as those of dissociated cell cultures, depend on several variables, such as the species and age of animal used and the anatomical origin of the tissue used (Gähwiler *et al.,* 1991).

The majority of nerve cells have been found to innervate their normal target cells in a culture. However, because of the loss of normal afferent projections as well as target areas, the intrinsic neuronal connections do reorganise themselves and even gain some additional synaptic sites (e.g. Zimmer & Gähwiler, 1984; 1987). The nerve cells have been also found to differentiate and mature in the slice cultures. In the co-cultures of the different brain areas the extrinsic afferent fibres are damaged during the slicing, but they are also found to regenerate rapidly during the first few days *in vitro* (Gähwiler *et al.,* 1991).

Organotypic slices of neuronal tissue can be maintained in cultures from several weeks to months. The basic requirements for such cultures are sterility, a substratum, culture medium and sufficient oxygenation and incubation. Under suitable conditions the cells continue to differentiate and develop, and the slices progressively thin over time due to the cell death and migration and provide an ideal slice culture for physiological and pharmacological studies (Gähwiler *et al.*, 1997).

1.5.2. Dissociated Cell Cultures

Dissociated cell cultures are prepared from a single-cell suspension of the experimental tissue. The suspension is prepared using enzymatic and mechanical dissociation and the plated on an appropriate substrate (For methods see chapter 3.4.7.). The cells extend processes and form extensive networks within days. These cultured have mainly be used in the determination of the synaptic organisation and synapse formation (Chuah *et al.*, 1991).

The anatomical clues used for specific identification of neuronal populations are lost in dissociated cell cultures. When culturing dissociated cells on MEAs, it is impossible to perform immunohistochemical staining on neurones due to the high cost of the arrays. Therefore the information of different cell types growing on these cultures must be interpreted from studies of these cultures grown on coverslips (e.g. Fracek *et al.*, 1994a). Ideally this information would be gained

by immunostaining on MEAs themselves with antibodies raised against specific neuronal, glial, neurotransmitter or enzyme markers (Withers & Banker, 1998). The real strength of the MEAs lays, however, in recording from slice cultures, which maintain some of the *in vivo* circuitry, or from anaesthetised animals *in vivo*.

1.6. ELECTROPHYSIOLOGY

1.6.1. Extracellular Recordings

Extracellular recordings have been widely used to study the electrical activity within the central nervous system. Until recently, the majority of the work has been done concentrating only on one or two neurones, i.e. single-unit or field potential recordings. Extracellular microelectrodes, fine, often etched and wire insulated almost to the tip or capillary tubes filled with a salt solution (Purves, 1981), are placed close to the nerve cell to gain information whether the neurone in question is depolarising (firing) or inactive and whether the rate of the possible firing is increasing or decreasing. The main advantage of using just one electrode is the possibility to associate the signals from the firing neurone to its anatomical location allowing examining the firing of an individual neurone and its influences on other neurones.

1.6.2. Multielectrode Arrays (MEAs)

The recordings from a single neurone provide little information on how the neurones process information in a network. It is necessary to be able to record simultaneously from several cells within a network to be able to understand the

interactions of neurones and the behaviour of an entire neuronal network. Verzeano (1956) was the first to use more than one (two rigidly coupled) microelectrodes. More recently the technological advances (intergrated-circuit fabrication, such as microetching and metal deposition) have enabled the production of multielectrode devices on planar substrates (e.g. Gross, 1979).

The essential requirements for a multiple microelectrode used to record the electrical activity from biological tissue *in vitro* include:

(a) a conductor to form the active electrode including the tracks, for example indium-tin oxide (e.g. Gross *et al.*, 1985) or titanium nitride (Egert *et al.*, 1998),

(b) an electrical insulation layer, for example polysiloxane (Gross *et al.*, 1985), polyimide (Oka *et al.*, 1999), silicon nitride (Egert *et al.*, 1998) or aluminium oxide (Jimbo *et al.*, 1993), and

(c) biocompatibility, resistance to the physiological saline environment and sterilisation of the microelectrode.

The microelectrode also needs to be sustainable in cell culture conditions for several weeks (Cotman *et al.*, 1994). One of the earliest groups to manufacture such a device were Hanna and Johnson (1968) who used photoetching to form an array consisting of 20 or 30 electrodes on a plastic sheet. However, due to the large size of the electrodes, the device was only suitable for recording field potentials. The first microelectrode array with integrated buffer amplifiers reducing the amount of electrostatic interference was designed by Jobling *et al.* (1981) to record from acutely prepared hippocampal slices *in vitro.*

One of the earliest successful MEA designs was by Thomas *et al.* (1972), a device manufactured to record 'bioelectrically active tissues and single cells' and used to record the spontaneous activity from cultured embryonic chick cardiac myocytes. Some of the more modern designs still use the basic photolithographic fabrication methods used by Thomas *et al.* Later, Gross *et al.* (1977) were the first group to record multiple single-unit neuronal activity *in vitro* using MEA consisting of 36 photoetched microelectrodes and gold-coated titanium tracks on glass substrate, initially covered by a thin insulating layer of either thermoplastic or thermosetting polymer. This design of an array allowed the activity of several neurones to be recorded simultaneously, the MEAs to be used for long-term cell cultures (12DIV) for the first time. The design was successfully applied to record from several types of tissues, including acutely excised snail brain ganglion (Gross *et al.*, 1977; Gross, 1979), cultured mouse spinal cord (Gross *et al.*, 1982; Gross & Lucas, 1982), auditory cortex (Gopal & Gross, 1996) and the suprachiasmatic nucleus (Herzog *et al.*, 1997). In addition of allowing simultaneous extracellular recording of several neurones in long-term cell cultures, this design of array also permitted optical monitoring of the growth of the cultures allowing the growth of the neuronal networks to be correlated with the appearance of electrical activity (Gross & Lucas, 1982). This was due to the 'invisibilty' of the indium-tin oxide electrode patterns caused by their high light transmission (Gross *et al.*, 1985).

The MEAs constructed by Gross *et al.* were further modified, the construction of the more modern arrays is described by Gross & Kowalski (1991). The modern MEAs allow, in addition to what is described previously, electrical

stimulation of the neurones through the recording electrodes (Gross *et al.*, 1993). More recently, the focus of the studies on neuronal networks grown on MEAs has been on using them as a system for neurone-based biosensors (e.g. Gross *et al.*, 1995). This MEA system, created and constructed by Gross *et al.* was used in this experimental study. The MEAs were supplied by Prof. G.W. Gross (CNNS, University of North Texas, USA) and the multineuronal acquisition processor and software developed by H. Wiggins (Plexon Inc., USA). The MEA construction, the use of the MEAs and the system in general is fully described in chapter 2 as they formed a major part of the experimental methods in this study.

Other research groups have independently produced similar MEAs for *in vitro* (e.g. Jimbo *et al.*, 1993; Stoppini *et al.*, 1997) and *in vivo* (Nicolelis *et al.*, 1997) recordings. The MEA designed by Stoppini *et al.* (1997) was specially designed for use with organotypic slice cultures. It is an array of 30 polyimide-insulated, gold-plated copper electrodes, which can be placed on top of a mature organotypic slice for the recording period. Similar device produced by Egert *et al.* (1998) enables an organotypic slice to be grown on the electrode array allowing long-term recording. The most recent design of the MEAs for organotypic slice cultures is that of Duport *et al.* (1999).The array is placed underneath the cultures rather than being on top, providing extra support for the cultures and preventing mechanical perturbation that occurs as a result of a contact between the electrodes and the tissue.

MEAs are now also being designed as biosensors allowing many physiological parameters to be measured (Pickard, 1999). Spontaneously active neuronal networks grown on MEAs are an efficient model for examining the neurotoxic properties of chemicals (e.g. Gross *et al.*, 1997). Furthermore, Gramowski *et al.* (2004) showed that the activity changes of cultured neuronal networks induced by a variety of toxins are repeatable, quantifiable as well as comparable with the available *in vivo* data. The possible use of MEAs as biosensors was of special interest in this experimental study.

1.7. EXPERIMENTAL AIMS AND OBJECTIVES

The original aim of this study was to develop a long-term *in vitro* sensory processing culture system and to be able to monitor chemo-sensory input, e.g. cannabinoids, odours, gap junction blockers etc. The cultures were to be grown on multielectrode arrays (MEAs) using electrophysiological extracellular single unit recordings, proceeding to multielectrode arrays to monitor neuronal activity.

Two sensory system preparations were investigated comprising of co-cultures of

(1) olfactory epithelium with olfactory bulb and

(2) dorsal root ganglia with spinal cord.

These preparations were characterised morphologically and electrophysiologically.

After 18 months dedicated to the development of the olfactory system culture, the preparation proved to be unsuitable for electrophysiological study. Subsequently research effort was then directed to developing single- and co-cultures of dorsal root ganglia and spinal cord *in vitro* sensory processing systems. In addition, dissociated frontal cortex cultures were used for comparative purposes. These objectives of the thesis then became:

1. To grow long-term (>21DIV) spinal cord (and dorsal root ganglia) slice and dissociated cell cultures on both, glass cover slips and MEAs. To grow long-term (>21DIV) frontal cortex cultures on MEAs.

2. To record the spontaneous activity of both, spinal cord and frontal cortex cell cultures grown on MEAs. To assess the firing characteristics of these cultures.

3. To compare the effects and mode of action of gap junction blockers on the spontaneous network dynamics in spinal cord and frontal cortex cultures. To compare these effects on mice and rat cultures. To assess the importance of gap junctions on the synchronised firing of the different types of neuronal networks.

CHAPTER 2

IN VITRO NEUROPHYSIOLOGY METHODS

The techniques for cell culture preparation, characterisation and *in vitro* electrophysiology used in this study are all well established (e.g. Gähwiler, 1981; Stoppini, 1991) and have been previously used in several studies (e.g. Gähwiler *et al.*, 1991; Gross *et al.*, 1995). These techniques enable the position of neurones in the microcircuits and neuronal networks to be characterised on the basis of morphology, chemistry and connections they form. This characterisation performed provides a framework within which the functional data collected later on can be interpreted. This chapter concentrates on the methods used in the investigation of the effects of gap junction blockers on the spontaneous network dynamics of dissociated cell cultures. Additional techniques, e.g. electron microscopy, used in this experimental study are described in chapter 3.

2.1. MICROELECTRODE ARRAYS

2.1.1. MEA Construction

The microelectrode arrays (MEAs, model #3; Fig. 2.1.) used in this study were supplied by Professor G.W. Gross, CNNS, University of North Texas, USA. The construction of MEAs is fully described by Gross *et al.* (1985) and briefly explained below.

Glass plates (50x50x1.1mm) were thinly coated with indium-tin oxide (ITO) and a pattern of 64 centrally located electrodes, arranged in four rows of 16, in 0.64mm^2 recording area with conducting tracks to the sides of the glass plates. The manufacturing procedure used standard positive photoresist photolithographic techniques (Cooper, 1999). A photomask, a negative image of the electrode tracks, was produced and the unwanted ITO was removed from the glass plates using an etchant. This resulted in an ITO cover over only the electrodes and conducting tracks. The recording area, in which the electrodes terminate, was insulated with polysiloxane or methyl silicone resin (Dow Corning DC648, USA). The thickness of the insulation layer was 1.5-2μm. The recording area of the MEA was then laser-deinsulated with single UV laser (wavelength=337nm) 12ns pulses forming 'a crater' in the insulation layer exposing individual ITO tips approximately 8x10μm. Low electrode impedance of 1-2MΩ was achieved by electrolytic deposition of gold to the individual electrodes. The tips of the electrodes were spaced 40μm between the columns

and 200μm between the rows. The width of the conductors was 8μm in the recording area and widened gradually reaching 32 contact pads at either side of the glass plate. The contact with the preamplifier was made via carbon elastomere 'zebra strip' connectors (Fujipoly, USA).

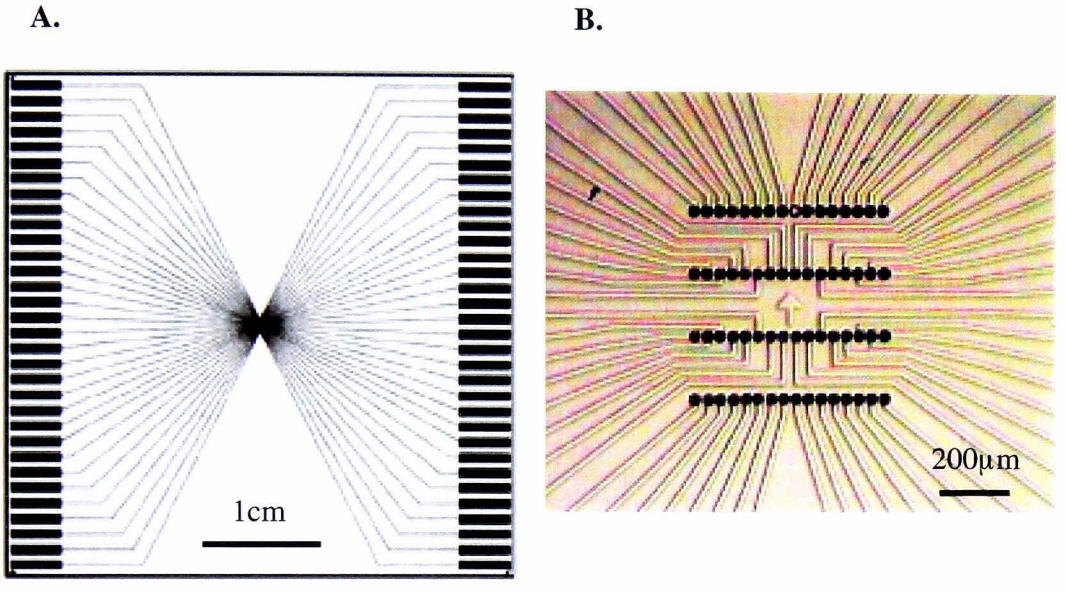

Figure 2.1. A photomigrograph of a MEA. (A) The recording area is located in the centre of the glass substrate plate and the conducting can be seen on the left and right edges of the MEA. These were connected to the preamplifiers. (B) A higher magnification photomicrograph of the recording area. The 64 conductors terminate in a 0.8 mm^2 recording area in 4 rows of 16 columns. Electrodes are spaced 40 μm between columns and 200 μm between rows. Electrode area is roughly 200 μm^2.

2.1.2. MEA Preparation for Culturing

The MEA preparation started two days prior to culturing. The MEA surfaces were carefully cleaned using a soft brush and diluted (10%) tissue grade glassware detergent (Liqui-Nox, Alconox Inc, USA). After the initial cleaning, the MEAs were left to soak in a beaker containing 5ml of diluted detergent and 200ml ultra-pure water (UPW) for 20 minutes and then rinsed with running UPW for another 20 minutes. The MEAs were sterilised by autoclaving at 120°C for 20 minutes and then dried at 70°C overnight. The silicon stopcock greased (G-9020, Nusil, USA) gasket seals were sterilised together with the MEAs. The gasket seals were custom made from a 4mm thick silicon rubber sheet (University of Nottingham, Medical School Workshop, UK). The external dimensions of such a gasket were 32x48mm and the internal dimensions 27x22mm.

One day prior the culturing, the polysiloxane insulation layer of the MEAs was exposed to a one second pulse from a propane flame to make it hydrophilic (Lucas *et al.*, 1986). After the flaming, the greased gasket seals were placed on each MEA and 500µl poly-D-lysine (PDL; Sigma, UK) solution (100µg ml-1 in a sodium borate buffer, 0.1M, pH 8.5) was applied onto the matrix area of the MEAs and left overnight. In the morning, at least two hours prior to dissection, the PDL was removed using a sterile small pore plastic pipette. Once the MEAs were completely dry of PDL, 100ml laminin solution (20µg ml^{-1} laminin (Sigma, UK), in neurobasal medium (Life Technologies, UK)) was applied on

the matrix area together with 50µl heat-inactivated foetal bovine serum (FBS, Life Technologies, UK). The MEAs were placed in a 5% CO_2 incubator at 37°C for 45 minutes.

2.1.3. Microincubation/Recording Chamber

The cultures grown on MEAs needed to be maintained in a controlled, constant temperature, pH and cell culture media volume environment in order to make valid electrophysiological recordings. A microincubation/recording chamber that allows this control over the environmental factors was developed by Gross & Schwalm (1994) and used in the present studies. The chamber (Fig. 2.2.) consisted of an anodised base plate, four power resistors (10W, 3Ω) designed to heat the MEA, and a stainless steel chamber containing the cell culture media.

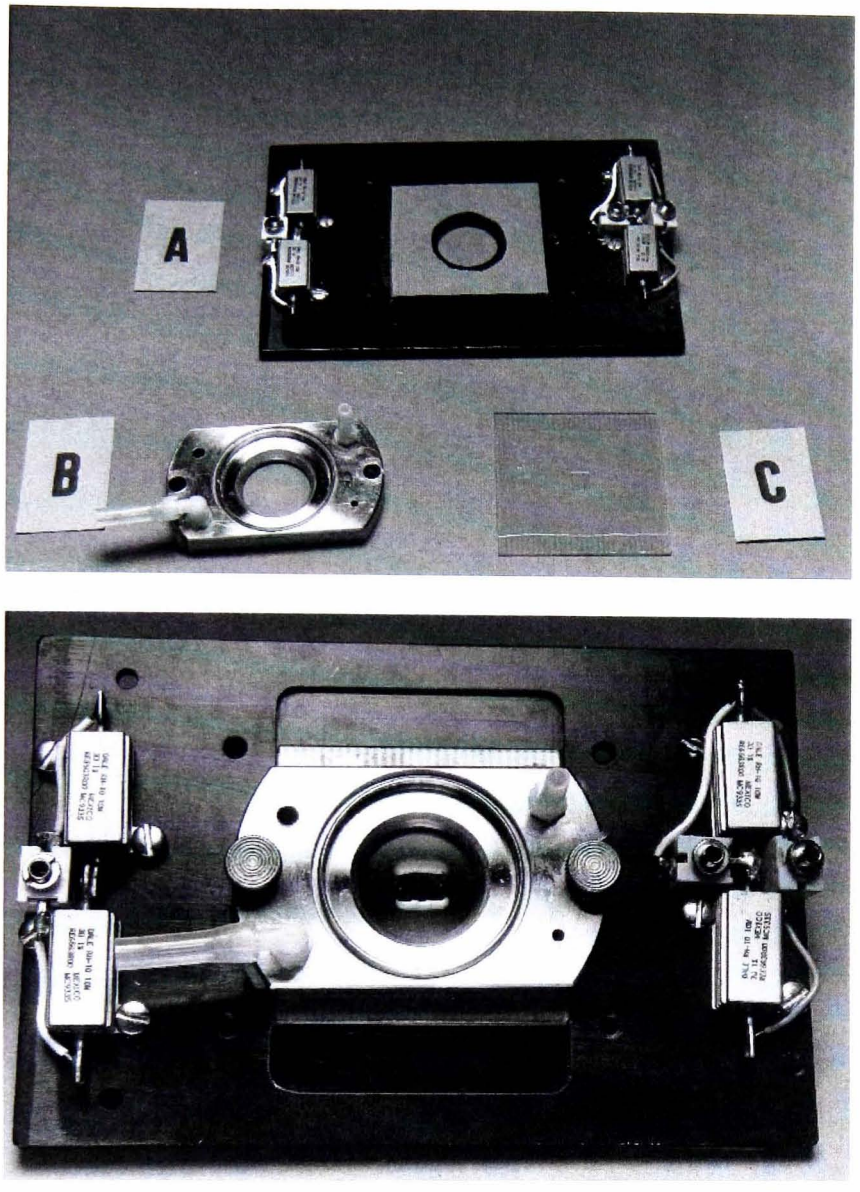

Figure 2.2. A microincubator/recording chamber exploded (top) and assembled (bottom). (A) Aluminium base plate with four power resistors, (B) stainless steel chamber and (C) MEA.

The temperature was maintained at 37°C during the recordings using a TC-344 Dual Heater Controller (Warner Instruments Corp., USA) with electronic feedback from a thermistor (Warner Instruments Corp., USA) inside the stainless steel chamber. The chamber was covered with a thin film ITO coated

plastic heater cover that a) allowed short-term (up to 24 hours) aseptic recordings, b) helped to maintain the temperature, c) reduced condensation which would have led to increases in the osmolarity of the cell culture media and, d) allowed visual observation of the culture during the recordings. The pH was maintained via an inlet in the heater cover using sterile moist 95% O_2/5% CO_2 and the volume of the culture medium was constantly kept at 1ml. The drugs were added through inlets in the stainless steel chamber using sterile syringes.

The use of MEAs provided a possibility of studying the neuronal networks as a whole. It enabled the study of connections between neurones (e.g. gap junctions) in culture compared to multiple single-unit recordings.

2.2. CELL CULTURE

2.2.1. Animals

In this study both male and female foetal rats (*Rattus rattus)* were used. While establishing the tissue culture methods, several rat strains were initially evaluated, finally to use only Charles River United Kingdom (CRUK) rats. Rats were obtained via the Biomedical Sciences Unit at the University of Nottingham. Animals were bred in the unit following timed matings, brought up until the required age (E14-16) and kept in a 12h light:12h dark cycle maintained at 23±2°C with food and water available *ad libidum.*

Experiments conducted on dorsal root ganglia attached to spinal cord used foetal CRUK rats (E15). At this stage of development the dorsal root ganglia remained attached to the spinal cord when removed and the ganglia contain few fibroblasts and Schwann cells.

Foetal ICR mice from Harlan Spraque-Dawley (*Mus musculus*) and foetal CRUK rats were used in the gap junction blocker study. The gestation time for spinal cord preparations was E14-15 days at the time of dissection. For the frontal cortex preparations the gestation time was one day longer, E15-16 days. The gap junction blocker study was performed at the CNNS, University of

North Texas, USA (for mice cultures only) and at the University of Nottingham (for rat cultures only).

2.2.2. Dissection

All the work was carried out in a laminar flow hood using aseptic technique with sterile equipment and solutions to maintain sterility. The contents of the dissection solution varied depending on the way the material was to be used and are described in chapters 2.2.2.1. and 2.2.2.2.

The work was carried out under the UK Home Office project license for primary culture of mammalian cells (40/1955). The gap junction blocker study on mice was carried out under the project licence of CNNS, University of North Texas, USA.

2.2.2.1. *Dorsal Root Ganglia and Spinal Cord*

Fifteen day - pregnant rats (and 14-15 day – pregnant mice for the gap junction blocker study), were killed by an inhalation of overdose of an anaesthetic (Halothane BP 100% v/v, Rhodia Organique Fine Ltd, UK or Chloroform, Sigma, USA for the gap junction study) in an anaesthetic jar, followed by

cervical dislocation. The abdomen was then rinsed with 70% ethanol. An incision was made through skin, the underlying fat and muscle in the midline of the low abdomen. The cut was continued up to the ribs along each side to create a large flap to expose the uterine tract. The uterine tube was cut at one end and the cut was continued through fat connecting the tube to the animal without actually cutting the tube. The uterine tube was removed and placed into a beaker containing dissecting solution with antibiotics (1.13g 10mM HEPES and 800 µl penicillin/streptomycin in 500ml Ca^{2+}/Mg^{2+} free HBSS for rats / 12.3mM HEPES, 8.7mM sucrose, 42mM glucose, 137mM NaCl, 5.4mM KCl, 0.6mM Na_2HPO_4 and 0.2mM KH_2PO_4 for mice). From there the uterine tube was moved to a Petri dish containing dissection solution without antibiotics and the tube was cut along its length to reveal each embryo. Great care was taken not to burst embryonic sacks. Each embryonic sac was cut or teased from the uterine tube and transferred into a Petri dish containing antibiotic-free dissection solution. The membrane of each sac was pulled or cut away and the sacs were detached by cutting umbilical cord. The embryos were transferred into a Petri dish containing antibiotic-free dissection solution. The heads, tails and intestines of the embryos were removed and the vertebra was cut through from top to bottom (rostral to caudal) leaving the meninges uncut. Then the vertebral column was spread in order to loosen the surrounding tissue, spinal cord and dura at the medulla were hold while pulling straight up and holding the vertebral column down with forceps. The spinal cord and attached dorsal root ganglia were then removed. The tissue was kept moist throughout the process using antibiotics-free dissection solution.

2.2.2.2. *Frontal Cortex*

Fifteen to sixteen day – pregnant mice were killed by an inhalation of an overdose of Chloroform (Sigma, USA) in an anaesthetic jar, followed by cervical dislocation. The embryos were removed as before and placed in a Petri dish containing dissection solution. The embryos were decapitated and the heads placed in another dish. The head was placed to rest on the dorsal surface and the dorsal side of the foramen magnum was grasped using sterile forceps. The skin was peeled laterally, proceeding rostrally and flipping the head onto its ventral surface. After the removal of the skin, the cartilaginous skull was removed using the same method and the dorsal surface of the brain was exposed. The brain was lifted out and placed into clean Petri dish with its ventral side up. The meninges were removed and the hemispheres separated from the rest of the brain by placing the tines of the forceps along the rostral and caudal edges of mid-brain and squeezing them together. The hemispheres were orientated so that medial surfaces were upwards. The olfactory bulbs were removed and a cut was made through the dorsal margin of the cortex at a point approximately one-third of the way from the rostral edge to the caudal edge. The cut was continued from this incision along the dorsal margin to the hole left by the removal of the olfactory bulb and ventrally to the caudato-pallial angle, the line where the cortex and caudate nuclei meet. Finally, the cut was continued rostrally along the line to the hole left by the olfactory bulb. The tissue circumscribed by these cuts contained the frontal cortex.

2.2.3. Dissociated Cell Culture Preparation

For the cell dissociation studies the tissue was removed as described in section 2.2.2. After the isolation of the tissue, it was placed into a small conical flask containing 4ml of Ca^{+}/Mg^{+} free Hanks' balanced salt solution (HBSS). One ml of 2.5% trypsin-EDTA solution (Sigma, UK), diluted in Ca^{2+}/Mg^{2+} free HBSS, was added and the flask sealed with a sterile bung. The flask and its contents were incubated at 37°C for 45 minutes after which the trypsin solution was removed and the tissue rinsed twice for 5 minutes with 20% new born calf serum (NCS) in 0.1M PBS. The tissue was then dissociated carefully using Pasteur pipettes. The tissue suspension was centrifuged at 1000rpm for 5 minutes and the formed cell pellet was re-suspended with 5ml of full culture medium (FCM; 10% MEM (Life Technologies, UK), 10% NCS (Life Technologies, UK), 5% sodium bicarbonate (Sigma, UK) and 1% L-glutamine (Sigma, UK) in 74% SUPW). A cell count was then performed with haemocytometer (Weber Scientific International, UK) and 1ml of suspension was placed on to PDL-coated cover slips. Desired cell concentration ($4{,}5x10^{5}$) was pipetted on the central matrix area of the MEA, and the MEAs incubated for one hour at 37°C, 5% CO2 to allow attachment on the MEA matrix. After the incubation, 1.5ml of media was added and the MEAs returned to the incubator.

After 24 hours *in vitro*, the full culture medium was replaced with 1ml of serum-free neurobasal medium (Sigma, UK). The medium was thereafter changed twice a week, adding some serum every second week, until the cultures had matured. During this period the slices were examined daily under a microscope to follow the development of the cells.

For the gap junction blocker study a modified neural dissociation procedure was used. After the isolation of tissue, the dissection solution was removed and the tissue was mechanically dissociated using sterile scalpel blades. For spinal cord dissociation, 30µl DNAse I (4.16mg/ml PBS; Roche, Switzerland) and 3ml of 0.7mg/ml papain (diluted into dissection medium; Roche, Switzerland) was added onto the cells and the suspension was incubated for 15 minutes at 37 ° in 10% CO_2. DNAse I is used to prevent clumping of the cells in the suspension, papain is added for enzymatic dissociation. After the incubation, the mixture was centrifuged for 5min at 900 r.p.m. The supernatant was removed and the cell pellet resuspended into 5ml of spinal cord seeding medium (MEM 10/10: 20% FBS (Life Technologies, USA) and 20% horse serum (Life Technologies, USA) in MEM (Life Technologies, USA). The centrifuging and resuspension were repeated and 12.5µl DNAse I was added into the final cell suspension. Cell count was performed and desired cell concentration ($4.5x10^5$) was pipetted on the matrix area and the MEAs were incubated for one hour at 37°C, 10% CO_2 to allow attachment on the MEA matrix. After the incubation, 2ml of media was added and the MEAs returned to the incubator.

Frontal cortex was not treated with papain, but 12.5µl DNAse I was diluted further into 5ml of frontal cortex seeding medium (DMEM 5/5: 0.2% ascorbic acid, 4mg/ml in H_2O (Sigma, USA), 2% B-27 supplement (Life Technologies, USA), 5% FBS (Life Technologies, USA) and 5% horse serum (Life Technologies, USA) in 88% DMEM (Life Technologies, USA) and added on the cell suspension. Mechanical dissociation was continued using a sterile large bore plastic pipette. A cell count was performed and the desired cell concentration ($4.5x10^5$) was pipetted on the central matrix area and the MEAs incubated for one hour at 37°C, 10% CO_2 to allow attachment on the MEA matrix. After the incubation, 2ml of media was added and the MEAs returned to the incubator.

A 1ml medium change was carried out three times per week, until the cultures reached the age of 30 days *in vitro*. At this age, FBS was left out of the medium and changes continued as before until the cultures were used for electrophysiological experiments. During the medium changes, the slices were examined under a microscope to follow the development of the cells (Fig 2.3.).

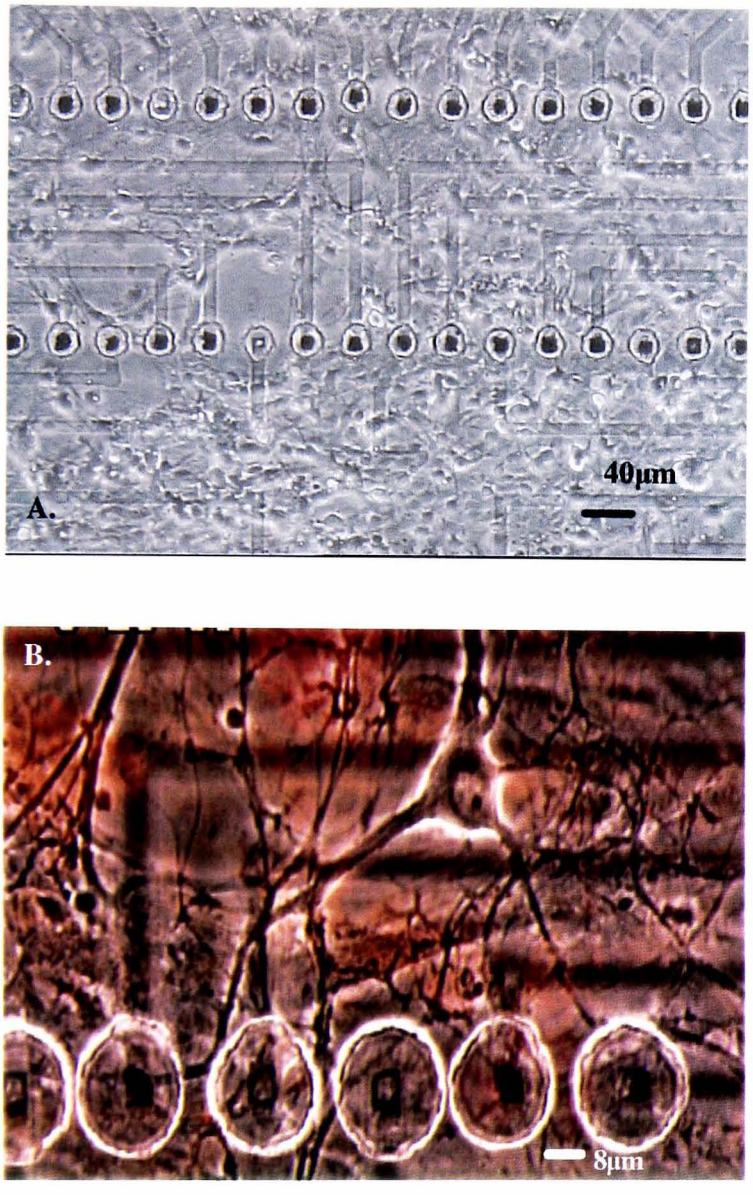

Figure 2.3. A photomicrograph of dissociated spinal cord cell cultures (22DIV) grown on MEAs. The electrodes can be seen as dark dots surrounded by a lighter electrode crater. (A) Individual neurones are not clearly visible possibly due to the glial proliferation. (B) The higher magnification photomicrograph reveals a neurone with processes growing over the electrodes.

2.3. DATA ACQUISITION AND ANALYSIS

Two different data acquisition systems were used in this experimental study: the majority of the work was carried out using the Multichannel Acquisition Processor (MAP, Plexon Inc., USA; chapter 2.3.1.). Due to the limited availability of a second MAP system at Nottingham, the gap junction blocker study on rats was done using the Recorder system (Plexon Inc., USA; chapter 2.3.2).

2.3.1. Multichannel Acquisition Processor (MAP)

MAP enabled simultaneous recording of timestamps and waveforms from MEAs. The MAP provided programmable amplification, filtering, switching and digital signal processing of the electrical signals recorded from the neuronal cell cultures grown on MEAs. The hardware architecture of the MAP (Fig. 2.4.) comprises modular system of plug-in circuit boards in a stand-alone box from 16 to 128 channels. A serial line and an external parallel bus formed a connection from the MAP system to a host PC workstation.

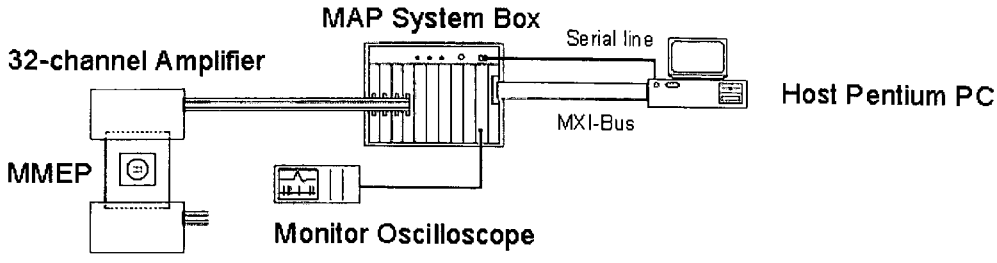

Figure 2.4. The 64-channel MAP system architecture used for the data acquisition.

Two 32-channel preamplifiers (gain x100) were connected to the ITO contact strips on either side of the MEA with 'zebra strips', made of carbon elastomere. Four 16-way ribbon cables were used to connect the preamplifiers to the four signal input boards (SIGs) in the MAP system box. Each of the SIGs provided a programmable gain, filtering and analogue to digital (A/D) conversion for 16 signals. The total number of SIGs used in Nottingham was four, allowing 64 inputs in total. The number of SIGs in the University of North Texas, USA, was only two, and therefore the maximum number of inputs was 32.

After filtering (500Hz-50kHz, with a 50Hz notch filter) and additional gain producing typically a final gain of x10000, the digital signal processor (DSP) board captured the waveforms with A/D conversion performed simultaneously by sampling 12-bit at 40kHz (25μs) per channel. Alternatively, the time stamped threshold waveform segments were saved for off-line sorting and further analysis. The spike sorting was performed so that up to four different action potential waveforms per channel could be discriminated. To visualise and

monitor the action potentials, the signals were led to an oscilloscope via an output board (OUT).

2.3.1.1. *Data Acquisition Using the MAP System*

Real-time spike discrimination was performed on-line using RASPUTIN (Real-time Acquisition System Programs for Unit Timing in Neuroscience; Plexon Inc., USA) spike separation algorithm based on a hierarchical analysis: the spike must first cross a user-defined amplitude threshold followed by the waveform passing through a pair of again user-defined graphical time-voltage windows. The spikes and timestamps of all active channels were then sent to the host PC (Dell Pentium 2GHz, running Windows XP platform) through a 16-bit parallel bus. The data was recorded in Plexon file format (.plx), which contained the timestamps for all of the spikes as well as waveforms.

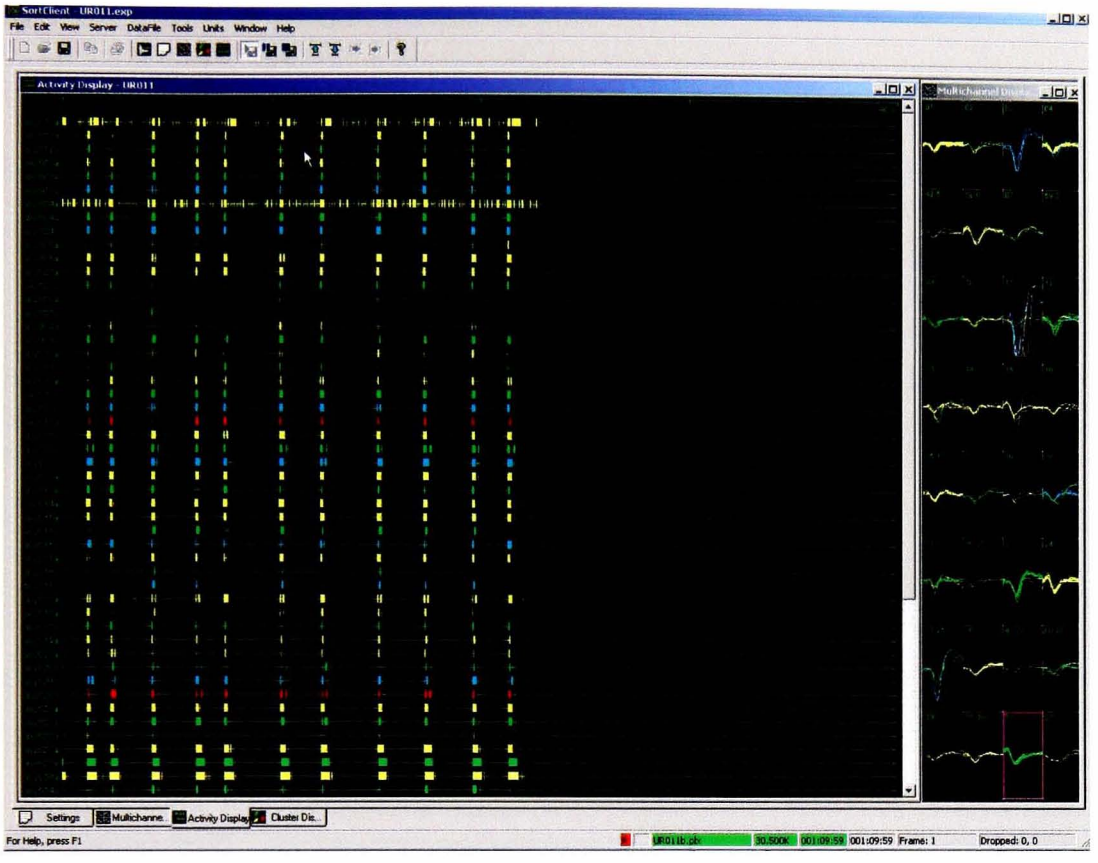

Figure 2.5. Representative on-line computer monitor display from the MAP sort client. Left – spike trains for the active electrodes. If more than one active neurones are discriminated per DSP, a suffix (a,b,c and d) and change of colour indicate the different neurones. Right – Multichannel digital oscilloscope shows the waveforms collected from individual electrodes.

The software interface for the MAP system box, RASPUTIN, enables the experimenter to view and acquire action potential waveforms around a voltage-threshold crossing, sort them in real time according to their shape and record continuous analog signals, such as field potentials. The main components of the Rasputin are the Server, the Sort Client, the Plate Client (PLAC).

The Server is a low-level interface to the MAP system box transferring commands (e.g. gain changes) from the various clients to the MAP box. It also delivers the data from MAP system box to the client programs. The Sort Client is an interface allowing the configuration of the spike sorting parameters of the MAP system box. It also allows on-line waveform discrimination and sorting. The Sort Client also includes a display of all the captured waveforms and a raster display of the spiking activity (Fig. 2.5.). The Sort Client and the PLAC provide together user-controlled switching of the filters and adjustment of the gain on individual channels. The PLAC is also used for the assignment of DSP channels to input channels.

2.3.2. Data Analysis

Two different types of software were used for the data analysis. NeuroEXplorer (Nex; Nex Technologies, USA) is a well established software package allowing all the standard histograms, including rate histograms, interspike interval histograms, peri-event histograms, autocorrelograms and crosscorrelograms to be calculated. It is able to simultaneously calculate and display histograms for all the spike trains in a data file. In addition to histograms, Nex also offers a range of other analysis tools, including rasters, peri-event rasters and burst analysis. NACTAN (Network ACTivity ANalyzer, written in LabView), was developed in house for the University of North Texas, USA. It was used only in the gap junction blocker experiments conducted in the University of North

Texas. NACTAN averaged network spike and burst rates (Fig. 2.6.) and facilitated long term monitoring of network stability and network responses to neuroactive agents.

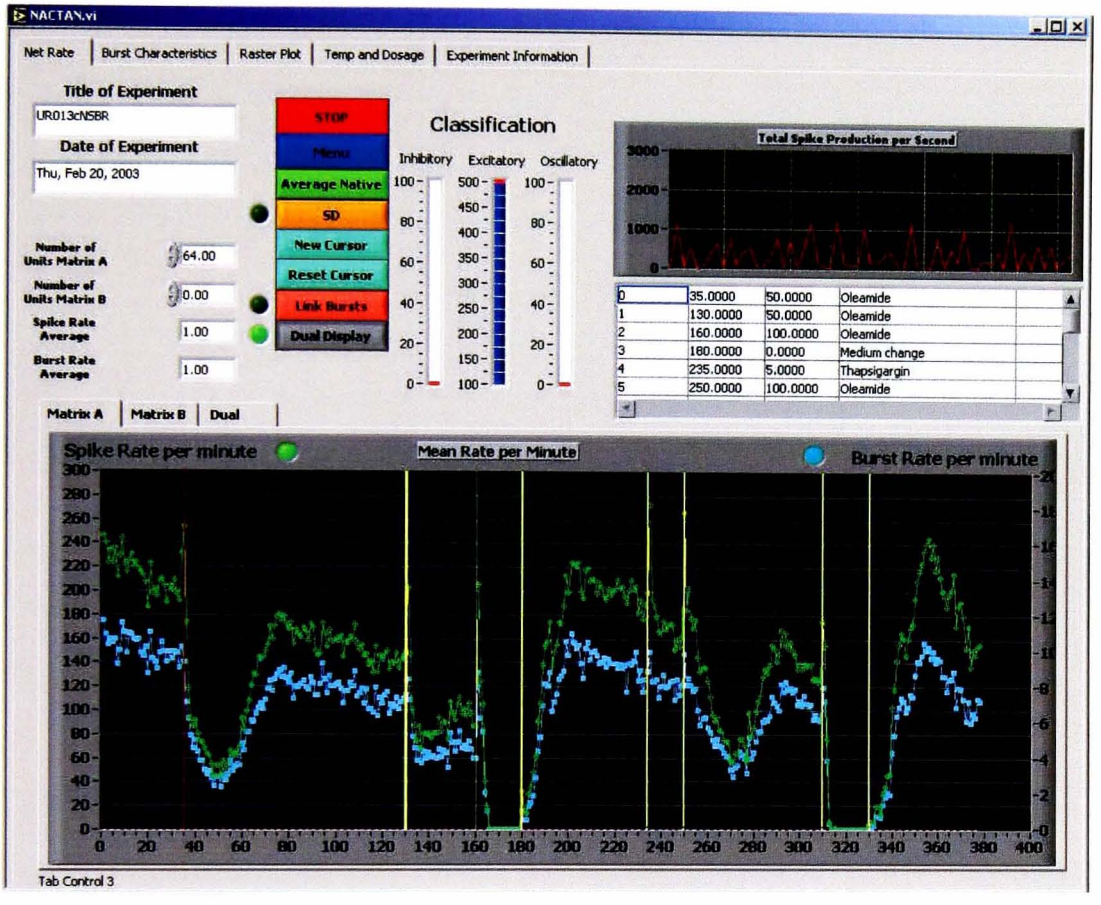

Figure 2.6. Representative on-line computer monitor display from NACTAN. Top – details of the experiment (name, date, number of active cells, total spike production/second, added drugs) and control buttons. Bottom – real-time display of the average spike/burst rate over the network.

For this experimental study, a burst was defined as a minimum of three spikes occurring with intrespike intervals <0.01s. When spikes and bursts of two or more simultaneously recorded neurones were compared (synchrony), they often appeared to be close, especially between neurones located by adjacent

electrodes, but bursts seldom started and ended simultaneously on two channels. Recurring bursts of one neurone were occasionally accompanied by timelocked bursts of other neurones suggesting the triggering role of some cells. If two neurones were found to exhibit a statistically significant covariation in firing probability, the cells were considered to be internally synchronised. Such episodes occurred both spontaneously during a response to a stimuli. These synchronised episodes manifested themselves at different timescales ranging from a few milliseconds to hundreds of milliseconds.

2.3.3. Data Acquisition Using the Recorder System

Recorder (Plexon Inc., USA) is a data acquisition software built for continuous recording of spikes, field potential and event data. It comprised of a National Instruments data acquisition card (PCI-6071E, 1.25M samples/sec, 64 channels, 12-bit resolution) in a host PC (Dell Pentium 1GHz, running Windows XP platform) and of two 32-channel preamplifiers (gain x100). Unlike MAP system box and RASPUTIN, Recorder does not allow spike sorting on-line, but provided additional gain (x1-100) and filtering (300Hz-4kHz). The unsorted spikes, non-spike analog signals and external digital events were saved to hard disk as .plx-filesand processed later with Offline Sorter (Plexon Inc., USA). After the sorting, the data were further anlysed with Nex. Recorder was originally developed for *in vivo* studies and has not previously been used for *in vitro* electrophysiological recordings.

CHAPTER 3

OLFACTORY EPITHELIUM AND BULBS

3.1. INTRODUCTION

Mammals use olfaction, the sense of smell, to find food and to protect them from predators in the wild. It is also used for protection from harmful substances, such as environmental contaminants (Jones & Rog, 1998). In addition it affects attraction, memories and emotions. The olfactory system is able to recognise and discriminate an immense number of different odour molecules – more than 400 000 compounds are said to be odorous to the human olfactory system. The central olfactory nervous system in mammals has evolved to construct an olfactory image of the object using the odour molecular information (Mori & Yoshihara, 1995).

In brief, the olfactory system consists of olfactory epithelium, olfactory bulbs and olfactory cortex. Odour recognition initiates in the nose, where odorants are detected by a large family of olfactory sensory neurones - in human, 339 intact olfactory sensory neurone genes and 297 olfactory sensory neurone pseudogenes have been identified (Malnic *et al.*, 2004). The olfactory sensory neurones are located in the olfactory epithelium (e.g. Allison, 1953; Graziadei,

1977) and the odour discrimination starts with the interaction of volatile odour molecules with these olfactory sensory neurones (Mombaerts *et al.*, 1996), from where the signals are transferred to the olfactory bulbs, which receive direct axonal input from the sensory neurones of the olfactory epithelium (e.g. Mori & Yoshihara, 1995) and project directly to the olfactory cortex. The olfactory bulbs themselves are outgrowths of the forebrain and are specialised in processing these molecular signals that give rise to the sense of smell (Shepherd & Greer, 1990).

The olfactory epithelium and the olfactory bulbs were of special interest to this part of the experimental study. Both, olfactory epithelium and olfactory bulbs have been, individually, successfully cultured *in vitro*. However, attempts to establish an *in vitro* model of the olfactory epithelium with its afferent connections to olfactory bulb, are few and there are no analysis of cellular organisation and morphology of these co-cultures *in vitro*. In this study, the anatomy and physiology of the olfactory epithelium and olfactory bulbs were investigated, both in individual cultures and in co-cultures, in order to develop an *in vitro* sensory system and in an attempt to understand sensory processing at a neural network level.

3.2. OLFACTORY SYSTEM

3.2.1. Olfactory Epithelium

The olfactory epithelium (Fig. 3.1.), where initial events in reception of odour molecules occur, lines the posterodorsal wall of the nasal cavity. It is formed of bipolar neurones, the olfactory sensory neurones (e.g. Allison, 1953; Graziadei, 1977). The olfactory sensory neurones have diverse protein sequences but can be assigned to subfamilies - members of the same subfamily have related sequences and are likely to recognize structurally related odorants (Malnic *et al.*, 2004). Several tens of millions of these neurones are distributed over the thin epithelium, which is developed over delicate bones, the turbinal bones, in both the right and left side of the nasal cavity. Each one of the individual sensory neurones extends a single, bipolar and round bodied dendrite to the surface of the epithelium and at the tip of the dendrite they emit 10-23 cilia, up to 200μm long, into the mucus layer that covers the olfactory epithelium (Jones & Rog, 1998). The dendritic knobs and sensory cilia sit in the air space of the nose, covered by layers of mucus (Pixley, 1996).

The olfactory sensory neurones express odour receptor proteins on the surface membrane of the cilia and thus expose the extracellular portion of the olfactory receptor protein to odour molecules carried by the stream of inhaled air and dissolved in the mucus. The unmyelinated axons, with an approximate diameter

of 0.2μm (Shepherd & Greer, 1990), of the olfactory sensory neurones project through the basal lamina and the lamina propria towards the olfactory bulb. They fasciculate into mesaxons surrounded by glial cells and penetrate the anterior and ventral surfaces of the olfactory bulbs. The axons then terminate in the second layer of the bulbs, glomerular layer, where they participate in the formation of the olfactory glomeruli. The other two cell types present in the olfactory epithelium are supporting (sustentacular) cells and basal (stem) cells (Mori & Yoshihara, 1995).

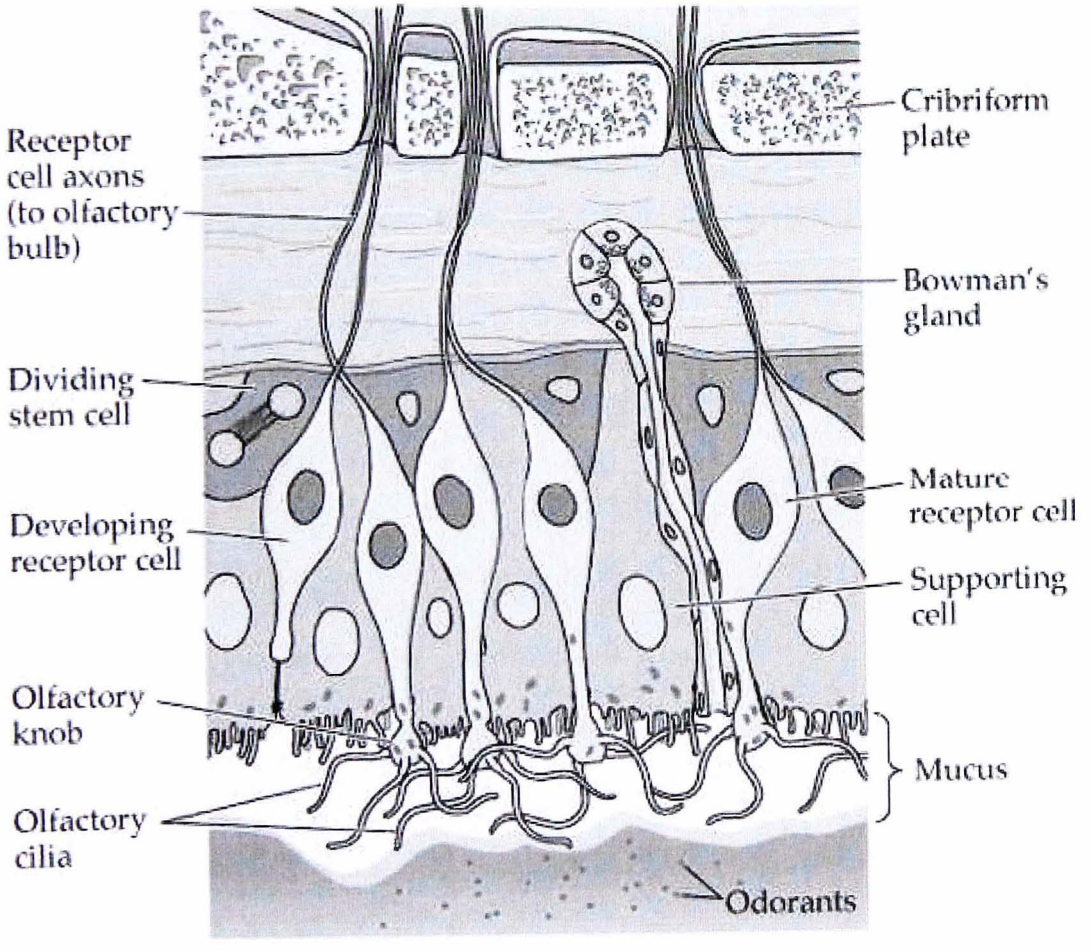

Figure 3.1. A line diagram of the olfactory epithelium.

The olfactory sensory neurones are continually generated by mitotic division of the small, polygonal basal cells (Graziadei & Monti-Graziadei, 1979), even in adult mammals. These basal cells distinguish into immature olfactory receptor neurones and eventually into olfactory marker protein expressing neurones. The neurogenesis, neuronal differentiation, maturation and death occur in the olfactory epithelium at all times (e.g. Graziadei & Monti-Graziadei, 1978). The whole epithelium is replaced approximately every 40 days (Jones & Rog, 1998). This generation takes place in the deep layer of the olfactory epithelium. The new sensory neurones are able to extend their dendrites with specific odour receptor proteins towards the epithelial surface, as well as projecting unmyelinated axons to form synaptic connections with the olfactory bulb neurones at specific target sites called glomeruli (Mori & Yoshihara, 1995). The specificity of the olfactory sensory neurone connections in the glomeruli is maintained despite the constant remodelling (Singer *et al.,* 1995). Such degree of plasticity is unique among the central nervous system and is inherent to the olfactory sensory neurones and relies on trophic factors (Royal *et al.,* 2002).

The supporting sustentacular cells of the olfactory epithelium are non-myelinating glial cells. They ensheath the olfactory receptor neurones and maintain the normal extracellular potassium levels needed for neuronal activity (Kimmelman, 1993). *In vivo* they express a mixture of astrocyte-specific and Schwann cell-specific phenotypic features with the former cellular phenotype predominating, but *in vitro* they can assemble a myelin sheath when co-cultured with dorsal root ganglion neurones. Thus, certain *in vitro* conditions induce ensheathing cells to express a phenotype more like that of a myelinating

Schwann cell (Devon & Doucette, 1992). The Schwann cell-specific phenotype are primarily bipolar spindle shaped cells with long, thin processes and are easily mistaken for olfactory sensory neurones whereas the astrocyte-specific phenotype have a characteristic stellate morphology with thin, short and often extensively branched processes (Pixley, 1992).

The olfactory epithelium is unique in the central nervous system, not only because of its ability to regenerate, but also being the only part in direct contact with the external environment (Firestein *et al.,* 1996). The cells exposed to the environment, and therefore to the pollutants, have a shorter lifespan than the approximate of one month (Williams, 1995). It is likely that apoptosis is important in maintaining a balance between cell proliferation and death (Calof *et al.,* 1996). Schwob *et al.* (1992) have also shown that the prolonged survival of the olfactory sensory neurones is trophically dependent on the olfactory bulb.

Degeneration of the olfactory epithelium is evident in the cellular arrangement and topographic distribution. There are also extensive changes in the cellular organisation in the adult epithelium compared to that of a foetus (Nakashima *et al.,* 1984). During the early mammalian development, some of the olfactory sensory neurone axons terminate transiently in the deeper layers but recede back and all sensory systems are within the glomeruli in the adult (Farbman, 1994).

Olfactory sensory neurones can be identified from the other cell types in the olfactory epithelium using polyclonal antibodies against olfactory marker protein (Chuah & Zhengh, 1987). Basal cells are small and polyclonal,

contacted with the underlying basement membrane in response of regeneration, both normally and after damage caused to the olfactory epithelium. The cells of the epithelium undergo apoptosis in all stages, whether they are neuronal precursors, immature or mature olfactory sensory neurones (Holcomb *et al.*, 1996). This has a likeness in other parts of the nervous system where many more neurones are produced than remain after they have made synaptic connections (Oppenheim, 1991).

The olfactory receptors of the olfactory epithelium have amino acid sequences expressed from about 500 genes and are likely to be interacting with several different odours each. It is still unknown, whether the large number of compounds which these receptors can recognise can be matched by the number of specific receptor proteins, each being a product of a separate gene and expressed on different genes (Lancet, 1994), or whether each olfactory receptor is able to express several receptor proteins, or whether each of these receptor proteins has broad binding specificities (Jones & Rog, 1998). It is also uncertain, if individual odorants have exact receptors to distinguish them (Bargmann, 1996), even though electrophysiological studies by Sicard and Holley (1984) have shown that, given a concentration high enough, each olfactory sensory neurone is able to recognise a range of different odours.

There are only a few electrophysiological recordings made from isolated mammalian olfactory sensory neurones due to their fragility and small size. In general, stimulation of the olfactory receptor cells of the olfactory epithelium leads to the generation of a depolarising receptor current which triggers spike

firing of increasing frequency with increasing odour concentration (Reisert & Matthews, 2001). The few electrical recordings made reveal that the isolated mammalian olfactory receptor cells respond to odour exposure with an inward current (e.g. Maue & Dionne, 1987; Lowe & Gold, 1995), which is mainly carried by an excitatory Ca^{2+}-activated Cl^- conductance (Lowe & Gold, 1993).

The isolated mammalian olfactory sensory neurones have high input resistances, and action potentials can be elicited by picoampere amounts of depolarizing current. The receptor neurones respond to applied odorant molecules with increases in membrane conductance (Maue & Dionne, 1987). The odour reception is dependent on a combination of molecular, cellular and network interactions in the olfactory system. Synchronised activity in networks of neurones is known to be an important feature of odour processing - the reception and discrimination of odorants is associated with fast (20-70 Hz) oscillations, even though the cellular mechanisms underlying these fast network oscillations have not been defined (Friedman & Strowbridge, 2003).

Ca^{2+} plays an essential role in olfactory transduction (e.g. Menini, 1999). It enters the cell through cyclic nucleotide-gated channels (e.g. Frings *et al.,* 1995) during the odour response and activates an excitatory Ca^{2+}-activated Cl^- conductance and thereby increases the magnitude of the total receptor current (e.g. Lowe & Gold, 1993). Ca^{2+} also has negative feedback effects on various stages of olfactory signal transduction including the cyclic nucletide-gated channel, adenylyl cyclase and phosphodiesterase (e.g. Borisy *et al.,* 1992). Therefore it is crucial to control the intracellular Ca^{2+}-levels in olfactory

receptor cells, not only for determining the response duration but also in the process of adaptation (Reisert & Matthews, 2001).

The mechanism for removal of the Ca^{2+} from the intraciliary space is still unknown in rodents. In frog olfactory receptors Ca^{2+} is believed to be removed by Na^{+}-Ca^{2+} exchangers across the ciliary membrane (Reisert & Matthews, 1998). In rat olfactory epithelium the presence of these exchangers has been reported in the soma, dendrite and possibly in the cilia (Noe *et al.,* 1997), but their functional significance is still unclear (Reisert & Matthews, 2001).

3.2.2. Olfactory Bulb

The olfactory bulb, a spherical cortical structure, is the first relay station for olfaction and consists of only a few types of neurones (Fig. 3.2.). It is an outgrowth of the forebrain receiving all of the input from the olfactory sensory neurones and sending its output directly to the olfactory cortex (Sepherd & Greer, 1990). As a relatively simple organisation of the neuronal connections, olfactory bulb makes an ideal system for the study of relations between neurones (Frosch & Dichter, 1984).

The olfactory bulb is attractive region for experimental analysis because of several reasons. It is easily accessible because of its position in front of the rest of the brain. Its sensory nerves are completely separated from its output fibres to

the brain, which enables each one to be manipulated independently. The olfactory bulb is a distinctly laminated structure containing greatly differentiated cell types (Shepherd & Greer, 1990) - the outmost layer of the olfactory bulb is the olfactory nerve layer, followed by glomerular layer, external plexiform layer, mitral cell layer and granule cell layer. The individual types of the olfactory bulb neurones are most easily identified by their location within the lamina (Fracek *et al.*, 1994a).

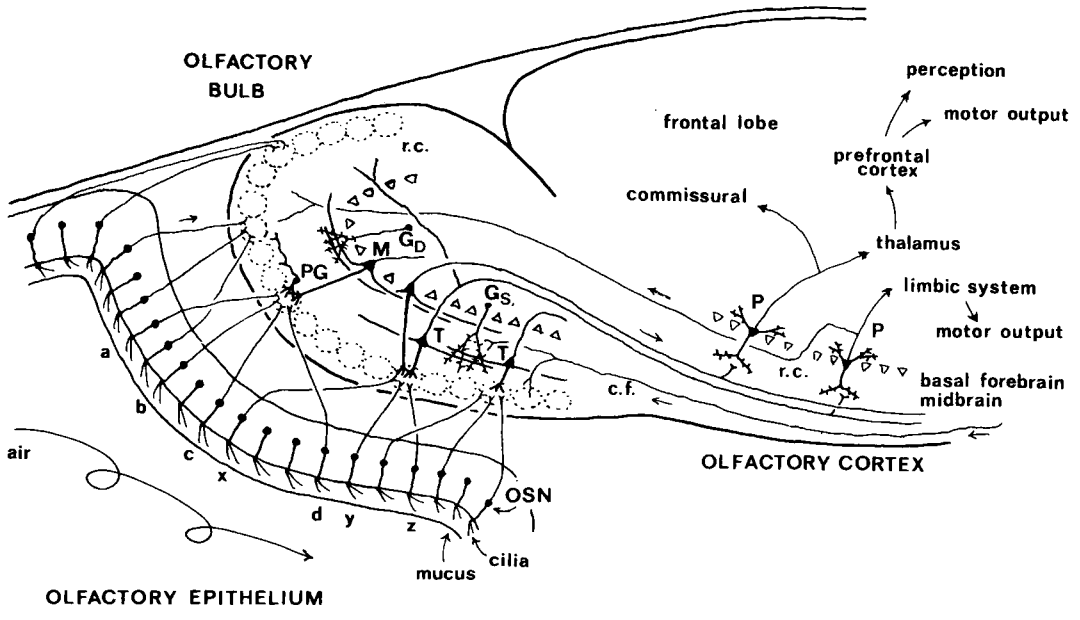

Figure 3.2. An overview of the olfactory pathway (From Sepherd & Greer, 1990). The olfactory bulb receives input from the receptor neurones in the olfactory epithelium and projects to the olfactory cortex. In the figure: a-d, x-z = overlapping populations of receptor neurones, OSN = olfactory sensory neurone, PG = periglomerular cell, M = mitral cell, T = tufted cell, G_S = superficial granule cell, G_D = deep granule cell, r.c. = recurrent axon collateral, c.f. = centrifugal fiber and P = pyramidal cell.

In addition to the great input from olfactory axons, the olfactory bulb contains a highly ordered synaptic organisation, which includes microcircuitry among principal neurones, local interneurones and centrifugal fibres (e.g. Mori, 1987; Shepherd & Greer, 1990) (Fig. 3.3.). At the surface of the olfactory bulb, bundles of olfactory axons show complex interweaving forming the olfactory nerve layer. These sensory axons are unmyelinated and their diameters vary

from 0.1 to 0.4 μm. They are tightly packed into Schwann cells and therefore provide an opportunity for ephaptic interactions between neighbouring axons (Sepherd & Greer, 1990). Beneath this layer lies the glomerular layer, which is formed of a number of glomeruli. This is where the olfactory axons terminate providing the only source of extrinsic excitatory input to mitral/tufted cells. In mammals, glomeruli are spherical with sharp borders. The olfactory sensory neurones only branch once inside the glomeruli. Each glomeruli is enclosed by glial membranes and the estimated mean numbers and diameters of glomeruli per main olfactory bulb are 1810 and 75-100 μm in mice, 2400 and 140 μm in rats and 1000 and 110 μm in man (e.g. Pinching & Powell, 1971; Royet *et al.*, 1988; Meisami & Sendera, 1993). The glomerular structure has been observed not only in mammals but also in a wide range of insects and arthropods (e.g. Boeckh *et al.*, 1990).

The two main cell types in the olfactory bulb are the mitral cells and the tufted cells. Mitral cells have their cell bodies, 15-30μm in diameter, in the mitral layer of the olfactory bulb, whereas somata of tufted cells, 50-150μm in diameter, are distributed in the external plexiform layer. An individual mitral/tufted cell projects its primary dendrite to a single glomerulus and receives synaptic inputs from a large number of olfactory axons. Mitral and tufted cells also have several secondary dendrites within the external plexiform layer and make several dendrodendritic synaptic connections, which are called reciprocal synapses, with granule cells, a type of interneurones in the olfactory bulb. Both cell types also send an axon to the olfactory cortices via the lateral olfactory tract (Mori & Yoshihara, 1995). The axons of the mitral cells serve as

the major outflow from the bulb (Frosch & Dichter, 1984). They proceed deep inside of the olfactory bulbs and pass caudally to gather at the posterolateral surface forming the lateral olfactory tract. Within the bulb, the axons give off recurrent collaterals. The collaterals remain within the granule layer and the internal plexiform layer (Orona *et al.*, 1984). The synapses formed by the mitral and tufted cells are asymmetrical and contain spherical vesicles in adult mammalian olfactory bulb (e.g. Pinching & Powell, 1971).

In mammals, the mitral cells can be divided into two subgroups based on their secondary dendrite branching patterns. The type one mitral cells send their secondary dendrites into the deepest region of the external plexiform layer, whereas the type two mitral cells send their secondary dendrites into the middle region of the external plexiform layer. The two types of the mitral cells form microcircuits with corresponding subtypes of interneurones (Macrides & Schneider, 1982).

The tufted cells can be divided into three subpopulations according to their laminar positions. The largest of the three groups is middle tufted cells, which lie in the middle of the external plexiform layer. Their dendrites terminate in the glomeruli and their axons give off collaterals confined to the internal plexiform layer then joining the lateral olfactory tract. External tufted cells have dendrites with distinctive branching patterns and give off collaterals in the internal plexiform layer and the granule cell layer of the olfactory bulb. Some of these send an axon to the lateral olfactory tract, those which do not, are classified as intrinsic neurones. The third subpopulation, internal tufted cells, overlaps in

distribution and morphology with outwardly displaced type two mitral cells (Mori & Yoshihara, 1995).

The mitral and the tufted cells differ in the detailed projection pattern of their processes (e.g. Macrides *et al.,* 1985). This suggests that these cell types are functionally differentiated (Mori & Yoshihara, 1995).

The afferent fibres of the olfactory bulb consist of the olfactory sensory neurones and the central or centrifugal inputs from higher brain centres. The olfactory sensory neurones transmit the primary stimulus to the olfactory bulb, the central inputs provide control to the olfactory bulb. The olfactory sensory neurones are grouped into bundles prior to entering the olfactory bulbs and splay out and interweave after entering the bulbs. The axons reorganise themselves into functionally related subsets (Mombaerts *et al.,* 1996; Treloar *et al.,* 1996) with the axons terminating in the glomeruli.

Local interneurones in the olfactory bulb can be classified into three main types; granule cells, periglomerular cells and short axon cells. Granule cells are small, axonless neurones, which are distributed in the layer called granule cell layer. Each of the granule cells also gives rise to a superficial process which branch out and terminate in the external plexiform layer. The granule cells can be divided into three subpopulations: superficial granule cells with peripheral dendrites in the external plexiform layer, deep granule cells with dendrites to the deep external plexiform layer and intermediate granule cells with dendrites everywhere in the external plexiform layer (Orona *et al.,* 1983). Periglomerular

cells are distributed in the periglomerular region of the glomerular layer. The cells surround the glomeruli and project their primary dendrites into glomerulus and receive direct input from olfactory axons. The dendrites of these cells also make reciprocal synaptic connections with mitral/tufted cells in the glomeruli. Periglomerular cells are among the smallest in the brain with a diameter of only 6-8μm (Sepherd & Greer, 1990). Short axon cells with several morphological types are present in the external plexiform layer, mitral cell layer and granule cell layer as a small population (Price & Powell, 1970). The cell synapses formed by local interneurones, including reciprocal synapses, are symmetric and have flattened, 'pleomorphic' vesicles, whereas synapses formed by mitral and tufted cells are asymmetric and contain spherical vesicles (e.g. Price & Powell, 1970; Pinching & Powell, 1971). Figure 3.3. shows the neural elements of the olfactory bulb and their location in the bulb.

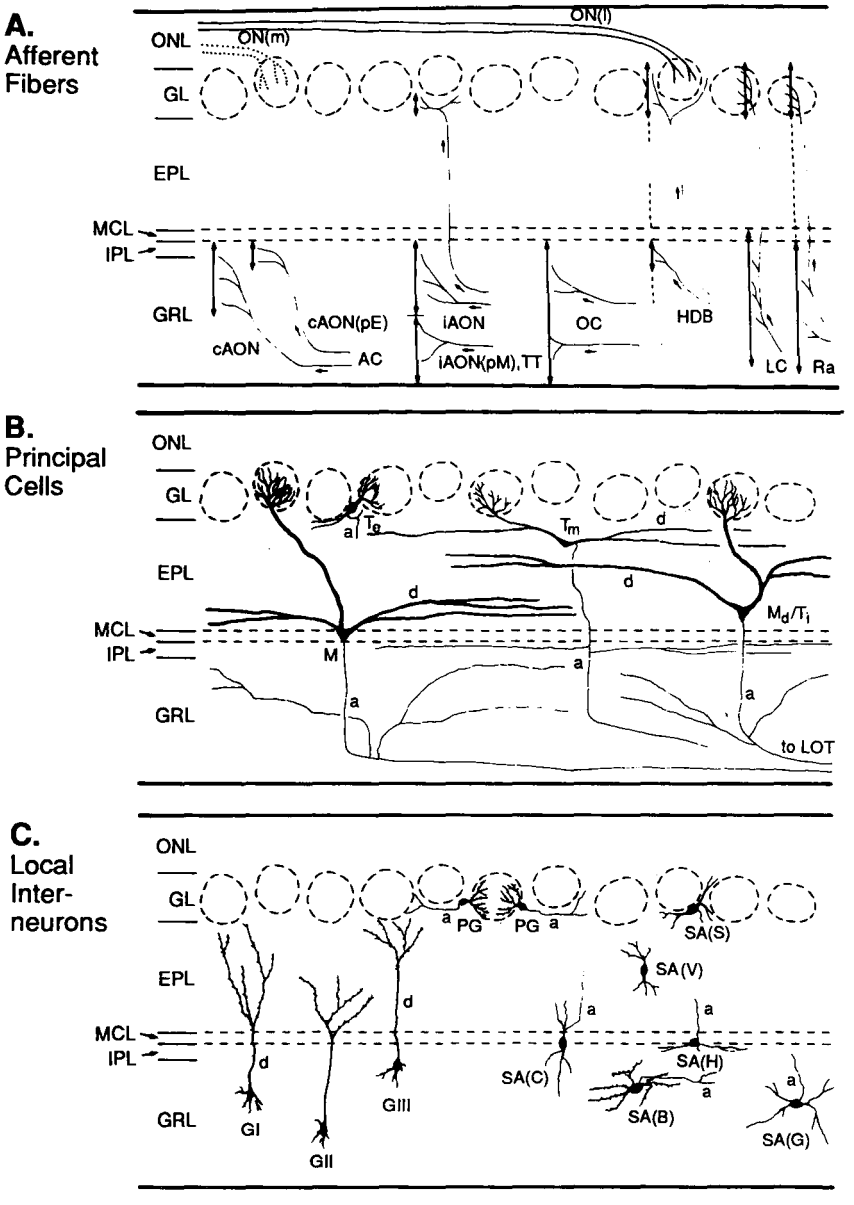
A.
Afferent
Fibers
ONL
GL
EPL
MCL
IPL
GRL
ON(m)
ON(l)
cAON
cAON(pE)
AC
iAON
iAON(pM),TT
OC
HDB
LC
Ra
B.
Principal
Cells
ONL
GL
EPL
MCL
IPL
GRL
a
T_g
T_m
d
M
M_d/T_i
to LOT
C.
Local
Inter-
neurons
ONL
GL
EPL
MCL
IPL
GRL
PG
SA(S)
SA(V)
SA(H)
SA(C)
SA(B)
SA(G)
GI
GII
GIII

Figure 3.3. The neural elements of the mammalian olfactory bulb (From Sepherd & Greer, 1990, modified from Mori, 1987). The elements are grouped according to the subdivision into inputs (afferent fibers), principal neurones and intrinsic neurones (local interneurones). In the figure: ONL = olfactory nerve layer, GL = glomerular layer, EPL = external plexiform layer, MCL = mitral cell layer, IPL = internal plexiform layer, GRL = granule cell layer. In part **A**, ON(m) and ON(l) idicate medial (m) and lateral (l) subtypes of olfactory nerve fibers. Centrifugal afferents are from the contralateral anterior olfactory nucleus (cAON), ipsilateral anterior olfactory nucleus (iAON), tenia tecta (TT), olfactory cortex (OC), horizontal limb of the diagonal band (HDB), locus coeruleus (LC) and raphe nucleus (Ra). pE = pars externa of the AON, pM = pars medialis of the AON. In part **B**, M = mitral cell, M_d = displaced mitral cell, T_i = internal tufted cell, T_m = middle tufted cell, T_e = external tufted cell, a = axon and d = dendrite. In part **C**, GI, GII and GIII = three types of granule cells, PG = periglomerular cell, SA(B) = Blanes' cell, SA(C) = Clandins' cell, SA(G) = Golgi cell, SA(H) = Hensen's cell, SA(S) = Schwann cell, SA(V) = van Gehuchten cell and LOT = lateral olfactory cell.

Development of the olfactory bulb in rodents takes place during the latter half of gestation and early postnatal life. In mice the final cell divisions, migrations and the formation of synaptic connections occur after the embryonic day 13 (Hinds, 1968a; 1968b; Hinds & Hinds, 1976). The main cell types undergo final division earlier than smaller interneurones (Frosch & Dichter, 1984). According to Hinds (1972a; 1972b), mitral cells acquire their adult position and neurite orientation by the day 15 of gestation. Judged by the presence of many axodendritic synapses and some dendrodendritic synapses, synaptogenesis is well underway (Hinds & Hinds, 1976). Frosch and Dichter (1984) also suggested, that cultures made from younger animals contain a larger fraction of cells other than granule cells. Mitral and tufted cells are differentiated prenatally, whereas periglomerular and granule cells continue to be generated late in the postnatal period and migrate to the olfactory bulbs via the rostral migratory stream (Kishi, 1987).

The cells of the olfactory bulb use several transmitters including GABA (γ-aminobutyric acid; e.g. Halasz *et al.,* 1979; Nicoll, 1971), dopamine (e.g. Halasz *et al.,* 1978; Priestley *et al.,* 1979) aspartate and/or glutamate (Harvey *et al.,* 1975). The summary of the putative neurotransmitters and neuromodulator substances of the main type of neurones is shown in figure 3.4.

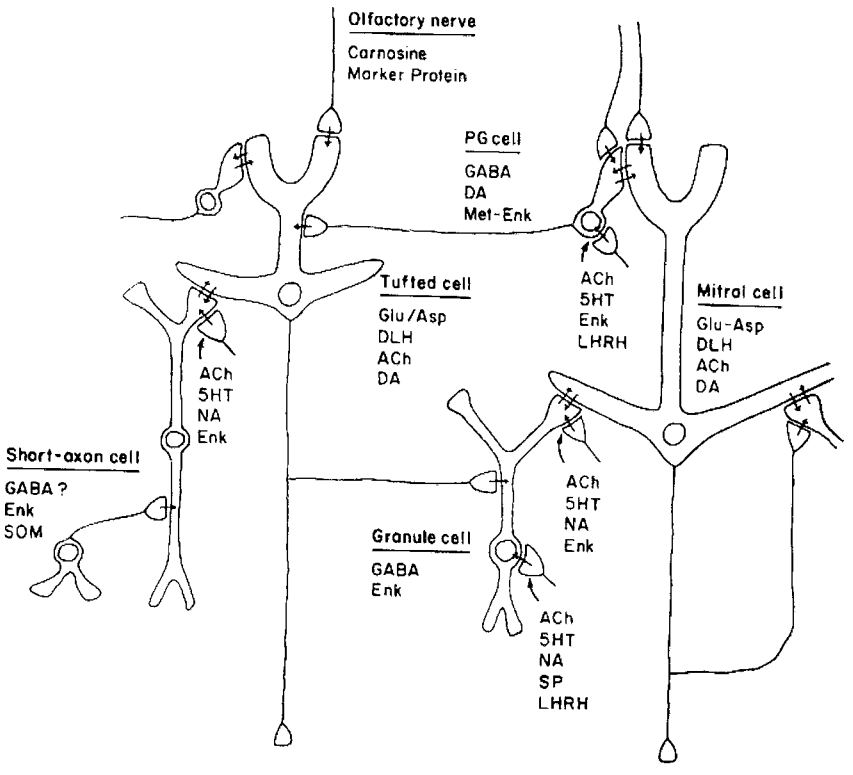

Figure 3.4. Schematic diagram of the olfactory bulb showing the synaptic circuits and the main neurotransmitter and neuromodulator substances (From Halaz & Shepherd, 1983). In figure, ACh = acetylcholine, DA = dopamine, DLH = DL-homocysteate, Enk = Met-enkephalin, Glu-Asp = glutamate-aspartate, 5HT = 5-hydroxytryptamine, LHRH = luteinizing hormone releasing hormone, NA = nor-adrenaline, SOM = somatostatin and SP = substance P.

The olfactory sensory neurones in the olfactory epithelium contain a special peptide (olfactory marker protein) and have a high concentration of the dipeptide carnosine (Margolis, 1988), but neither of these molecules has been shown to be neuroactive at the synapses formed by olfactory sensory neurones (Sepherd & Greer, 1990).

Halasz *et al.* (1977) showed in their study that some periglomerular cells of the olfactory bulb are positive for dopamine-synthesizing enzymes. The dopamine is known to be transneuronally regulated and degeneration and regeneration of olfactory nerves to be paralleled by changes in dopamine and dihydroxyphenylacetic acid levels in the olfactory bulb (Baker *et al.,* 1983). It is also known that some of the periglomerular cells and their dendrites contain glutamic acid decarboxylase, which is known as a GABA-synthesizing enzyme, as well as taking up GABA itself (Ribak *et al.,* 1977).

Action potential in mitral/tufted cells release glutamate from their lateral dendrites onto the dendrites of the granule cells evoking GABA-ergic recurrent and lateral inhibition of mitral cell activity (e.g. McLennan, 1971). Recurrent and lateral inhibition of these cells are thought to be important in odour discrimination (Yokoi *et al.,* 1995) and also in the generation and synchronisation of odour-evoked rhythmic cell activity (e.g. Shepherd & Greer, 1998).

The lateral dendrites of the mitral and tufted cells spread across large regions of the olfactory bulb (Orona *et al.,* 1984), which suggests that the granule cell mediated lateral inhibition also plays an important role in the patterning of activity across wide regions of the olfactory bulb. The dendrites of the mitral and tufted cells release not only glutamate but also possess a high density of AMPA, NMDA and metabotropic glutamate receptors (e.g. Montague & Greer, 1999). However, it has been suggested that the major part of the mitral cell dendrites is entirely free of glutamatergic inputs although their membranes are

provided with glutamatergic receptors (Salin *et al.,* 2001). This has raised a question whether or not these dendritic receptors are synaptically activated when glutamate is released? It has been shown that the NMDA glutamate autoreceptors of the mitral cell dendites are activated by glutamate from the dendrodendritic synapses (e.g. Aroniadou-Anderjaska *et al.,* 1999) and that in addition to NMDA receptors, the non-NMDA autoreceptors are activated by sodium and calcium spikes (Salin *et al.,* 2001).

Glutamate has been believed to be a transmitter of mitral cell somadendritic output (Cotman *et al.,* 1987) with NMDA receptors in the external plexiform layer of the olfactory bulb. It is also known that glutamate and aspartate are neurotransmitters of the mitral axons in the olfactory cortex (Sepherd & Greer, 1990). It is believed that the neurotransmitters of the tufted cells are the same as the neurotransmitters of the mitral cells. However, Halasz *et al.,* (1977) found, that some of the tufted cells are dopaminergic, which connects the tufted cells with the periglomerular cells instead of the mitral cells.

There have been several studies showing GABA to be neurotransmitter released by the dendrodendritic synapses of the granule cells. The GABA-synthesizing enzyme has been found in granule cells and it is known that the granule cells do take up GABA (e.g. Ribak *et al.,* 1977; Halasz *et al.,* 1978).

The neurotransmitters mentioned are just a small sample of all of the neurotransmitters and neuromodulators in the olfactory system. These are, however, the best known and play a major role in neurotransmission in the system.

The olfactory bulb receives a significant noradrenergic input from the locus coeruleus (e.g. Fallon & Moore, 1978; McLean & Shipley, 1991). These inputs play significant role in olfaction: olfactory cues increase the discharge of locus coeruleus neurones in behaving animals (Aston-Jones & Bloom, 1981) and trigger increases in norepinephrine levels in the olfactory bulb (e.g. Rangel & Leon, 1995). These locus coeruleus- norepinephrine projections to the olfactory bulb are important for the formation and recall of specific olfactory memories, pheromonal regulation of pregnancy and maternal behaviour (e.g. Brennan *et al.,* 1990). Both mitral and granule cells are potential targets of these projections. Noradrenergic fibers terminate mainly in the internal plexiform layer and the granule cell layer of the olfactory bulb, but also in the external plexiform and mitral cell layers (McLean *et al.,* 1989).

In the mammalian olfactory system, the olfactory sensory neurone activity is transformed by the neuronal circuitry of the olfactory bulb into odour specific action potential patterns. Olfactory sensory neurones then increase their activity monotonically with increasing odorant concentration (Duchamp-Viret *et al.*, 1999; 2000). The odour evoked responses in mitral and tufted cells are more specific and show both increases and decreases in the action potential firing rate with increasing concentrations of odorant (Kauer, 1991). This transformation of

the olfactory sensory neurone activity into mitral and tufted cell activity within the olfactory bulb depends on recurrent and lateral inhibition (Laurent, 1999), thus both recurrent and lateral inhibition are thought to be important to odour discrimination. They also play a role in the generation and synchronisation of the mitral cell activity (Shepherd & Greer, 1990).

The olfactory information is thought to be at least partly encoded by oscillating or bursting neuronal networks (e.g. Kauer, 1998) - olfactory bulb responds to a sensory input with fast network oscillations. Friedman and Strowbridge (2003) also showed that these synchronous fast oscillations can be evoked *in vitro* by brief electrical stimulation in the rat olfactory bulb. Stimulation induces periodic inhibitory synaptic potentials in the mitral cells and prolonged spiking in GABAergic granule cells. In case of repeated stimulation of the olfactory bulb cells, both granule cell activity and mitral cell inhibition are constantly enhanced. Stimulation also induces high-frequency activity throughout networks of olfactory bulb neurones. The synchrony of the network results from chemical and electrical synaptic interactions - both glutamate-receptor antagonists and gap junction inhibitors are shown to block oscillatory intracellular and field responses. Furthermore, Friedman and Strowbridge (2003) showed that the olfactory bulb can generate fast oscillations autonomously through the persistent activation of networks of inhibitory interneurones. The local circuit interactions are thought to be critically involved in odour processing *in vivo.*

In vivo studies have also indicated that odour stimulation of the olfactory nerve produces a burst of action potentials in some olfactory neurones (Wellis &

Scott, 1990). In vitro patch clamp recordings indicate that some olfactory neurones generate low-threshold spikes and bursts in response to depolarising currents (McQuiston & Katz, 2001). It was recently shown by Hayar et al. (2004) that the external tufted cells of the olfactory bulb also generate spontaneous (i.e. without stimulation or current injection) bursting at frequencies ranging from 0.5. to 9 Hz. The spontaneous rhythmic activity seen in these cells is generated by a persistent sodium current active at resting membrane potential. However, it is still unclear whether any other olfactory neurone subtypes generate spontaneous bursting (Hayar et al., 2004).

Each olfactory glomerulus contains a population of external tufted cells. They receive an input from the olfactory nerve and the spontaneous activity generated by these cells readily entrains to patterned olfactory nerve input at theta frequencies (3-10Hz). The frequency of the spontaneous bursting of the external tufted cells ranges overlaps substantially with the theta frequency band. This is the prominent band of activity in olfactory-limbic neuronal activity (Eeckman & Freeman, 1990) and includes the 5-10Hz rhythmic sniffing patterns used by rodents investigating novel odours (e.g. Uchida & Mainen, 2003). Synchronising glomerular activity during sniffing might be an important physiological function of the glomerular ET cell network.

The bursting of the external tufted cells is thought to be an intrinsic property of these cells. In patch clamp studies the bursting was found to deteriorate rapidly after the establishment of the whole cell configuration and the bursting activity of the olfactory neurones has been reported only in isolated patch clamp studies

(e.g. McQuiston & Katz, 2001). This rundown of bursting in patch clamp recordings could attribute to intracellular dialysis of an intracellular messenger important to maintain spontaneous activity (Alreja & Aghajanian, 1995).

It has also been shown that the burst frequency is voltage dependent and that blocking the glutamate and $GABA_A$ receptors does not block bursting. This rules out the glutamatergic input from the olfactory nerve, glutamatergic dendrodendritic interactions among the external tufted cells and/or mitral cells and disinhibition as a driving force behind the bursting (e.g. Aroniadou-Anderjaska et al., 1999). Moreover, suppressing Ca_2-dependent neurotransmitter release does not block the spontaneous bursting. This eliminates the possibility of involvement of other neurotrasmitters in the generation of the spontaneous bursting of the external tufted cells (Hayar et al., 2004).

The external tufted cells of the olfactory bulb generate, as a response to a single olfactory nerve stimulus, a short latency excitatory post-synaptic current and burst of spikes. Hayar et al. (2004) recently showed that the sensory input from the olfactory nerve can override the intrinsic burst frequency of the external tufted cells. They also noted that the strength (spikes per burst) of the spontaneous bursting varies depending on the membrane potential. Hyperpolarised cells have a relatively stronger bursting activity whereas cells with low bursting frequency have a less regular bursting pattern. This could be due to the spontaneous excitatory post-synaptic potentials (EPSPs) which may have an important role in regulating the synchrony on the spontaneous bursting.

These EPSPs reset the bursting in the external tufted cells which, in turn, become entrained by the rhythm imposed by the pattern of the sensory input.

The ability of the external tufted cells to turn a single olfactory nerve input into bursts amplifies the sensory input sent into the glomeruli. This amplification, combined with the entrainment of the external tufted cells, is thought to synchronise the activity of the glomerular and mitral cells and to enhance the specificity of odorant profiles projected onto each glomerulus (Hayar et al., 2004).

3.3. PRIMARY CELL CULTURES OF THE OLFACTORY SYSTEM

Primary cell cultures of olfactory epithelium and olfactory bulb are used extensively to examine the physiological, anatomical and pharmacological properties of the olfactory system *in vitro* (e.g. Pixley, 1996; Muramoto *et al.*, 2001). The most commonly used methods include organotypic slice cultures, using either the Stoppini method or the Gähwiler method, and dissociated cell cultures.

Most olfactory sensory neurones innervate their normal target cells *in vitro*. Though it has been shown, that due to the loss of normal afferent projections and target areas, the intrinsic neuronal connections reorganise themselves and also take over some additional synaptic sites (Zimmer & Gähwiler, 1984).

Dissociated culture systems of the olfactory bulb have been used for various purposes (e.g. Carlson *et al.*, 1997; Puche & Shipley, 1999), even though the culture aspects (e.g. cellular organisation and morphology of cultured neurones) have been slightly different among the studies (Muramoto *et al.*, 2001).

There have been several studies attempting to analyse olfactory functions using dissociated culture systems of the olfactory bulb. Reciprocal synapses between the mitral/tufted cells and the interneurones have the most important role in the signal processing in the olfactory bulb, but it is still unclear, if these synapses are formed in the same way *in vitro* as they are *in vivo* (Muramoto *et al.*, 2001).

It has been shown by several researchers (e.g. Phillips *et al.,* 1963; Isaacson & Strowbridge, 1998) that lateral as well as recurrent (self-inhibition) inhibition could be mediated through these synapses. Also the formation of both excitatory and inhibitory synapses in these culture systems has been demonstrated by pharmacological and physiological studies (Trombley & Westbrook, 1990; Trombley & Shepherd, 1992). Moreover, it has been shown by Fracek *et al.* (1994a; 1994b) that the synapses formed *in vitro* have similar morphological characteristics to those in the intact olfactory bulb.

Cultured embryonic mouse olfactory bulb cells include mitral and tufted cells, granule cells, short-axon cells and other cell types, such as fibrous and protoplasmic astrocytes. The cells are found as individuals, clusters and aggregates (Fracek *et al.,* 1994a). They form asymmetric, symmetric, axodendritic and dendrodendritic synapses and they contain electron lucent, dense core and coated vesicles with similar diameters to those found in intact olfactory bulb. But even after these detailed studies, the existence of reciprocal synapses have not been confirmed (Fracek *et al.,* 1994b), even though it has been suggested by Muramoto *et al.* (2001) that the synapses in the culture show resemblance to *in vivo* synapses, including reciprocal synapses.

The anatomy and physiology of the olfactory bulb have been widely examined using dissociated culture systems, but several questions still remaining unanswered. Other methods, such as tissue slices, have not been used as widely as dissociated culture systems, especially in studies related to synaptic organization and synapse formation.

The olfactory epithelium and the olfactory bulbs can be co-cultured using organotypic slice cultures. This allows the axonal connections to be re-established *in vitro*. The axonal projections from the olfactory epithelium can penetrate the olfactory bulb already after four days *in vitro*. The formation of the synaptic connections is confirmed by generation of an inward current between olfactory sensory neurones and the olfactory bulb (Kanaki *et al.*, 2000).

3.4. METHODS

To develop an *in vitro* network, the basic anatomy and physiology of the olfactory system consisting of the olfactory epithelium and the olfactory bulbs needed to be understood. These were studied using basic morphological stainings (e.g. haematoxylin and eosin staining), immunohistochemistry and electron microscopic analysis. Furthermore, organotypic slice cultures (Stoppini method (Stoppini *et al.*, 1991) and Gähwiler method (Gähwiler, 1981)), dissociated cell cultures and co-cultures were used to study the behaviour of these tissues/cells *in vitro* and to establish the best possible circumstances to culture these tissues/cells. Electrophysiological methods, notably extracellular single unit recording and extracellular recording from cultures grown on multielectrode arrays (MEAs), were used to monitor the electrophysiological activity in these cultures.

3.4.1. Animals

While establishing the methods, postnatal and adult rats (*Rattus rattus)* from several strains were used but during the later parts of the study only Charles River United Kingdom (CRUK) rats were used. Both male and female rats were used. All rats were obtained from the Biomedical Sciences Unit at the University of Nottingham. Animals were bred in the unit following timed

matings, brought up until the required age and were kept in a 12h light:12h dark cycle maintained at 23±2°C.

Postnatal rats (day 1-4) were used when studying olfactory bulbs whereas adult rats (>200g) were used when studying olfactory epithelium. Postnatal animals were preferred because of the longer survival of neurones in tissue derived from younger animals (Gähwiler *et al.*, 1991). Adult rats were only used in case of the olfactory epithelium because of their much larger, and therefore easily accessible, nasal cavities. There were no problems caused by using tissue from different donors or ages.

3.4.2. Removal of the Olfactory Tissue

All the work was carried out in a laminar flow hood using aseptic technique with sterile equipment and solutions to maintain sterility. The contents of the dissection solution varied depending on the way the material was to be used.

3.4.2.1. *Olfactory Epithelium*

Animals were killed by an inhalation of overdose of an anaesthetic (Halothane BP 100% v/v, Rhodia Organique Fine Ltd, UK) in an anaesthetic jar followed by cervical dislocation and decapitation. The bones surrounding the nasal cavity were broken using bone clippers. The olfactory epithelium was then removed using sterile forceps. The tissue was kept moist throughout the process using dissection solution (1.13g 10mM HEPES (Sigma, UK) and 800 µl penicillin/streptomycin (Life Technologies, UK) in 500ml Ca+/Mg+ free Hanks Balanced Salt Solution (Ca+/Mg+ free HBSS; Life Technologies, UK)).

3.4.2.2. *Olfactory Bulbs*

Animals were killed by an inhalation of overdose of an anaesthetic (Halothane BP 100% v/v, Rhodia Organique Fine Ltd, UK) in an anaesthetic jar followed by decapitation by a quick scissor cut at the level of the foramen magnum. Using small dissecting scissors an incision was made through the skull horizontally on both sides at a level just above ears. The dorsal part of the skull was then removed. The ventral part of the skull was then removed under a dissecting microscope (Nikon, Japan) at x2 magnification and the olfactory bulbs were revealed. The cranial nerves were cut and the brain was eased out of the cranium and placed in a Petri dish containing dissection solution. The

olfactory bulbs were then removed from the rest of the brain using a sterile scalpel. The tissue was kept moist throughout the process using dissection solution (1.13g 10mM HEPES in 500ml Ca+/Mg+ free HBSS).

3.4.3. Light Microscopy

For light microscopy the tissue was fixed overnight with Bouins fixative (75% saturated picric acid, 20% formaldehyde and 5% glacial acetic acid). The fixed tissue was rinsed in 0.1M phosphate buffer (PBS, pH 7.4) and dehydrated using rising concentrations of ethanol and toluene. The concentrations used were 70% ethanol 2x30 minutes, 90% ethanol 30 minutes, 100% ethanol 2x45 minutes, 1:1 ethanol-toluene solution 60 minutes and 100% toluene 60 minutes. After the dehydration the toluene was removed and the tissue submerged into molten wax. The specimen was placed into a vacuum chamber for 5 minutes after which the wax was replaced with fresh wax and the specimen is placed into the vacuum chamber for 15 minutes. After that the wax was replaced with fresh wax once more and the specimen was vacuumed for further 45 minutes. Finally, the wax and the tissue were poured on a mould and left to solidify overnight. Once the wax had solidified the embedded tissue was sectioned at 8μm using microtome (Anglia Scientific, UK).

3.4.3.1. *Haematoxylin and Eosin Staining*

The glass slide mounted wax sections were dewaxed through xylene and decreasing concentrations of ethanol (100%, 95%, 90%, 70% and 50%). The sections were rinsed in xylene and 100% ethanol for 2x5 minutes and in less concentrated ethanol 1x5 minutes. After the dewaxing the slides were rinsed in running water and submerged for 3-5 minutes in filtered Harris' haematoxylin solution. Haematoxylin stained nuclei with purple colour and the tissue turned blue. The slides were washed in running water and then dipped 20 times in acid alcohol (1% hydrochloric acid in 70% ethanol) which turned the tissue red by removing haematoxylin stain mainly from cytoplasm as nuclei have more affinity for stain and retain it more easily. The slides were washed in running water and the dipped for a few seconds in lithium carbonate ($LiCO_3$). This made the tissue blue. The slides were now checked under a microscope to ensure that mainly only the nuclei were stained. If the cytoplasm was too blue, the slides were dipped again in acid alcohol. The slides were now immersed in filtered eosin for 30 seconds. This stained the cytoplasm with pink colour. The slides were washed in running water and then quickly dipped through the ethanol concentrations to xylene again to differentiate the eosin stain and improve resolution. A few drops of a synthetic resin (DPX) and cover slips were added immediately.

3.4.3.2. *Periodic Acid Schiff's Staining*

The glass slide mounted wax sections were dewaxed through xylene and decreasing concentrations of ethanol (100%, 90%, 70% and 50%). The sections were rinsed in xylene and 100% ethanol for 2x5 minutes and in less concentrated ethanol 1x5 minutes. After the dewaxing the sections were rinsed in distilled water for further 5 minutes and then treated with periodic acid solution for 5 minutes. The sections were washed with several changes of distilled water and then covered with Schiff's solution for 15 minutes. The slides were washed with running water for 5-10 minutes and the nuclei were stained with filtered Harris' haematoxylin for 3-5 minutes. The slides were again washed with running water and then dipped 20 times in acid alcohol. The washing was repeated and the slides were dipped in lithium carbonate for a few seconds. The slides were then checked under a microscope to ensure that mainly only the nuclei were stained. If the cytoplasm was too blue, the slides were dipped again in acid alcohol. The slides were washed with water and rinsed in absolute alcohol. A few drops of DPX and cover slips were added immediately.

3.4.4. Electron Microscopy

For the semi-thin (1-5μm) electron microscopy the tissue was fixed overnight with 1% paraformaldehyde, 3% glutarldehyde in 0.1M cacodylate buffer. After the fixative the tissue was placed in 6% sucrose in 0.1M cacodylate buffer and left for 30 minutes at room temperature. After the 30 minutes the tissue was post-fixed in 1% osmium tetroxide in Millonigs buffer (pH 7.4) at room temperature for 20 minutes. This results in tissue turning brown/black. After the post-fixation the tissue was processed through distilled water (2x5 minutes), 60% ethanol (20 minutes), 80% ethanol (20 minutes), 100% ethanol (3x20 minutes), acetone (20 minutes), 1:1 acetone-araldite mixture (overnight), araldite mixture (3x2 hours) and finally transferred into an embedding tray with fresh araldite mixture and polymerised at 60°C for 48 hours. The tissue was then ready for sectioning using ultramicrotome (Ultracut, Leica, Germany).

The sections were dried on a glass slide with gentle heat on a hotplate and stained with toluidine blue-mixture. The sections were then examined using a light microscope to examine the toluidine blue staining.

3.4.4.1. *Scanning Electron Microscopy*

Tissues and cells of the olfactory epithelium and the olfactory bulb were also studied using a scanning electron microscopy. For this, the tissue was fixed for 1-2 hours, depending on the size, with 1% paraformaldehyde, 3% glutarldehyde in 0.1M cacodylate buffer. For cells the fixation time was 20 minutes. After the fixation the tissue (cells) were placed into 7% sucrose solution and left at 4°C overnight. The tissue (cells) was now washed with 0.1M cacodylate buffer for 3x5 minutes and then post-fixed for 1 hour (cells for 45 minutes) in 1% osmium tetroxide. After the post-fixation, the tissue (cells) was dehydrated through rising concentrations of alcohol (20%, 40%, 60%, 70%, 80% and 90%, 10 minutes each, and 100%, where the tissue was kept 2x10 minutes). For cells the times were halved. Then the tissue (cells) was submerged to hexamethyldisilazane (HMDS; Sigma, UK) for 2x 10 minutes (for cells 2x5 minutes). The samples were critical point dried for 45 minutes, mounted on aluminium discs and gold-coated for 4 minutes.

3.4.5. Immunohistochemical Stainings for Confocal Microscopy

The cellular organisation of the cell cultures was determined using immunohistochemical staining. This is a method that allows accurate identification of different cell types through specific antibody-antigen interactions. Neurons in the cultures were stained using an antibody against neurofilament. This protein is the intermediated filament of nerve cells and it acts as an internal 'scaffold' maintaining the shape of the cell body and axon (Stevens & Lowe, 1997).

Glial cells were identified by using an antibody against glial fibrillary acidic protein (GFAP). GFAP is a specific form of cytoskeletal intermediate filament and is exclusive to astrocytic glial cells. The stained astrocytes are easily identifiable as they have a stellate morphology with numerous fine processes radiating in all directions (Stevens & Lowe, 1997).

Due to the autofluorescent problems experienced with immunofluorescent staining protocols (3.4.5.1. and 3.4.5.2), both immunofluorescent and immunoperoxidase methods were used.

3.4.5.1. *Immunofluorescent Neurofilament Staining for Wax Sections*

Every tenth glass slide of wax embedded and sliced tissue was picked for the immunohistochemical double staining and dewaxed through xylene and decreasing concentrations of ethanol (100%, 90%, 70% and 50%). The sections were rinsed in xylene and 100% ethanol for 2x5 minutes and in less concentrated ethanol for 1x5 minutes. After the dewaxing the sections were rinsed in distilled water for further 5 minutes. The sections then were incubated for 30 minutes in 0.01M KPBS containing 0.3% Triton X-100 to increase the penetration in the cell walls. After the Triton X-100 treatment the slides were incubated for 30 minutes in 0.3% hydrogen peroxide (H_2O_2) in methanol to block the endogenous peroxide activity. The sections were washed for 3x5 minutes in 0.01M KPBS and incubated for 20 minutes with normal horse serum (NHS; Sigma, UK) diluted 1:200 with 0.01M KPBS in order to decrease non-specific staining. The sections were washed for 3x5 minutes in 0.01M KPBS and incubated overnight at 4^0C with the primary antibody (neurofilament raised in mouse, Affinity, UK, 1:1000). The primary antibody was diluted with 0.01M KPBS containing 0.3% of Triton X-100 and 1% NHS.

The secondary antibody used was fluorescence horse anti-mouse (Vector, USA, 1:200), in which the sections were incubated for 2 hours in room temperature. Also the secondary antibody was diluted with 0.01M KPBS containing 0.3% of Triton X-100 and 1% NHS. The sections were washed for 3x5 minutes in 0.01M KPBS between the antibodies and after the secondary antibody.

After the washings the sections were briefly rinsed three times in super ultra pure water (SUPW). A drop of Vectashield mounting medium (Vector, USA) was placed on each section to prevent loss of fluorescence during the microscopic examination and the sections were covered with cover slips. Prior to viewing, the samples were wrapped in aluminium foil to prevent UV light penetration and stored at 4°C.

The sections were viewed using an upright fluorescent microscope (Leica, UK). Neurofilament immunoreactivity was visualised using a fluorescein green filter at a wavelength of 456nm.

3.4.5.2. *Immunofluorescent Double Staining for Neurofilament and Glia*

Tissue slices, either straight after dissection or after culturing, were selected for staining and fixed with 5% acid alcohol (95% ethanol, 5% glacial acetic acid). The slices were washed 3x5 minutes in 0.01M potassium phosphate buffered saline (KPBS, pH 7.4) and then incubated for 30 minutes in 0.01M KPBS containing 0.3% Triton X-100 to increase the penetration in the cell walls and 1% normal goat serum (NGS, Sigma, UK) to decrease non-specific staining. The slices were washed in 0.01M KPBS again and incubated overnight at 4°C in primary antibody (GFAP raised in rabbit, Dako, UK, 1:1000). The primary antibody was diluted with 0.01M KPBS containing 0.3% Triton X-100 and 1% NGS. The control slices were incubated without the primary antibody.

The secondary antibody used was Texas red goat anti-rabbit (Vector, USA, 1:200) in which all the slices were incubated for 2 hours at room temperature. Also the secondary antibody was diluted with 0.01M KPBS containing 0.3% of Triton X-100 and 1% NGS. The slices were washed for 3x5 minutes in 0.01M KPBS between the antibodies and after the secondary antibody. The staining was continued by incubating slices for 30 minutes in 0.01M KPBS containing 0.3% Triton X-100 and 1% NHS. After the incubation the slices were washed 3x5 minutes in 0.01M KPBS and then incubated overnight at 4^0C in second primary antibody (neurofilament raised in mouse, 1:1000). The primary antibody was diluted with 0.01M KPBS containing 0.3% Triton X-100 and 1% NHS. The control slices were incubated without the primary antibody.

The second secondary antibody used was fluorescence horse anti-mouse (Vector, USA, 1:200) in which all the slices were incubated for 2 hours at room temperature. Also the secondary antibody was diluted with 0.01M KPBS containing 0.3% of Triton X-100 and 1% NHS. The slices were washed for 3x5 minutes in 0.01M KPBS between the antibodies and after the secondary antibody.

After the washings the sections were briefly rinsed three times in SUPW. A drop of Vectashield mounting medium was placed on each section to prevent loss of fluorescence during the microscopic examination and the sections were covered with cover slips. Prior to viewing, the samples were wrapped in aluminium foil to prevent UV light penetration and stored at 4°C.

The sections were viewed using an upright fluorescent microscope with a fluorescein green filter at a wavelength of 456nm for neurofilament immunoreactivity and a rhodamine red filter at a wavelength of 568nm for GFAP immunoreactivity.

3.4.5.3. *Immunoperoxidase Staining for Neurogilament/Glia*

This method was used for the immunohistochemical staining for both, neurofilament and GFAP after the problem with autofluorescence was discovered. The protocol is the same as 3.4.5.1. up to the wash after the incubation with the primary antibody. Following the wash, the secondary antibody, biotinylated goat anti-rabbit (Vector, USA, 1:200), was added and the samples incubated for 1 hour in room temperature. The secondary antibody was diluted into 0.01M KPBS containing 1% NGS.

Following the incubation, the samples were washed 3x5 minutes in 0.01M KPBS. After the washes, horseradish peroxidase sreptavidin (Vector, USA, 1:200) diluted in 0.01M KPBS with 1% NGS was added and the samples were incubated for 30 minutes. The samples were washed 3x5 minutes in 0.01M KPBS and transferred into a fume hood. 200µl of 3,3'-diaminobenzidine (DAB, Vector, USA) substrate was added on all samples for maximum of 5 minutes. The samples were the washed for 3x5 minutes in SUPW and a drop of

Vectashield mounting medium was placed on each section and the sections covered with cover slips.

3.4.6. Organotypic Slice Cultures

3.4.6.1. *The Stoppini Method*

For the Stoppini method (Stoppini *et al.,* 1991) the tissue was removed as described earlier (see 3.4.2.). After isolation the olfactory bulb were sectioned at 400µm using a McIlwain tissue chopper (The Mickle Laboratory Engineering Co, UK). The resulting slices were placed in chilled dissection solution without antibiotics (1.13g 10mM HEPES in 500ml Ca+/Mg+ free HBSS), inspected, separated and trimmed for excess tissue under a microscope. The slices were then placed on membrane inserts (Millicell culture plate inserts, PICM03050, pore size 0.4 µm, Millipore, UK), approximately five slices per insert. The inserts are transferred to plastic 6-well well plates (Becton & Dickinson, USA). Each well contained 1ml of full culture medium (FCM; 10% MEM (Life Technologies, UK), 10% NCS (Life Technologies, UK), 5% sodium bicarbonate (Sigma, UK) and 1% L-glutamine (Sigma, UK) in 74% SUPW). The well plates were placed in an incubator (ICN Biomedicals, USA) at 37°C with 5% CO_2. After 24 hours *in vitro*, the culture medium was replaced with 1ml of serum-free neurobasal medium (NBM; 400µl B-27 supplement (Life

Technologies, UK) and 200µl L-glutamine in 20ml neurobasal medium (Life Technologies, UK)) containing several hormones, fatty acids and free radical scavengers including glutathione and vitamin E. The medium was thereafter changed twice a week until the cultures have matured. During this period the slices are examined daily under a microscope.

The olfactory epithelium did not require chopping. The pieces of epithelium were stretched on membrane inserts, approximately five pieces per insert. The inserts were transferred to plastic 6-well well plates, each well containing 1ml of serum-containing FCM. In case of the olfactory epithelium 400µl of penicillin/streptomycin and 200µl of fungizone (Life Technologies, UK) were added per 100 ml of FCM to prevent bacterial and fungal infections. The olfactory epithelium slices were taken care of the same way as slices of the olfactory bulbs.

3.4.6.2. *The Gähwiler Method*

For the Gähwiler method (Gähwiler, 1981) the tissue was removed as described earlier (see 3.4.2.). After isolation the olfactory bulb was sectioned at 400µm using a McIlwain tissue chopper. The resulting slices were placed in chilled dissection solution without antibiotics (3ml 50% D-glucose (Sigma, UK) and 400µl penicillin/streptomycin in 300ml Gey's balanced salt solution (GBSS;

Sigma, UK), inspected, separated and trimmed for excess tissue under a microscope.

The slices were now mounted on glass cover slips (10x12mm, thickness 0, SLS, UK), which were pre-cleaned with alcohol and sterilised at 200°C for at least two hours. 20µl of chicken plasma (Cocalico Biologicals, USA) was placed in the centre of each cover slip and then, using a fine spatula, one tissue slice was placed in the centre of each drop. Carefully the plasma was spread over the surface of the cover slip and the tissue slice. Thirty microliters of thrombin (75units/ml activity, Serva, Germany) was added on each cover slip and thoroughly mixed with the chicken plasma. The plasma-thrombin mixture was left for approximately 60 seconds to allow them to form a jelly-like clot. Once the clot had formed, the cultures were placed in flat bottomed screw top culture tubes (SLS, UK) containing 750µl of pre-warmed (37°C) culture medium (20ml HBSS, 20ml heat-inactivated horse serum (Life Technologies, UK), 400µl L-glutamine and 400µl 50% D-glucose in 40ml Eagles' basal medium (BME) with Hanks' salt solution and without L-glutamine). The test tubes were then placed in a custom made roller drum (Medical School Workshop, University of Nottingham, UK) at an angle of 5° rotating at 10 revolutions per hour in an air incubator (Leec, UK) at 37°C.

After 48 hours *in vitro*, the metabolised medium was replaced with new medium. Thereafter the cultures were fed once or twice per week by tipping out the old medium leaving a drop (approximate volume 250µl) of 'old', conditioned medium in to the tube and adding 500µl of fresh medium. This was

done until the cultures had matured. During this period the slices were examined daily under a microscope to follow the maturation of the cultures.

3.4.7. Dissociated Cell Cultures

For the cell dissociation the tissue was removed as described earlier (see 3.4.2.). After the isolation of the tissue needed, it was placed into a small conical flask containing 4ml of Ca^{+}/Mg^{+} free HBSS. 1 ml of 2.5% trypsin-EDTA solution (Sigma, UK, diluted to Ca+/Mg+ free HBSS) was added and the flask was sealed with a sterile bung. The flask and its contents were incubated at 37°C for 45 minutes after which the trypsin solution was removed and the tissue rinsed 2x5 minutes with 20% new born calf serum (NCS) in 0.1M PBS. The tissue was then dissociated carefully using Pasteur pipettes. The tissue suspension was centrifuged at 1000rpm for 5 minutes and the formed cell pellet was re-suspended with 5ml of FCM. Cell count was then performed with haemocytometer (Weber Scientific International, UK) when handling olfactory bulb cells, and 1ml of suspension was placed on to poly-D-lysine (PDL; Sigma, UK)-coated cover slips. When dissociating olfactory epithelium, the cells were placed in 6-well well plates (1ml/well) and into a CO_2 incubator for two hours. This purifies the neurone suspension by decreasing the number of glia-cells as glia are stickier than neurones and adhere to the bottom of well plates. After this incubation a cell count was performed and 1ml of suspension was placed on to PDL-coated cover slips.

After 24 hours *in vitro*, the full culture medium was replaced with 1ml of serum-free neurobasal medium. The medium was thereafter changed twice a week until the cultures had matured. During this period the cells were examined daily under a microscope.

3.4.8. Co-culture of Olfactory Epithelium and Olfactory Bulb

Several different types of co-cultures of olfactory epithelium and olfactory bulb were used in order to trace the growth of the olfactory sensory neurons from the epithelial tissue to the olfactory bulbs. A specific anterograde lectin, *Phaseolus vulgaris* Leucoagglutinin (PHA-L; Sigma, UK) was introduced into the epithelial tissue as a part of the tissue processing. PHA-L was then taken up by the olfactory sensory neurons and transported axonally in the anterograde direction (Leavitt *et al.*, 1977; Gerfen & Sawchenko, 1983). The transported marker was later visualised using immunohistochemical methods.

3.4.8.1. *Co-culture of Dissociated Olfactory Epithelium and Dissociated Olfactory Bulbs – Type 1*

The tissues were removed and dissociated as described earlier (see 3.4.2.; 3.4.7.). Long cover slips were coated with poly-L-ornithine (P-ORN; Sigma,

UK) and placed into Petri dishes. The seeding areas of the cover slips were further treated with 100µl laminin and 50µl FCS and placed into an incubator at 37°C with 5% CO_2 for 45 minutes. A volume of 3 x 10^5 of dissociated olfactory bulb cells was seeded on one end of each cover slip and a volume of 3 x 10^5 of dissociated olfactory epithelium cells treated with PHA-L on the other. The cultures were incubated for 24 hours prior to an addition of 1ml of culture medium for PHA-L labelling (600mg D-glucose, 1ml L-glutamine, 500µl 1M HEPES, 340µl 7.5% $NaHCO_3$ (Life Technologies, UK), 50µl penicillin/streptomycin and 20ml heat inactivated FCS in 80ml MEM). The medium was thereafter changed twice a week until the cultures had matured. During this period the cells were examined daily under a microscope.

For the PHA-L labelling, the dissected olfactory epithelium was placed into a conical flask containing 0.05% trypsin in 33mM glucose-PBS (GPBS; 3g D-glucose in 500ml of PBS) and incubated in a 37°C water bath for 20 minutes. After the incubation the trypsin solution was removed and the tissue was washed three times in a wash solution of 20% FCS in GPBS. After the final wash, the tissue was dissociated mechanically in 2ml of the was solution using plastic pasteur pipettes. The cell solution was centrifuged twice at 500rpm for 5 minutes and the supernatant was removed. The cells were resuspended into 200µl of GPBS and the cell suspension was then placed into a siliconised conical flask. For labelling, 200µl of 1% PHA-L solution in Dulbecco's phosphate buffered saline (DPBS; Sigma, UK) was added and the cell solution was incubated for one hour at 37°C with 5% CO_2. After the incubation the labelling solution was removed and the tissue was washed three times in a wash

solution of 20% FCS in GPBS. The cell solution was centrifuged twice at 500rpm for 5 minutes and the supernatant was removed. The cell pellet was resuspended in 2ml of culture medium for PHA-L labelling.

3.4.8.2. *Co-culture of Dissociated Olfactory Epithelium and Dissociated Olfactory Bulbs – Type 2*

The tissues were removed and dissociated as described earlier (see 3.4.2.; 3.4.7.). Round cover slips were coated with P-ORN and placed into well plates. The cover slips were further treated with 100µl laminin and 50µl FCS and placed into an incubator at 37°C with 5% CO_2 for 45 minutes. A volume of 3 x 10^5 of dissociated olfactory bulb cells was seeded on the cover slips and the same volume of dissociated olfactory epithelium cells treated with PHA-L was added. The cultures were incubated for 24 hours prior to an addition of 1ml of culture medium for PHA-L labelling. The medium was thereafter changed twice a week until the cultures had matured. During this period the cells were examined daily under a microscope.

3.4.8.3. *Co-culture of Dissociated Olfactory Epithelium and Organotypic Slice of Olfactory Bulbs – Type 3*

The required number of organotypic olfactory bulb slice cultures cultured using the Gähwiler method (see 3.4.6.2.) were removed from the test tubes and placed into a well plate. $3x10^5$ PHA-L labelled olfactory epithelium cells in 500µl of culture medium for PHA-L labelling were added on each olfactory bulb slice and the cultures were placed into and incubator at 37°C with 5% CO_2. The medium was thereafter changed twice a week until the cultures had matured. During this period the cells were examined daily under a microscope.

3.4.9. Immunohistochemical Staining for PHA-L Labelled Olfactory Sensory Neurons

Several different protocols were used to stain the PHA-L labelled olfactory sensory neurons in the co-cultures of olfactory epithelium and olfactory bulb. The immunofluorescent protocol was modified twice to reduce the background autofluorescence in the samples prior to using the immunoperoxidase method.

In the final protocol for immunofluorescent staining for PHA-L labelled olfactory sensory neurons, the samples were treated for autofluorescence before staining. They were photo-bleached using fluorescent (494nm) light (Neumann

& Gabel, 2002) for 1.5 hours eradicating the autofluorescence. However, the staining needed to be done within 24 hours from the photo-bleaching to prevent the autofluorescence from returning.

After the photo-bleaching, the samples were incubated in 0.5% H_2O_2 in methanol for 30 minutes. They were then washed 3 x 5 minutes in 0.05M tris buffered saline (TBS, pH 7.5) with 0.3% Triton X-100. The samples were then, to decrease inappropriate antibody reactions, incubated in a blocking serum consisting of 20% NHS in 0.05M TBS for 20 minutes at room temperature. The samples were washed 3 x 5 minutes in 0.05M TBS and then incubated for 15 minutes with avidin (Vector, USA) and, after a subsequent wash, for 15 minutes with biotin (Vector, USA). The avidin-biotin was used in order to reduce endogenous avidin-biotin activity. The samples were washed and the samples were then incubated at 4°C for 24 hours in the primary antibody (biotinylated anti-PHA-L, Vector, USA, 1:200). The primary antibody was diluted in 0.05M TBS and 1% NHS, the control samples were treated without the primary antibody.

The samples were washed 3 x 5 minutes in 0.05M TBS and the secondary antibody (fluorescein avidin, Vector, USA, 1:200) added to all samples for 20 minutes at room temperature. The samples were again washed 3 x 5 minutes in 0.05M TBS followed by SUPW. A drop of Vectashield mounting medium was placed on each section to prevent loss of fluorescence during the microscopic examination and the sections were covered with cover slips. Prior to viewing,

the samples were wrapped in aluminium foil to prevent UV light penetration and stored at 4°C.

For the immunoperoxidase staining for PHA-L (Gerfen & Sawchenko, 1983), the samples were incubated overnight at 4°C in 0.02M KPBS containing 0.3% Triton X-100 and 2% normal rabbit serum (NRS, Sigma, UK). The solution was removed and avidin was applied for 15 minutes followed by a wash with 0.02M KPBS. Biotin was then applied for 15 minutes, washed and the primary (anti-PHA-L, Vector, USA, 1:1000) in 0.02M KPBS and 2% NRS was added to the samples for 48 hours at 4°C with constant agitation. The negative control was treated without the primary antibody. The samples were then washed 2 x 10 minutes in 0.02M KPBS containing 2% NRS. The secondary antibody (biotinylated rabbit anti-goat, Vector, USA, 1:200), in 0.02M KPBS and 2% NRS was added to the samples followed by an incubation of 1 hour at room temperature.

The samples were washed 2 x 10 minutes in 0.02M KPBS and 2% NRS and SA-HRP (Vector, USA, 1:200) in 0.02M KPBS and 1% NRS was added for 45 minutes at room temperature. The samples were washed 2 x 10 minutes in 0.02M KPBS and 2% NRS and the secondary antibody was reapplied for further 30 minutes. The samples were washed 2 x 10 minutes in 0.02M KPBS and the SA-HRP was applied as before for 30 minutes. The samples were washed 3 x 10 minutes in 0.02M KPBS and transferred into a fume hood where 200µl of DAB was added for 30 minutes. This was followed by 3 x 5 minutes washes in SUPW and the samples were mounted with Vectashield mounting medium.

3.4.10. Electrophysiology

The electrical properties of the dissociated olfactory bulb and olfactory epithelium cultures were studied using extracellular electrophysiological recording methods.

3.4.10.1. *Single-Unit Recordings*

In this experimental study single-unit recording was used in order to prove that the organotypic slice cultures of olfactory bulb expressed spontaneous electrical activity. The microelectrodes were prepared from glass-coated tungsten (2-5MΩ; see Merrill & Ainsworth, 1972) following the protocol of Bullock *et al.* (1988). The electrodes were then inserted into the olfactory bulb slice cultures using a manually controlled micromanipulator (MM-33, Marzhauser, Germany) and a hydraulic microdrive (Clarke Electromedical, UK). Action potentials were amplified (x10K-20K) using an AC preamplifier (NL104; Neurolog, UK), filtered (300Hz-3kHZ) and displayed on an oscilloscope.

3.4.10.2. *Multiple Single-Unit Recordings utilising MEAs*

Multielectrode arrays, MEAs, were used to collect electrophysiological data from neuronal networks. MEAs allow the electrophysiological activity of several neurones to be observed simultaneously. For the MEA methods used see Chapter 2.

3.5. RESULTS

3.5.1. Light Microscopy

The morphology of neurons of the olfactory epithelium and the olfactory bulbs was first identified at the light microscopic level. The information gained is important for characterisation of the microcircuitry and neuronal networks of the region in question since individual neuronal elements or populations of neurones in the microcircuitry can be analysed. The light microscopy studies were carried out successfully for fresh and cultured tissues of olfactory epithelium and olfactory bulbs and also for dissociated cell cultures of both tissues.

Haematoxylin and eosin and periodic acid Schiff's staining revealed the cellular organisation of the tissues in question and therefore provided important information for this study. This method also provided crucial information of the development of the tissues in question, which enabled the study to concentrate on material of certain age. Haematoxylin and eosin stained olfactory bulbs of 1-4 days old rats are shown as figure 3.5. and the PAS-stained adult rat olfactory epithelium as figure 3.6. The laminated structure of the olfactory bulb is clearly formed already on day one and therefore the tissue is matured enough for purposes of this study.

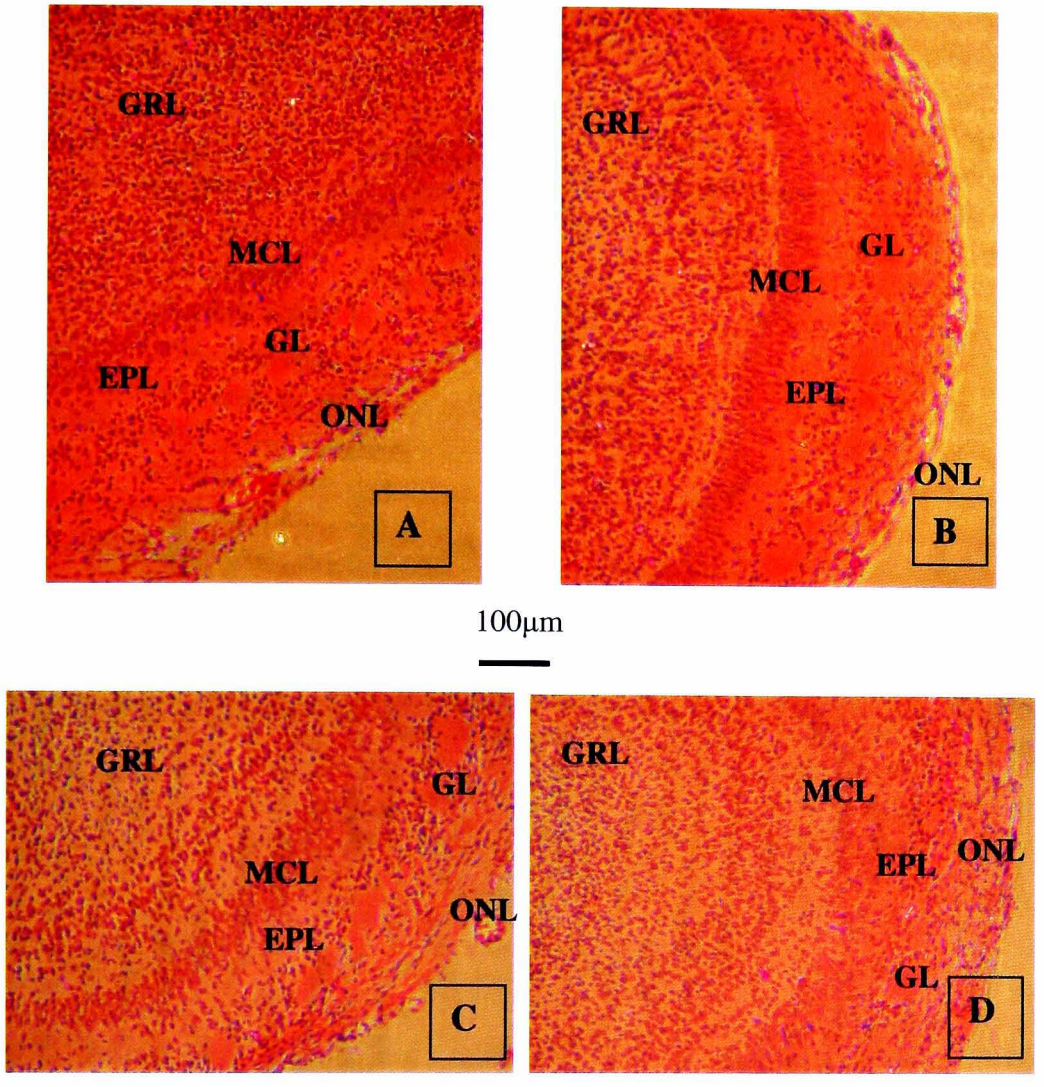

Figure 3.5. Photomicrographs of haematoxylin and eosin stained olfactory bulbs of 1-4 day old rats (x10) (**A-D**). Abbreviations: ONL = olfactory nerve layer, GL = glomerular layer, EPL = external plexiform layer, MCL = mitral cell layer and GRL = granule cell layer.

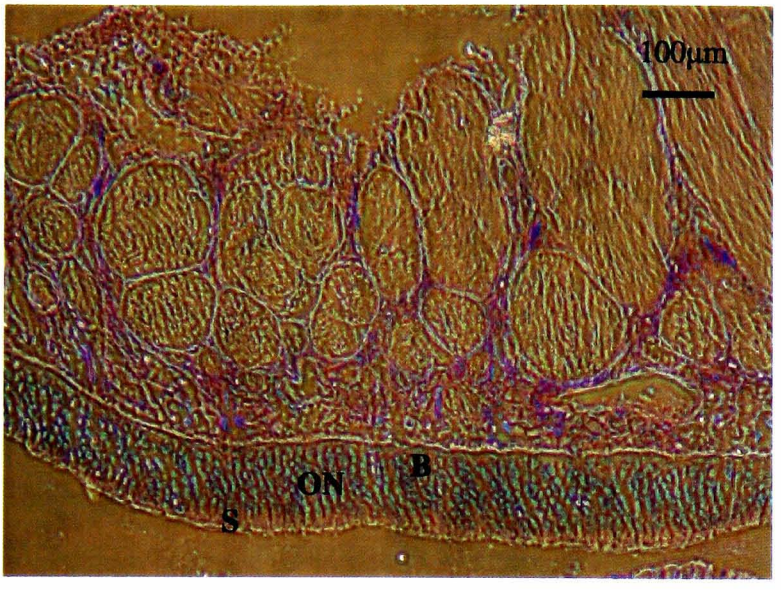

Figure 3.6. Photomicrograph of a periodic acid Schiff's stained adult rat olfactory epithelium (x10). Purple colour represents glycogen. Abbreviations: S = supporting cell layer, ON = olfactory sensory neurone layer and B = basal cell layer.

3.5.2. Electron Microscopy

The chemistry, origin and pattern of afferent synaptic input can be identified at the electron microscopic level and the information gained can be used when characterising the microcircuitry and neuronal networks of the region of interest. Electron microscopy allows the tissue to be examined at the ultrastructural level and the synaptic interactions of individual neurones or populations of neurons to be analysed. The electron microscopy was carried out on fresh tissue of the olfactory epithelium and olfactory bulbs using the

toluidine blue-staining. Scanning electron microscopy was carried out besides on fresh tissues of the olfactory epithelium and olfactory bulbs, also on dissociated cell cultures of both tissue types. A toluidine blue-stained olfactory bulb of a 4-day old rat is shown as figure 3.7. The structure of the olfactory bulb is clearly visible.

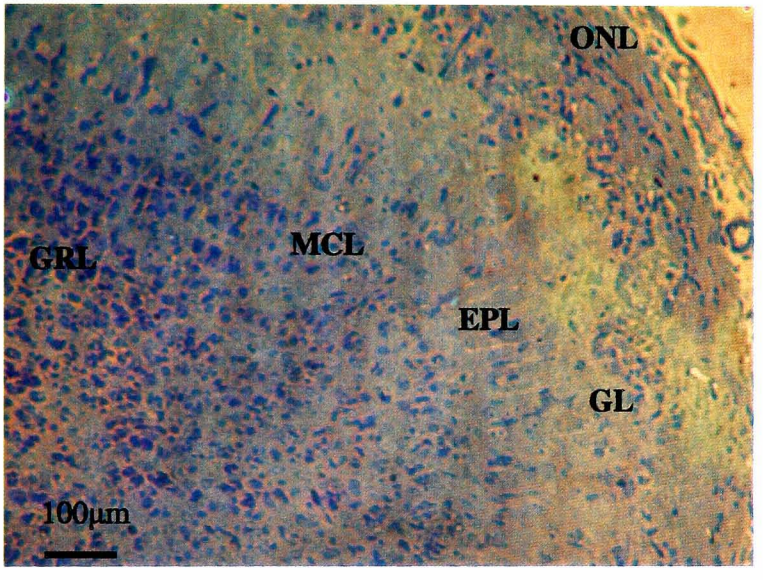

Figure 3.7. Photomicrograph of a toluidine blue-stained olfactory bulb of 4-day old rat (x10). Abbreviations: ONL = olfactory nerve layer, GL = glomerular layer, EPL = external plexiform layer, MCL = mitral cell layer and GRL = granule cell layer.

An overall structure of *ex vivo* (A) and cultured (B, C) olfactory epithelium can be seen in figure 3.8. The dissociated olfactory epithelium culture was fixed on day 14DIV. The olfactory sensory neurones were found to generate *in vitro* and the cultures had olfactory neurones in different stages of maturation present at all times.

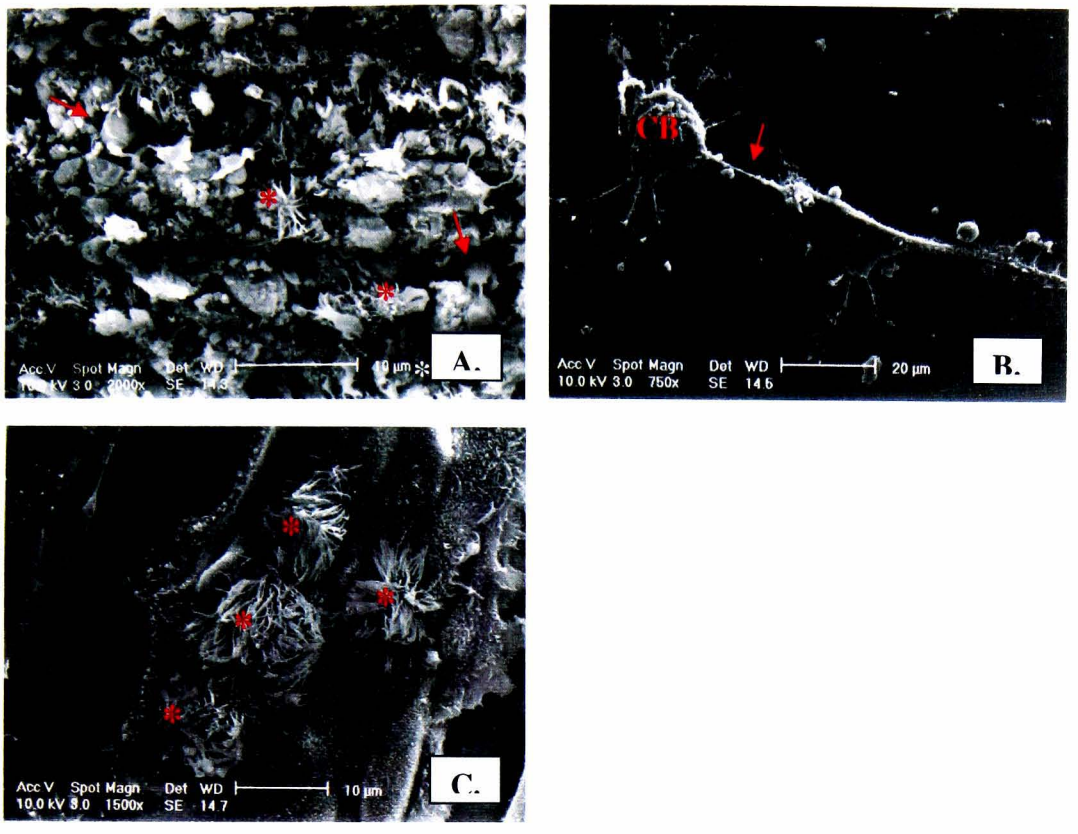

Figure 3.8. Scanning electron photomicrographs of the rat olfactory epithelium. (**A**): Structure of freshly removed olfactory epithelium (2000x). (**B**): Mature olfactory sensory neurone towards the end of its lifespan. The axons and the dendrite can be seen around the cell body. Dissociated cell culture, 14DIV (750x). (**C**): A healthy population of olfactory sensory neurones in dissociated olfactory epithelium culture, 14DIV (1500x). The three dimensional growth of the neurones in the culture is clearly illustrated. In the figures the stars indicate some of the olfactory receptor neurones and the arrows some of the supporting cells (A) and the dendrite (B), CB = cell body. Scale bars A 10µm, B 20µm and C 10µm.

A representative dissociated olfactory bulb cell culture fixed on day 6 *in vitro* is shown in figure 3.9. Different sizes and shapes of olfactory bulb cells can be

seen and these are thought to represent the cell types of the olfactory bulb. The cilia are clearly visible around these cells. The cells are clearly forming connections in this dissociated cell culture.

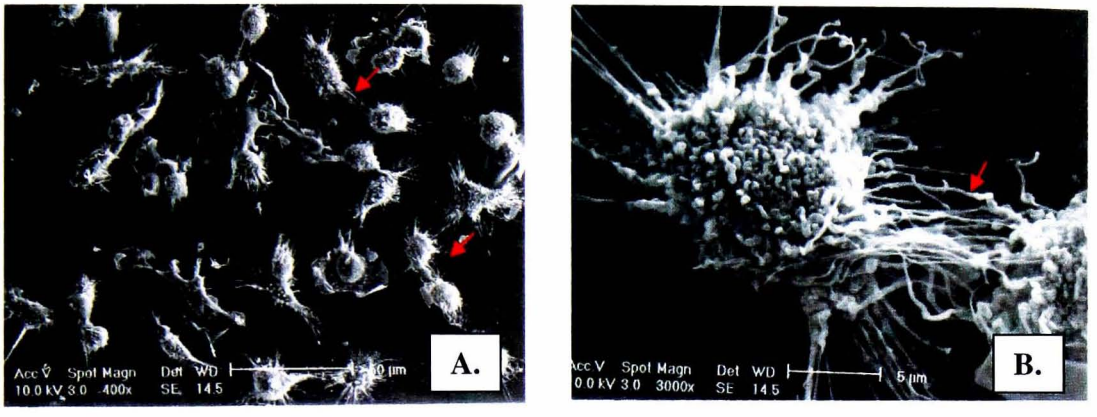

Figure 3.9. Scanning electron photomicrograph of a dissociated cell culture of the rat olfactory bulb, 6DIV. (**A**): (400x). (**B**): Higher magnification, 3000x. The structure of a single olfactory bulb cell can be clearly seen. Also a connection with the neighbouring cell is clearly visible. In the figures the arrows indicate some of the connections formed between the cells of the olfactory bulb.

3.5.3. Organotypic Slice Cultures

An organotypic slice cultures of rat olfactory epithelium were cultured using the Stoppini method. A typical representative cultures are illustrated at 9DIV (Fig. 3.10. A) and 28DIV (Fig. 3.10.B).

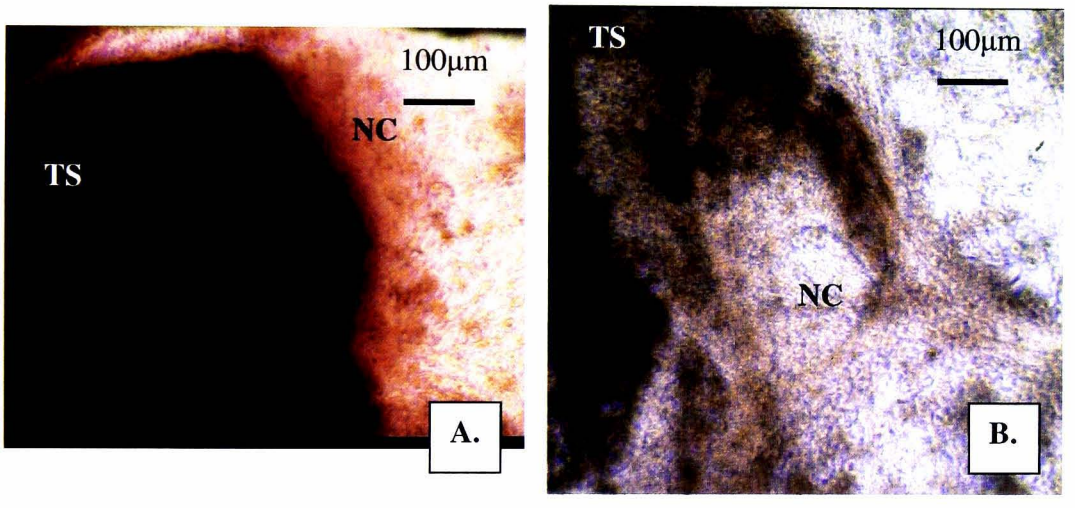

Figure 3.10. Light photomicrograph of an organotypic slice culture of rat olfactory epithelium (10x). (**A**): 9DIV, new cells are clearly visible surrounding the original tissue. The tissue itself is turning slightly dark as a result of cell death. (**B**): The same culture 28DIV. Growing cells have filled the area surrounding the original tissue slice. Cells of the slice itself are mainly dead and turned completely black.In the figure TS = tissue slice and NC = new cells surrounding the tissue slice. In the figure TS = tissue slice and NC = new cells surrounding the tissue slice.

An organotypic slice cultures of rat olfactory bulb were cultured using the Stoppini method. A typical representative cultures of a 2-day old rat olfactory bulb are illustrated at 1DIV (Fig. 3.11.A) and 5DIV (Fig. 3.11.B). The organotypic cell cultures of olfactory bulb were found to grow faster *in vitro* than those of olfactory epithelium but to survive shorter periods (~20 days) than those of olfactory epithelium (~40 days).

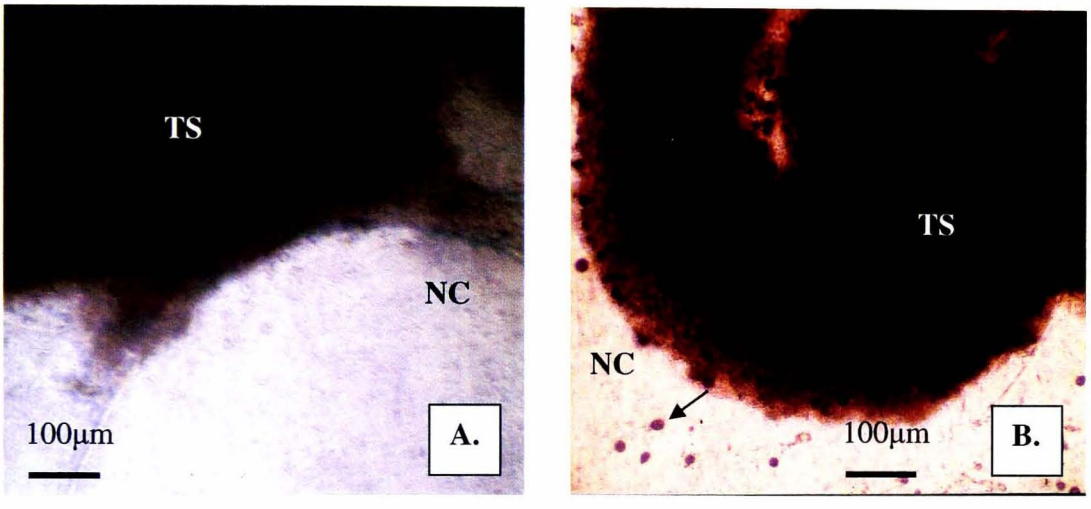

Figure 3.11. Light photomicrograph of an organotypic slice culture of rat olfactory bulb (10x). (**A**): 1DIV, new cells are already developing around the original tissue slice, and (**B**): 5DIV, new cells are clearly visible surrounding the original tissue slice. The slice itself is turning slightly dark as a result of cell death. Dark spots around the tissue slice are dead cells. In the figures TS = tissue slice and NC = new cells surrounding the tissue slice and the arrow indicates one of the dead cells (B).

3.5.4. Dissociated cell cultures

The dissociated olfactory epithelium and the olfactory bulb cells were grown on glass cover slips for up to 40DIV. Figure 3.12. shows a dissociated cell culture of rat olfactory epithelium at 10DIV. Cells of different stages can be seen forming connections in the culture. The elongated cells are believed to be olfactory sensory neurones (e.g. Graziadei, 1977).

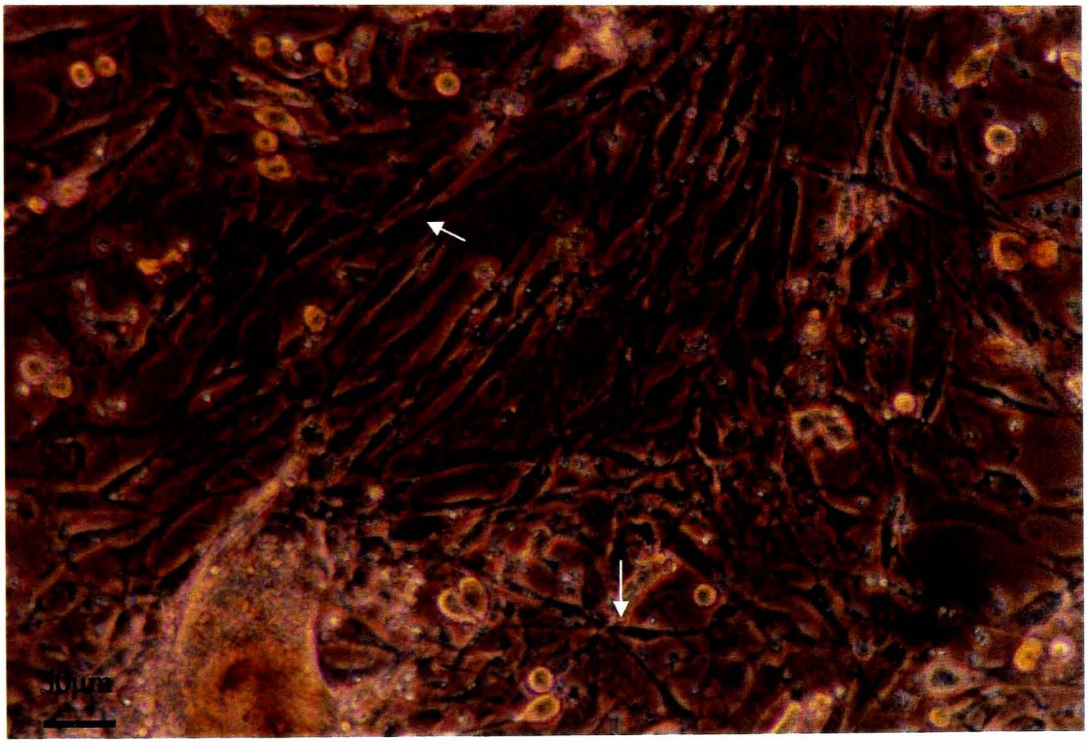

Figure 3.12. Light photomicrograph of a dissociated cell culture of rat olfactory epithelium 10DIV (20x). In the figure the arrows indicate some of the connections between the olfactory sensory neurones.

Figure 3.13. shows a dissociated cell culture of rat olfactory bulb 4DIV. Several different shapes and sizes of cells can be seen in this culture. These cells are clearly forming connections (arrows). The proportions of different cell types in a dissociated cell culture of the olfactory bulb are not known as yet.

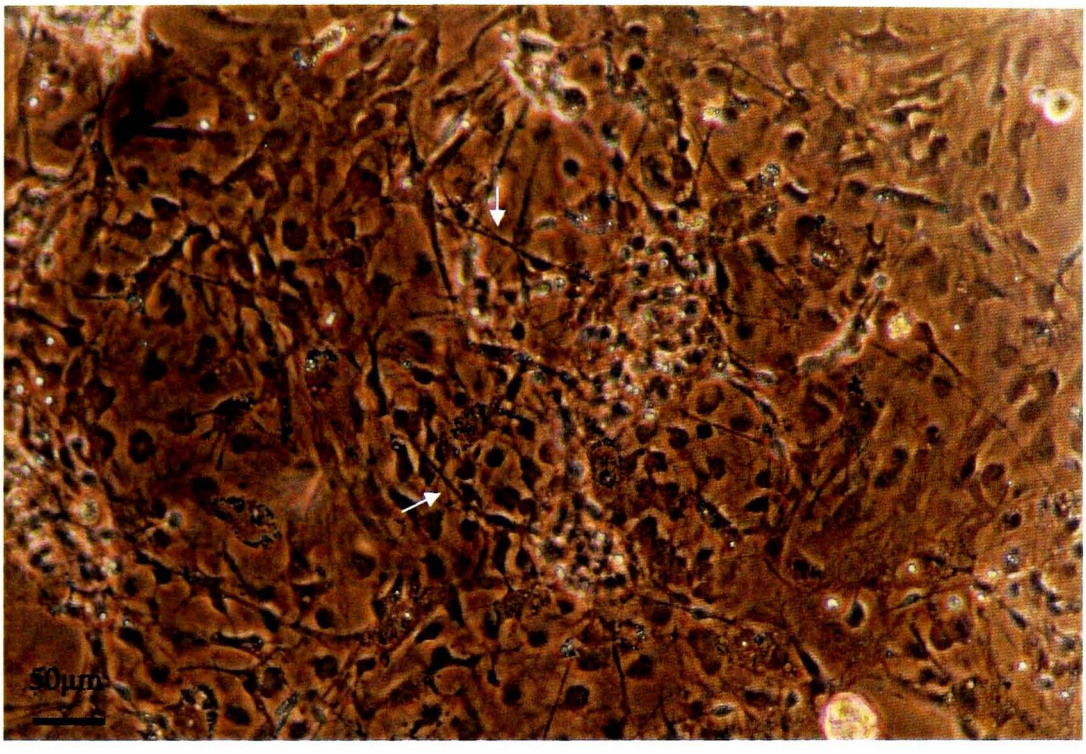

Figure 3.13. Light photomicrograph of a dissociated cell culture of rat olfactory bulb 4DIV (20x). In the figure the arrows indicate some of the connections between the cells.

3.5.5. Immunohistochemistry

A series of immunohistochemical stainings were performed on wax embedded and fresh, as well as cultured olfactory epithelium and olfactory bulb tissue. Wax embedded slides were stained for neurofilament and fresh tissue was stained for neurofilament and glia. Single stain for PHA-L was carried out for the labelled co-cultures of olfactory epithelium and olfactory bulb. All staining techniques used enable qualitative and quantitative characterisation of different cell types in tissue.

3.5.5.1. *Immunofluorescent Protocol*

The immunofluorescent methods described in part 3.4.5. were carried out successfully but background autofluorescence was discovered in both olfactory epithelium and olfactory bulb (Fig 3.14.), and prevented conclusive morphological results from being obtained.

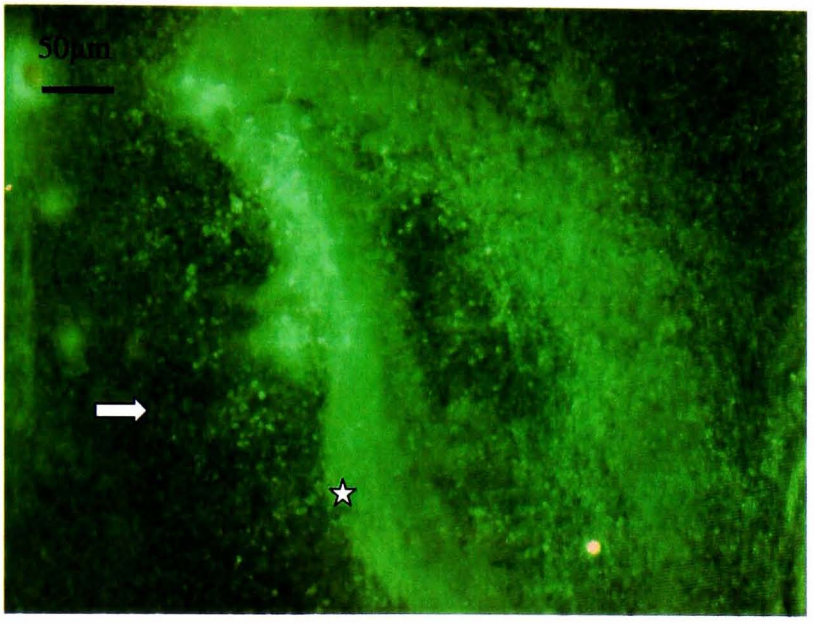

Figure 3.14. Fluorescent photomicrograph showing autofluorescence in a co-culture of an olfactory bulb slice (star) and dissociated olfactory epithelium (arrow) fixed in 4% paraformaldehyde prior to the staining (x20). Out of 5 samples stained, all 5 exhibited autofluorescence.

The results from the immunofluorescent staining for neurofilament and glia were invalid to certain extent due to the autofluorescence. The negative controls, as well as the actual stained samples, displayed a dull green (neurofilament) and red (GFAP) fluorescence throughout. However, close inspection of the samples illustrated the layered structure of the olfactory bulb in the stained samples, as well as a brighter peripheral band and a few brighter spots (Fig. 3.15.). In the samples stained for neurofilament, the brighter spots were also evident in the negative control and therefore can not be attributed to stained neurons. However, in the case of the GFAP stain the bright spots were not present in the negative sample. Therefore, these spots could be due to either stained astrocytic glial cells or to endogenous peroxidase activity of

erythrocytes and leucocytes. The lack of the brighter spots in the negative sample is probably due to the brighter autofluorescence of the negative controls blocking them out. These results (Fig. 3.15.) provide an example of a double stained olfactory bulb. All the other immunofluorescent staining studies carried out provided similar non-conclusive results, n=8.

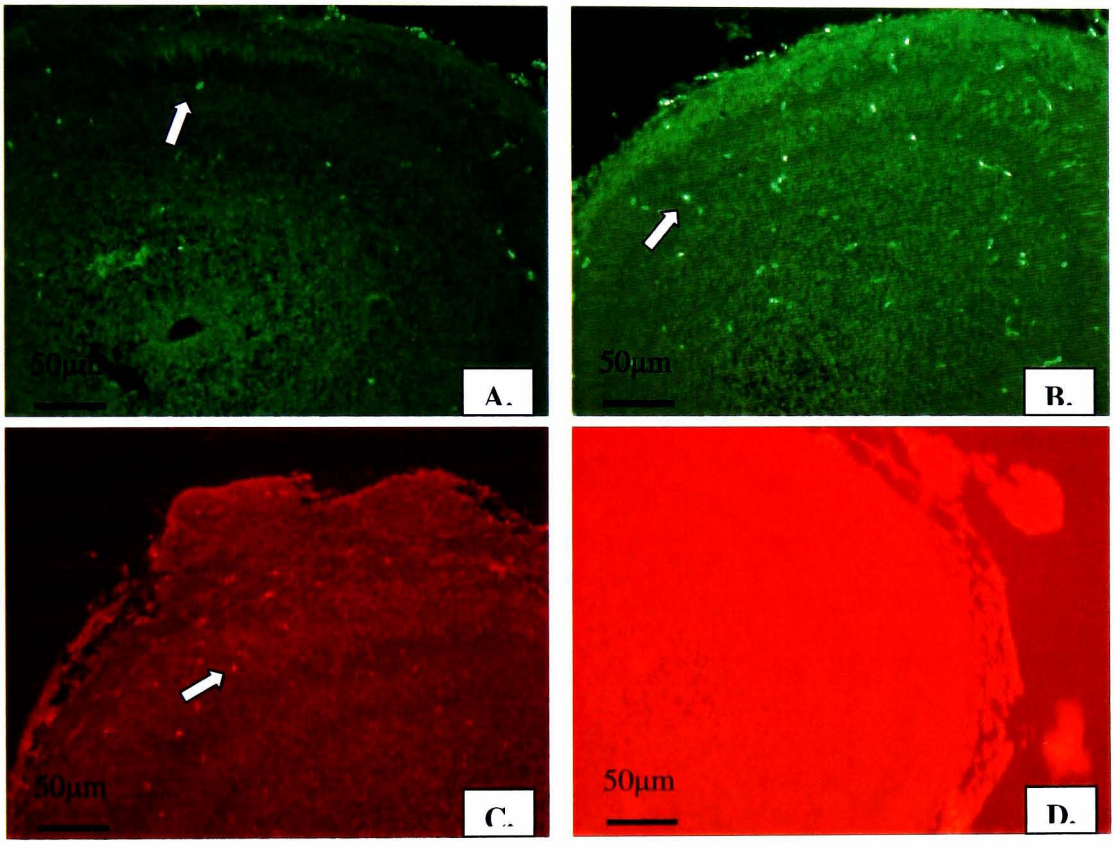

Figure 3.15. Immunofluorescent double staining for neurofilament and GFAP on wax embedded sections of 2-4 day old rat olfactory bulb. (**A**): Neurofilament stain (x20). (**B**): Negative control for neurofilament (x20). (**C**): GFAP stain (x20). (**D**): Negative control for GFAP. Arrows indicate suspected endogenous peroxidase activity of erythrocytes and leucocytes. Note: Most autofluorescent molecules emit in the green-yellow part of the spectrum and are not so prominent in the red fraction (Kasparov *et al.*, 2002). Therefore the GFAP stain

visualised with red fluorescent light is noticeable higher quality that that of neurofilament.

3.5.5.2. *Immunoperoxidase Protocol*

The high level of autofluorescence displayed masks the true result of specific antibody mediated immunofluorescence. As autofluorescence does not interfere with antibody antigen interactions, it does not have effect on the immunoperoxidase protocols (see 3.4.5.3.). Autofluorescence only masks the results of fluorescent images and not the DAB images which are visualised through a normal light source.

The immunoperoxidase DAB staining was successful in highlighting the presence of neuronal cells (Fig. 3.16.) and astrocytic glial cells (Fig. 3.17.) in the rat olfactory bulb. The difference between negative controls and actual samples is distinctive, even though the negative control has a very low level of background staining throughout the samples. The laminated structure of the olfactory bulb is clearly visible in the stained samples and the layers can be distinguished.

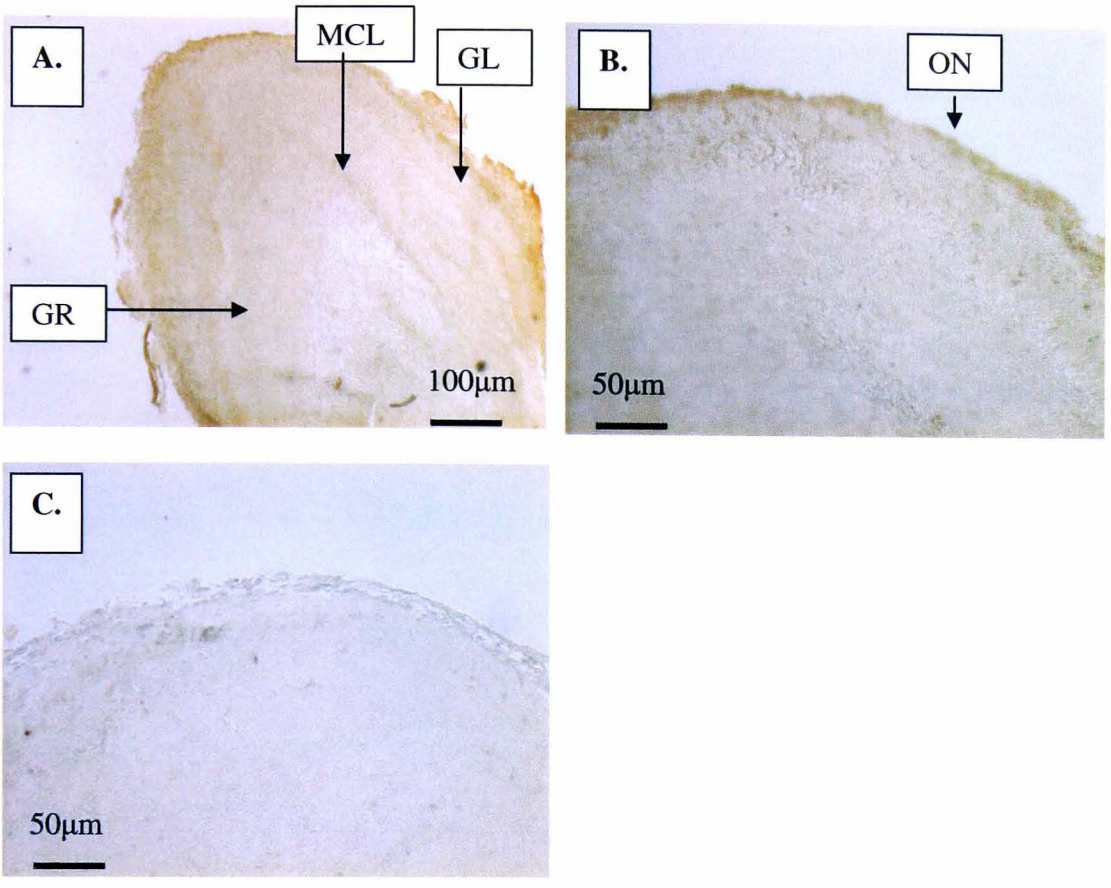

Figure 3.16. Immunoperoxidase stain for neurofilament on wax embedded sections of 2-4 day old rat olfactory bulb. (**A**): Neurofilament stain (x10), the layers of the olfactory bulb are clearly visible. (**B**): Neurofilament stain (x20). (**C**): Negative control (x20) showing negligible DAB staining. In the figures, GL = glomerular layer, MCL = mitral cell layer, GRL = granule layer, and ONL = olfactory nerve layer, n = 6.

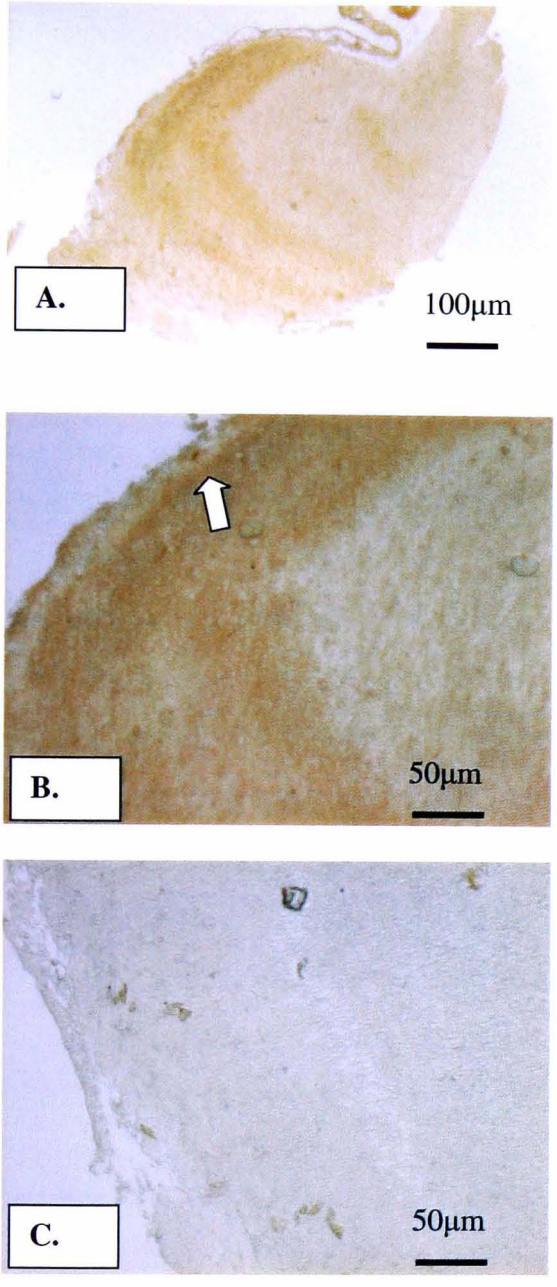

Figure 3.17. Immunoperoxidase stain for GFAP on wax embedded sections of 2-4 day old rat olfactory bulb, n = 5. (**A**): GFAP stain (x10). (**B**): GFAP stain (x20) showing a darkly stained peripheral band (arrow) of astrocytic glial cells. (**C**): Negative control (x20) showing negligible DAB staining.

3.5.5.3. *Single Stain for PHA-L*

The fixed co-cultures of PHA-L labelled dissociated olfactory epithelium cells and olfactory bulb slices were stained against PHA-L in order to visualise the axonal projections from the olfactory sensory neurons. To overcome the problem of autofluorescence, the samples were photo-bleached prior to staining. The photo-bleaching was found to only partly reduce the autofluorescence of the olfactory tissue. Bright staining of cellular particles (arrows) was found in the PHA-L stained samples but not in the negative controls (Fig. 3.18.).

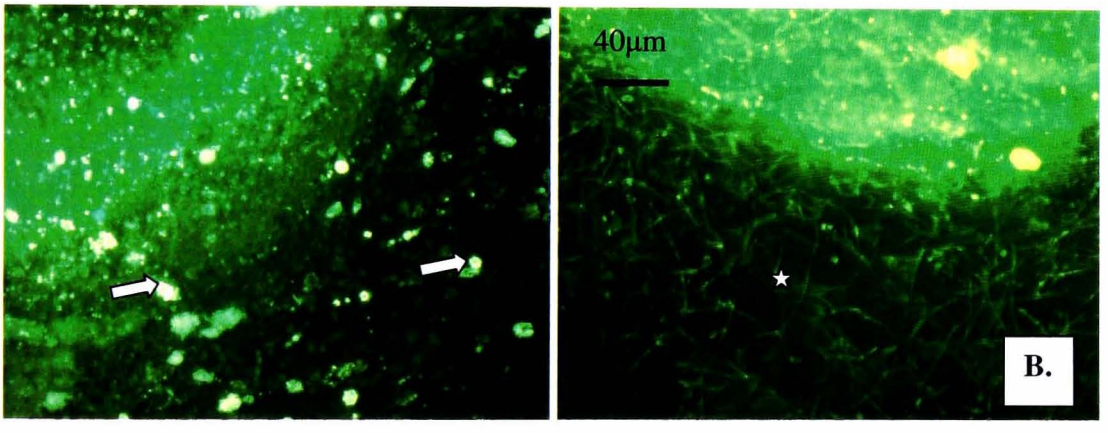

Figure 3.18. Immunofluorescent stain for PHA-L labelled co-cultures of dissociated olfactory epithelium and olfactory bulb slice. Out of 6 samples stained, all 6 exhibited autofluorescence. (**A**): The olfactory sensory neurons (arrows) are intensely highlighted (x25). (**B**): Negative control showing axonal projections from the olfactory sensory neurons (star) to the olfactory bulb, probably highlighted by autofluorescence alone (x25).

To overcome the problem of autofluorescence, the co-cultures were stained against PHA-L using immunoperoxidase method (Fig. 3.19.). The negative controls have similar staining patterns to the actual samples: the peripheral band of the olfactory bulb is stained strongly and the olfactory epithelium cells are highlighted. The nuclear and axonal staining of the olfactory sensory neurons is visible to some extend also in the negative control, but much weaker than in the stained samples.

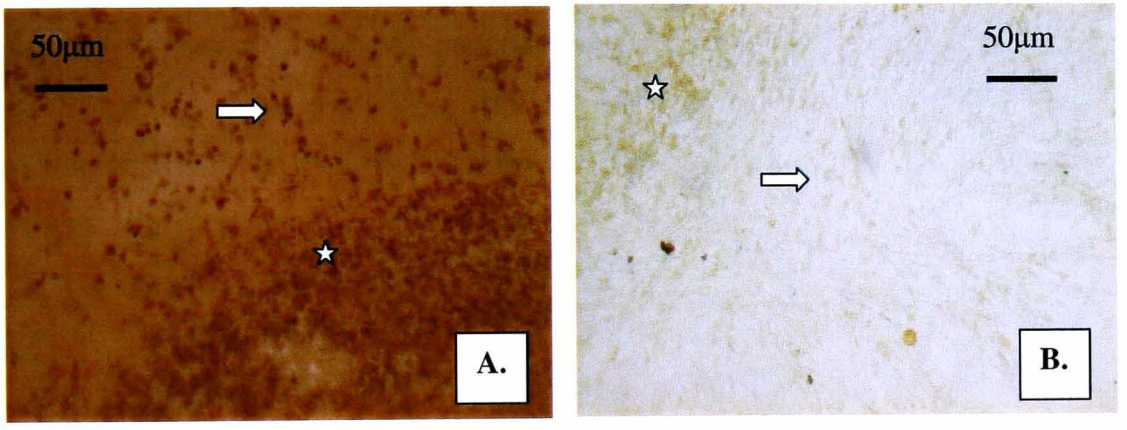

Figure 3.19. Immunoperoxidase stain for PHA-L on co-cultures of dissociated olfactory epithelium and olfactory bulb slice, n = 4. (**A**): Strong DAB stain of olfactory sensory neurons (arrow) and olfactory bulb cells (star) (x20). (**B**): Negative control showing weak DAB staining of olfactory sensory neurons (arrow) and olfactory bulb cells (star) (x20).

3.5.6. Electrophysiology

A small number (n[cells]=3, n[cultures]=12) of spontaneously electrically active neurons in the olfactory bulb cultures were discovered first with single unit recording from the organotypic slice cultures and later using dissociated cell cultures grown on MEAs. The electrically active cells were few and far apart, and the firing rates of these cells were very low (less than 20 spikes/minute, no bursts). This was found to be the case even after stimulation with the selective competitive $GABA_A$-receptor antagonist bicuculline methobromide (40μM; BIC, Sigma, UK). Bicuculline has been shown to induce synchronised epileptiform-like burst discharges in dissociated neuronal cell cultures grown on MEAs (e.g. Gross *et al.*, 1995). No statistical analyses were conducted due to the small number of active cells and the low firing rates.

3.6. DISCUSSION

The purpose of this part of the experimental study was to evaluate the olfactory system as a model for the *in vitro* sensory system to be developed and to be used for gaining greater understanding of sensory processing at neural network level. The aims for this part of the study consisted of three points and, based on the results, these points and questions can now be answered.

1: The behaviour of olfactory tissue (cells) *in vitro* vs. *in vivo*.

The olfactory system was studied using the organotypic slice and dissociated cell cultures. Good progress was made in understanding the behaviour of the olfactory system in a culture. The model *in vitro* olfactory system developed was compared with that of *in vivo* using immunohistochemical staining of neurofilament, GFAP and PHA-L, which highlighted neurones, glia (astrocytes) and olfactory sensory neurone respectively. The model chosen was dissociated olfactory epithelium cell culture combined with an organotypic slice culture of olfactory bulb. This is thought to be morphologically the most comparable system to that of *in vivo* (Hajos *et al.*, 1992). Axons from the olfactory sensory neurones were seen to penetrate the olfactory bulb from 1DIV onwards demonstrating the plasticity of the olfactory system. This also indicates the possibility of presence of chemotropic factors released from the olfactory bulb encouraging the olfactory sensory neurones to proliferate and to form synapses with the bulb itself (Graziadei *et al.*, 1979).

Production of the model cultures was successful throughout the study. Unfortunately, the immunohistochemical studies were not successful – largely due to the autofluorescence exhibited by both, olfactory epithelium and olfactory bulbs. However, some immunohistochemical results were achieved using the immunoperoxidase method and the immunofluorescent protocol for PHA-L. After successful removal of some of the autofluorscence by photo-bleaching, bright cellular structures, olfactory sensory neurones, were discovered in the olfactory epithelium surrounding the olfactory bulb. These olfactory sensory neurones projected penetrating axons to the olfactory bulb slice. To be able to analyse the connections formed further, the autofluorescence of the olfactory epithelium and olfactory bulbs needs to be completely eradicated.

Autofluorescence itself can be intrinsic to the tissue or induced by the fixation process (Del Castillo *et al.*, 1989; Noonberg *et al.,* 1992). The reason behind the autofluorescence can only be determined by comparing the tissue in a culture environment to freshly dissected tissue (without any contact with chemicals) to see whether the autofluorescence is caused by the culture medium, the fixative or if it is naturally present in the tissue.

Autofluorescence was not a problem with the samples stained using the immunoperoxidase method as a normal light source instead of fluorescent light is used to visualise the resulting staining. The DAB staining achieved using this method was present in the GFAP and neurofilament stainings and negligible in the respective negative samples.

The other types of co-cultures of olfactory epithelium and olfactory bulb (see 3.4.8.1. and 3.4.8.2.) also showed axonal projections from the olfactory sensory neurones to the olfactory bulb and the olfactory sensory neurones formed synapses with the dissociated olfactory bulb cells. The success in forming co-cultures of dissociated olfactory epithelium and olfactory bulb seeded on the opposite ends of long cover slips and later grown together forming synaptic connections is worth noting. There is no evidence of this particular co-culture type in the current literature and this is a novel arrangement to study olfactory system *in vitro*. However, further studies need to be done to confirm the presence of the synaptic connections between the two tissue types. In this study, the assumption of the presence of these connections is based only on the ultra-structural details of the live cultures.

Dissociated olfactory epithelium and olfactory bulb cells were also cultured on their own. With olfactory bulb, this was achieved easily, the cells attached to the culturing surface by 1DIV and continued to proliferate showing dendritic projections by 4DIV. Interestingly, the olfactory epithelium cells did not attach to the culturing surface, and consequently did not proliferate. Several preparations confirmed this with large amounts of detached free floating cells not proliferating after 1DIV. In fact, most cellular structures necrosed within the first 24 hours, indicated by the large amounts of floating cellular debris. The cultures did not improve within 9DIV.

The lack of cell attachment onto the cover slips was the primary reason for the rapid cell necrosis seen in the cultures. Several studies suggest that the problem

may be the surface coating of the cover slips. Grill & Pixley (1997) and McEntire and Pixley (2000) successfully cultured dissociated olfactory epithelium on an astrocyte feeder layer derived from the central nervous system providing metabolic support. Ronnett *et al.* (1991) used cover slips containing MDV and laminin. They found the cells to attach within 2 hours *in vitro*, to distribute axonal and dendritic processes with 24 hours and to reach uniform distribution within 72 hours *in vitro*. Similar pattern of proliferation was seen in this study when culturing dissociated olfactory epithelium together with the olfactory bulb, explanted on laminin coated cover slips. This suggests that a substratum such as laminin is required for the adhesion of olfactory epithelium cells.

There have been several studies *in vivo* and *in vitro* (e.g. Schwob *et* al., 1992; Holcomb *et al.*, 1995) suggesting that the survival of the olfactory sensory neurones is tropically dependent on the presence of the olfactory bulbs. Holcomb *et al.* (1995) showed that bulbectomy dramatically increases the number of apoptotic cells in the olfactory epithelium *in vivo*. They also suggested that the apoptotic death of olfactory sensory neurones *in vitro* can be inhibited by the presence of certain members of neurothropin family of growth factors released by the olfactory bulb. However, there have been several studies culturing olfactory epithelium successfully without the presence of olfactory bulb (e.g. Chuah *et al.*, 1991; Ronnett *et al.,* 1991; Pixley, 1996). All of these studies show, however, an increase in neurone degeneration compared to those with the presence of the olfactory bulbs. In comparative studies of olfactory epithelium cultured with and without olfactory bulb, it was discovered that a

greater number of mature olfactory sensory neurones was present (after 9DIV) in olfactory epithelium cultured with olfactory bulb. It can be concluded that the olfactory bulb plays an important role in the maturation of the olfactory sensory neurones (Grill & Pixley, 1997).

The number of cell present in the *in vitro* cell culture may also have an effect on the survival of the olfactory epithelium cell cultures (Pixley, 1996). In the preparations used in this study, the cell counts were performed straight after the dissociation rather than at the end of the procedure. A large proportion of cells may have been lost through centrifugation and washing resulting as lower than desired cell concentration in the final cell suspension.

2: The adaptability of electrophysiological methods for the olfactory system.

The long-term culture methods, such as the methods used in this experimental study, allow the basal properties of the network development to be investigated. They also enable the investigation of the effects of physical and pharmacological manipulation and set a stage for analyses of regulatory factors influencing neuronal generation and differentiation. The culture methods described (see 3.4.) were used successfully throughout the study and the resulting model, in theory, offers an optimum base for electrophysiological studies of neuronal network. Dissociated monolayer cultures are ideal, not only for MEA studies (e.g. Gross & Kowalski, 1991; Streit *et al.*, 2001) but also for patch clamp (e.g. Maue & Dionne, 1987).

Previously there have been good results in electrophysiological experiments of olfactory bulb (e.g. Ciombor *et al.*, 1999; Desmaisons *et al.*, 1999), but mainly only using extracellular single unit recording methods. The olfactory bulb network cultures on MEAs were found to only rarely exhibit electrical activity.

The possibility to be able to record electrical activity from the co-cultures of olfactory epithelium and olfactory bulb is very small. There have been only a few electrical recordings made from isolated mammalian olfactory receptor cells (e.g. Maue & Dionne, 1987). This is generally due the difficulties caused by the small size and fragility of these cells. The technical difficulties of stimulating and recording from mammalian olfactory receptor cells have limited most electrophysiological studies to the larger and tougher olfactory receptor cells of amphibians (e.g. Lowe & Gold, 1991; Reisert & Matthews, 1999). Those few studies that have recorded single unit discharges from rodent olfactory receptors, have been carried out in the intact olfactory epithelium *in situ* using relatively brief air-borne stimuli (e.g. Duchamp-Viret *et al.*, 2000).

It is still largely unknown, whether or not the olfactory neurones are capable of generating spontaneous (i.e. without stimulation or current injection) bursting activity. In 2004, Hayar *et al.* showed that the external tufted cells of the olfactory bulb exhibit spontaneous bursting generated by a persistent sodium current active at resting membrane potential. However, it is still unclear whether any other olfactory neurone subtypes generate spontaneous bursting. The olfactory bulb cultures used in this study consisted of a heterogeneous population of olfactory bulb neurones and glia. The lack of spontaneous activity observed may be due to a lack of generation of spontaneous bursting by

different subtypes of olfactory neurones. To study this further, a homogenous population of external tufted cells should be used as a positive control.

3: Suitability of the olfactory system for the purposes of this research.

After the lack of success with the electrophysiological recording from the dissociated olfactory bulb cells grown on MEAs and discussions with other research groups working in the area, notably Prof. G.W. Gross (CNNS, University of North Texas, USA) and Dr E.D. Herzog (Department of Biology, Washington University, USA), it was decided the olfactory system was an unsuitable preparation for the purposes of this study. Therefore, the main focus of the study was moved from the olfactory system to the spinal cord and dorsal root ganglia.

CHAPTER 4

CO-CULTURES OF SPINAL CORD AND DORSAL ROOT GANGLIA

4.1. INTRODUCTION

The spinal cord is formed of 31 segments, which form during embryonic development. The dorsal medial sulcus and the ventral medial fissure are continuous from the medulla to the spinal cord. Lateral to the dorsal medial sulcus is called the dorsal root entry zone, a continuous line along the spinal cord. This is where small fascicles or bundles enter the spinal cord forming the dorsal root. The cells giving rise to the dorsal root axons, pseudounipolar sensory neurones, form clusters of nerve cells that are wrapped into connective capsules forming small nodules. These lie in two chains on either side of the spinal cord and are a part of the sympathetic system of the autonomic nervous system. These clusters, associated with each dorsal root, are the dorsal root ganglia (Kingsley 2000).

The motoneurones of the spinal cord send preganglionic axons to the autonomic ganglia, where they synapse on principal neurones, the ganglion cells. The

ganglion cells send their postganglionic axons to various different target tissues (Adams & Koch 1990).

The autonomic dorsal root ganglia neurones require the presence of their peripheral target for survival. The target tissue produces trophic factors, which are essential for the survival of the dorsal root ganglia neurones. It is also likely, because of their central projections, that the spinal cord is also required especially during the early development. Adding nerve growth factor-supplement into the cultures and thus providing these essential trophic factors prolongs the survival of the dorsal root ganglia *in vitro* (Wetts & Vaughn 1998).

Dorsal root ganglia also contain the cell bodies of primary afferents of the somatosensory system. These neurones are functionally heterogenous and signal sensory receptor-transduced stimuli of diverse sensory modalities including touch, temperature, nociception (pain) and proprioception (Namaka *et al.,* 2001). Somatosensory signals normally originate at peripheral afferent axon endings (Amir *et al.,* 2002).

During development, the dorsal root ganglia are derived from precursors in the neural crest (e.g. Greenwood *et al.,* 1999). Both in the mouse and rat the neurogenesis of the dorsal root ganglia ends several days before birth (e.g. Kitao *et al.,* 1996). However, Namaka *et al.* (2001) suggest that early postnatal dorsal root ganglia continue to house a population of neuronal precursors which remain their capability for neurogenesis beyond embryological development.

The ability of the dorsal root ganglia to fire repetitively depends on their ability to generate subthreshold membrane potential oscillations. Action potentials are triggered when the amplitude of oscillations reaches the threshold and therefore only neurones with oscillations fire repetitively. Other neurones may fire at the onset of a depolarizing pulse but the activity can not be sustained (Amir *et al.*, 2002).

In the experiments involving dorsal root ganglia attached to spinal cord, foetal rats (embryonic day 15) were used. At this stage of development the dorsal root ganglia remain attached to the spinal cord when removed and the ganglia contain few fibroblasts and Schwann cells.

4.2. METHODS

The spinal cord and attached dorsal root ganglia were removed and sliced as described in chapter 2. Co-cultures were established using the Stoppini method (see 3.4.6.1.) for the morphology studies and dissociated cell cultures (see 2.2.3.) in order to observe the electrical activity of the co-cultures. Dissociated spinal cord cells were seeded on one end (Ch 1-32), and dissociated dorsal root ganglia cells to the other (Ch 33-64), of dual MEAs (Fig. 4.1.) using the methods described in chapter 2.

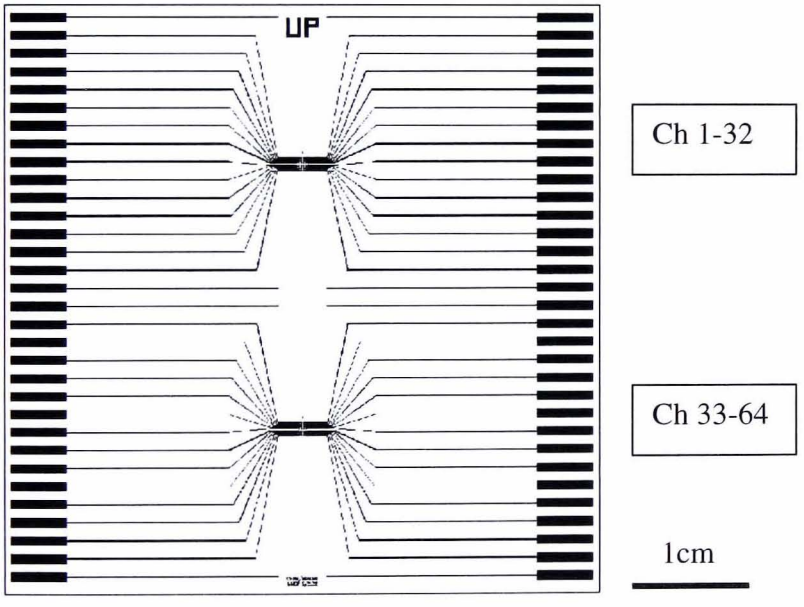

Figure 4.1. A dual MEA for co-cultures (model #4, supplied by Professor G.W. Gross, CNNS, University of North Texas, USA). The distance between the two groups of 32 electrodes is 2.5cm.

The cells were allowed to grow in a controlled, constant temperature, pH and cell culture media volume environment (see 2.2.3.) for a minimum of 21 days

and connect between the two groups of electrodes. Some preliminary electrophysiological recordings were made from the dissociated spinal cord cultures. Compounds tested included a potent cannabinoid agonist, HU210 (50nM-5μM; n=3).

4.3. RESULTS

An organotypic slice culture of rat dorsal root ganglia attached to the spinal cord on day 7 *in vitro* is shown in Figure 4.2. New cells have filled up the space surrounding the tissue slices and connected two separated slices of dorsal root ganglia attached to the spinal cord.

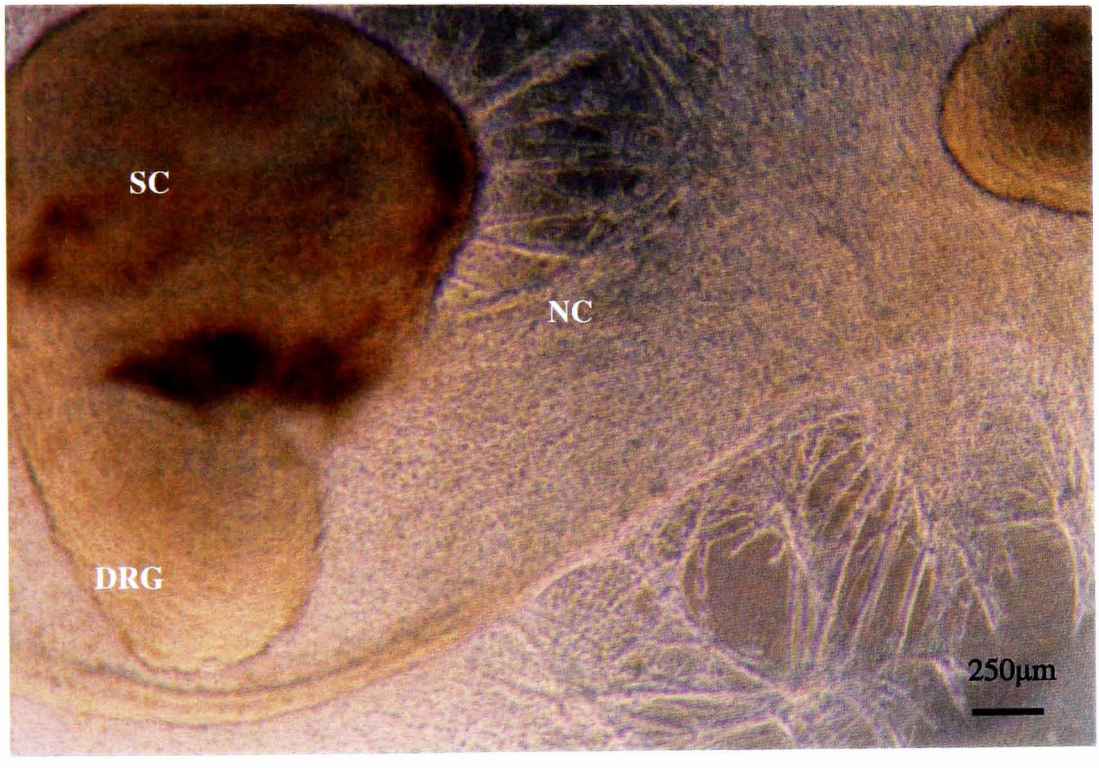

Figure 4.2. Light photomicrograph of an organotypic slice culture of rat dorsal root ganglia attached to the spinal cord grown on a cover slip on day 7 *in vitro* (4x). In the figure SC = spinal cord, DRG = dorsal root ganglia and NC = new cells connecting the two separated slices of dorsal root ganglia attached to the spinal cord.

The dissociated cell cultures of spinal cord and dorsal root ganglia seeded in the opposite ends of dual MEAs were grown for three weeks. Unfortunately, it was discovered that the cells were not able to connect over the distance of 2.5cm between the two arrays of electrodes. Therefore no electrophysiological data was recorded from the co-cultures of spinal cord and dorsal root ganglia. Spinal cord cultures (n=18) were used for some basic pharmacological tests to test the reliability of the cultures. The effect of the cannabinoid agonist HU210 (50nM-5μM; n=3) on the spontaneous activity of the spinal cord cultures was tested. HU210 was found to decrease the spontaneous electrical activity of *in vitro* neuronal networks concentration dependently comparably to what has been found *in vivo* (e.g. Chapman, 2001).

4.4. DISCUSSION

Neuronal networks of the developing spinal cord are spontaneously active and able to generate rhythmic activity. This activity consists of on-off patterns, bursts, directed to flexor and extensor muscles and used for locomotion (Chub & O'Donovan, 1998; Tscherter *et al.*, 2001). Several pharmaceutical compounds can be used to manipulate the types of rhythmicity, e.g. NMDA has been used to induce fictive locomotion (Kudo & Yamada, 1987). The dissociated cell cultures of spinal cord grown on MEAs, therefore, formed an ideal model for pharmaceutical physiological studies. The addition of co-cultured dorsal root ganglia added a possibility of studying the interactions between these two tissue types.

The dissociated cell cultures of spinal cord and dorsal root ganglia have been previously successfully cultured for electrophysiological studies (e.g. Crain & Peterson, 1981). Crain & Peterson also showed that the isolated foetal mouse dorsal root ganglia cells are able to grow across a collagen substrate and, more importantly, then selectively innervate specific dorsal horn and dorsal column nuclei regions (but interestingly not ventral cord (Peterson & Crain, 1981)) in co-cultured spinal cord. The cultured dorsal root ganglia cells form connections in central nervous system target zones generating characteristic primary afferent network responses to sensory stimuli.

In this experimental study, however, the co-cultures of spinal cord and dorsal root ganglia were not able to connect from the two sides of the dual MEAs. The dual MEAs are unsuitable for studying co-cultures of this type, even though

there is a distinct possibility that some other cell types would be able to connect over the distance of 2.5cm. However, the electrophysiological properties of co-cultures of spinal cord and dorsal root ganglia should be studied using an alternative method.

The role of cannabinoids in pain control is one of the ongoing major interests in the School of Biomedical Sciences, University of Nottingham. HU210 is a potent cannabinoid agonist and has been shown to have an effect on somatosensory transmission in neuropathic pain (Chapman, 2001). The effect of HU210 on the spontaneous activity of the spinal cord cultures grown on MEAs was tested to assess the reliability of the MEA spinal cord cultures. HU210 was found to decrease the spontaneous electrical activity of *in vitro* neuronal networks concentration dependently comparably to what has been found *in vivo* (e.g. Chapman, 2001). It can be concluded that the data collected form the spinal cord neuronal networks is reliable.

CHAPTER 5

CHARACTERISATION OF RODENT SPINAL CORD AND FRONTAL CORTEX MONOLAYER NETWORKS ON MEAs

5.1. INTRODUCTION

5.1.1. Gap junctions

In the multicellular organisms, each cell type retains its individuality, even though all cellular functions must be co-ordinated with other cells. Organisms have several strategies to achieve this: the long range interactions are mediated by neural or endocrine mechanisms, whereas the short range interactions are mediated by direct cell to cell contact. The direct cell to cell contact involves communication through channels in a specialised cell surface membrane structure, the gap junction (Kumar & Gilula, 1996). The gap junctions (Fig. 5.1.) exist in all metazoans and in almost all the cell types in these organisms. Some exceptions are skeletal muscle, red blood cells and freestanding cells, such as circulating lymphocytes. The size and numbers of gap junctions are dependent on the cell cycle and the physiological state of the organism and exposure to environmental stress (Kumar & Gilula, 1996).

Gap junctions develop very early in rodent development, during compaction in the eight-cell stage (Houghton *et al.*, 2002). They play a crucial role in relaying developmental electrical and metabolic signals during embryogenesis (e.g. Guthrie & Gilula, 1989) and both in neural and extraneural development (Allen *et al.*, 1990). Intercellular channels present in gap junctions are the only channels that allow adjacent cells to share ions, second messengers and small metabolites. This intercellular communication permits co-ordinated cellular activity, which is critical for organ homeostasis of multicellular organisms (Bruzzone *et al.*, 1996).

The basic unit of a gap junction is transmembraneous channel termed a connexon, a hexamer formed of protein subunits (approx. 25 - 60 kDa) called connexins, at least 16 of which are encoded by distinct genes in rodents (Houghton *et al.*, 2002). Connexons permit intercellular diffusion of ions and small molecules up to the molecular weight of ~1 kDa or diameter of 10 to 14nm (Willecke *et al.*, 2002), although the permeability of gap junctions may vary in different tissues (Nicholson *et al.*, 2000). Two connexons are attached to each other via their extracytoplasmic extremities forming gated homo- and hetero-typic intercellular pores, through which the low molecular weight compounds are transported (Bruzzone *et al.*, 1996).

The connexon forming connexins are a group of integral membrane proteins. They are synthesised in the rough endoplasmic reticulum (Falk *et al.*, 1994) and then recruited into the formation of gap junctions (Windoffer *et al.*, 2000). The

connexins traverse the membrane four times and are expressed in many tissues. The connexin proteins consist of specialised regions so that they can be anchored into the lipid bilayer of the cell membrane, have external regions which allow coupling to the opposing connexon and have intracellular regions for specific regulatory control in the cytoplasm. In some cell types the hexameric connexons are formed by six identical connexins (homologous connexon) and in other cell types by a combination of two expressed connexins (hybrid or heterologous connexons) (Kumar & Kilula, 1996). The gap junctions formed by different connexins have different functional properties and are gated differently. They are differentially sensitive to transjunctional voltage, intracellular pH and effects of phosphorylating agents such as tyrosine kinases (Dermietzel, 1998). Also, the spread of the intercellular Ca^{2+} has been correlated with the expression of different connexin types (e.g. Toyofuku *et al.*, 1998). The connexins have attracted a lot of attention in recent years, mainly in connection to the identification of hereditary diseases (e.g. developmental malformations) caused by mutations in connexin genes and the involvement of gap junctional communication in tumour formation (Bruzzone *et al.*, 1996).

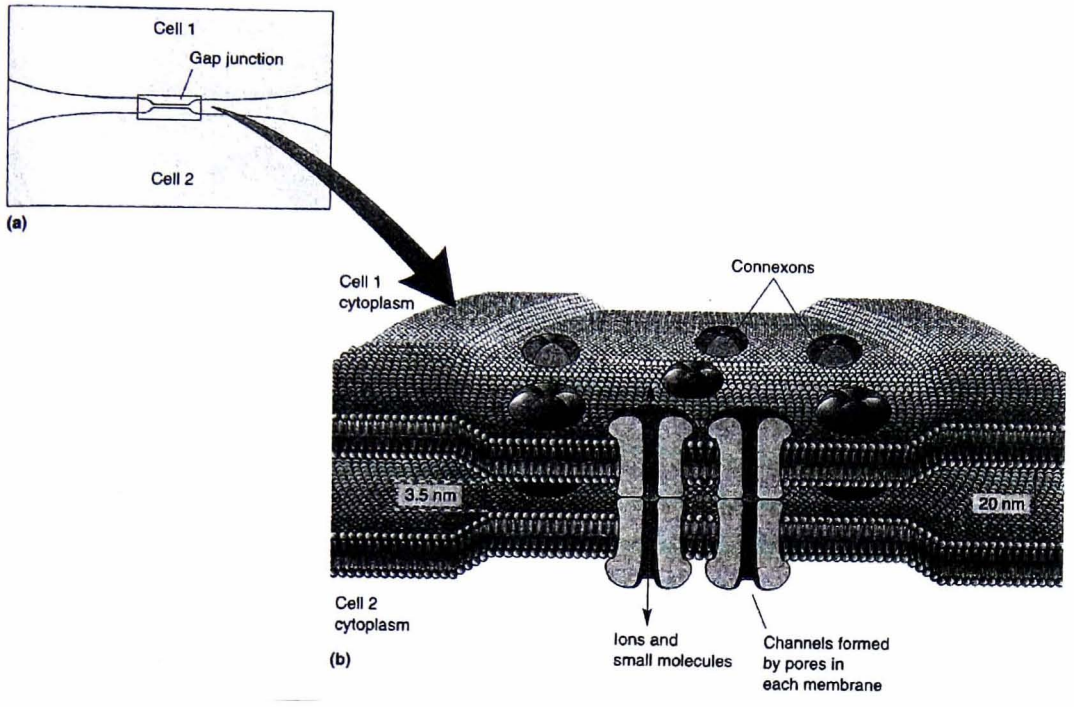

Figure 5.1. The basic structures of a gap junction and gap junction forming connexins (From Bear *et al.*, 2001). Two cells connected by a gap junction (a) and an enlargement showing connexons, each formed by six connexins bridging the cytoplasm of the two cells.

The connexins in the nervous system are developmentally regulated and differentially expressed (Dermietzel & Spray, 1993). The major connexin type in neuronal cells is connexin36 (Cx36). Cx36 is differentially expressed in various brain regions, including moderate levels in cortex and spinal cord (Condorelli *et al.*, 1998). Other connexin types, apart from the neuronal Cx36, are also found in the central nervous system. Astrocytes express predominantly Cx43, whereas oligodendrocytes express Cx32. In addition Cx26 and Cx43 are expressed by leptomeningeal and ependymal cells. These gap junction proteins are differentially expressed during the embryonic and postnatal maturation of the brain: Cx43 and Cx26 predominate in the neuroepithelium of embryonic

mammalian brain, whereas Cx32 is virtually absent. Cx26 largely disappears between 3 and 6 weeks after birth, whereas Cx32 increases. Expression of Cx43 remains high throughout the mammalian development (Dermietzel *et al.*, 1989). The exact extent and identity of connexins in neurones is still unclear. In total the cell-specific expression of eight different connexins has been demonstrated in the central nervous system (Dermietzel *et al.*, 1989; Dermietzel & Spray, 1998; Table 5.1.).

NEURONE	ASTROCYTE	OLIGODENDROCYTE
Cx36(mRNA)	Cx43	Cx32
Cx32	Cx45	Cx45
Cx26	Cx40	
Cx43	Cx30	

Table 5.1. Cellular pattern of connexin expression in brain tissue (According to Dermietzel, 1998).

The rhythmic electrical activity in the central neural networks is thought to arise from interneuronal electrical coupling via the gap junctions as well as from chemical synaptic inhibitory transmission (Tamas *et al.*, 2000). Also electrical field effects and ionic changes in the extracellular space are thought to synchronise neurones (Dudek *et al.*, 1999). The gap junctions do not only synchronise the outputs from coupled neurones, but also provide rapid impulse propagation between pre- and postsynaptic elements. Electrophysiological recordings support the importance of gap junctional communication in network behaviour. Direct coupling by electrical synapses is now accepted to provide a

second major pathway, in addition to chemical transmission, contributing to physiological rhythms (Zoidl & Dermietzel, 2002). Particularly the role of Cx36 is thought to be important in the generation of widespread, synchronous inhibitory activity (Deans *et al.*, 2001).

The gap junctions between the neurones can be soma-somatic, i.e. between the two somas of the adjoined cells, dendro-dendritic, i.e. between the dendrites of the two cells or dendro-somatic, i.e. formed between the soma of one of the cells and dendrite from the other cell. The gap junction types identified in the rodent spinal cord at the electron microscopic level are dendro-dendritic and dendro-somatic (Matsumoto *et al.*, 1988). Interestingly, work by Ishimatsu and Williams (1996) in the locus coeruleus suggests that also the dendro-dendritic gap junctions outside the cell bodies can lead to synchronous activity within the nucleus.

Like many ion channels, gap junctions are voltage dependent, although the magnitude of the dependency varies with the type of connexin (Spray, 1994). The voltage sensitivity of these channels is thought to involve the movement of electrical charges in the protein as a response to the potential differences across the cell membrane (Kumar & Gilula, 1996). The main cytoplasmic factor in the regulation of gap junction function is thought to be the intracellular Ca^{2+}. It is transferred through gap junctions by diffusion and also plays a role in gating the gap junction channels. The changes in intracellular Ca^{2+} concentrations serve a role in co-ordination of cell activities, including neurone-glia cell interactions: A change in intracellular Ca^{2+} levels in glial cells can cause a change in

intracellular Ca^{2+} levels of surrounding neuronal cells. This process is mediated by gap junctions between the two different cell types (Nedergaard, 1994).

The gap junctional communication is also modulated by neurotransmitters such as dopamine and noradrenaline in various parts of the central nervous system (Rörig & Sutor, 1996). For example, glutamate receptors have been shown to be involved in the generation of rhythmic locomotor activity in neonatal spinal cord. Also, the locomotor rhythm of the spinal cord can be depressed by the blockade of NMDA receptors, both *in vivo* and *in vitro* (e.g. Beato *et al.*, 1997). Early postnatal blockade of these NMDA subtype of glutamate receptors also delays the normal developmental decrease of the electrical coupling during the first postnatal weeks, which suggests that the increase in glutamatergic synaptic activity with the onset of locomotion leads to the loss of gap junctions between developing motoneurones (Mentis *et al.*, 2002).

The conductance of different gap junctions varies between 30 and 300pS. This means that these channels, when open, can have a great influence on the input resistance and activity of a cell. Since the connection between neurones is often an array of gap junctions, the junctions could, if open simultaneously, have an enormous effect on the input conductance and electrical behaviour of coupled neurones (Carlen *et al.*, 2000).

Each gap junction formed by specific connexins has different biophysical and modulatory properties. This means that gap junctions are able to mediate a great diversity of physiological and pharmacological responses (Carlen *et al.*, 2000).

Hence, when studying the gap junctions, it is important to characterise the specific gap junctions being affected.

A characteristic neuronal network exhibits spontaneous synchronised depolarisations detectable as bursting behaviour and/or intracellular calcium oscillations. The mechanisms causing this synchronisation are still under debate, but several non-synaptic mechanisms, such as electrical field effects, ionic changes in the extracellular space and electrical coupling through gap junctions, have been suggested (Dudek *et al.*,1999; McCormick & Contreras, 2001). The gap junctions provide the morphological basis needed for network organisation: they are formed by intracellular channels composed of connexins which make up a multigene family (Willecke *et al.*, 2002), whose members are separated according to their molecular mass (Bruzzone *et al.*, 1996). The permeability of gap junctions is controlled by endo- and exogenous compounds such as neurotransmitters, cytokines, growth factors (Rouach *et al.*, 2002) and other bioactive substances, such as CBX, a potent gap junction blocker.

The involvement of gap junction in generation of neuronal synchrony is supported by several studies. The presence of the gap junctions has been demonstrated in slices of several CNS structures (e.g. Rorig *et al.*, 1996), and it is also known that the network activity in neuronal *in vitro* assemblies can be abolished by gap junction blockers in several CNS regions (e.g. Margineanu & Klitgaard, 2001). Theoretical neuronal network models also suggest that the gap junctions play an important role in the neuronal synchronisation (Perez Velazquez & Carlen, 2000). Finally, it has been shown that the Cx36 knockout

mice display impaired gamma oscillations and high-frequency network oscillations (Buhl *et al.*,2003), 4-aminopyridine (4-AP) induced epileptiform discharges in the hippocampus (Maier *et al.*, 2002) and lack of synchrony on the inferior olive (Long *et al.*, 2002)

However, exactly how the gap junctions influence neuronal network activity remains unclear. The currently used gap junction blockers affect other ion channels and receptors as well as gap junctions themselves. The gap junction blockers have been shown to act not only in neurones, but also on astrocytes (Giaume & McCarthy, 1996). It has been suggested that the astrocytes play an essential part in the formation of the synchronous oscillatory and epileptiform activity seen for example in hippocampal neurones *in vitro* (Verderio *et al.*, 1999). It has also been shown that the astrocytes interact with neurones by responding to synaptically released neurotransmitters with changes in intracellular calcium concentration. This in turn affects the synaptic transmission (Kang *et al.*, 1998). It is likely that the astrocytic gap junctions have an important role in the neuronal network activity.

5.1.2. Gap junction blockers

Gap junction blockers, the uncoupling agents, have been widely used to study the biological role(s) of the gap junctions (e.g. Bannerman *et al.*, 2000). Despite the desirability of finding agents that would specifically block gap junctions

without other pharmacological effects, such agents are still unknown. The lack of natural gap junction toxins might be due to the inaccessibility of gap junction proteins to extracellular space. Generally, the uncoupling agents used were originally discovered as side effects of drugs that had been shown to affect other ion channels (Rozental *et al.*, 2001).

As a result of a working gap junction blocker, the single-channel current flickers rapidly between the open and closed states as a result of binding and unbinding of the drug to a site in the pore of the channel. Most of the known uncoupling agents elicit the closure of the channel by binding to the regions right outside the pore. In case of the blocker binding and unbinding fast, the single-channel current amplitude will seem to be reduced (Hille, 2001).

A large number of drugs are known to block gap junctions at least to some extent (Spray *et al.*, 2002). These include long-chain alcohols (e.g. octanol and heptanol), volatile general anaesthetics (e.g. halothane and ethrane), glycyrrhetinic acid derivates (18α- and 18β-glycerrhetinic acids), endogenous N-acylethanolamine oleamide, aminosulfonates (e.g. taurine), tetraalkylammonium ions and the antimalarial drug quinine (e.g. Johnston *et al.*, 1980; Davidson *et al.*, 1986; Burt & Spray, 1989; Bevans & Harris, 1999; Musa *et al.*, 2001; Srinivas *et al.*, 2001).

The most commonly used gap junction blockers include carbenoxolone (CBX), the volatile alcohol 1-octanol, halothane and, the endogenous N-acylethanolamine, oleamide. At least carbenoxolone and 1-octanol are known to

depress spontaneous bursting and antagonise spontaneous neuronal hypersynchrony in *in vitro* neuronal cultures. Burst depression occurs via a gradual decrease in burst amplitude, which, in turn, occurs via a decrease in the number of synchronised neurones in the network. Carbenoxolone has more specific interaction with the gap junction connexins than 1-octanol, halothane or oleamide, whose inhibitory actions conceivably derive from a profound perturbation of the membrane lipid bilayer (Margineanu & Klitgaard, 2001). Also, 1-octanol is thought to indirectly perturb gap junction function by altering the hydrophobic environment of the plasma cell membrane (Spray & Burt, 1990).

Halothane is a highly volatile general anaesthetic and also a potent and readily reversible gap junction blocker (e.g. He & Burt, 2000). However, the blocking action is not specific for gap junctions. In general, halothane depresses neuronal firing and excitatory synaptic transmission, and also potentiates synaptic inhibition. These actions are due to the inactivation of both voltage-dependent and agonist-triggered currents (Rozental *et al.*, 2001). The application of halothane leads to an increase in slow transitions between the open and closed stages of the gap junction channel, although also fast transitions have been occasionally observed. This pattern has also been detected in response to certain other gap junction blockers, such as heptanol and quinine (Srinivas & Spray, 2003). These slow transitions have been attributed to a distinct form of channel closure that presumably involves conformational rearrangements of the channel (Trexler *et al.*, 1996).

Oleamide is a sleep-inducing lipid which blocks gap junction communication without inhibiting intercellular calcium wave transmission in the cells. Oleamide exerts its action on some generalised structural property of the connexin oligomeres or channels in the lipid bilayer and is therefore able to block gap junction channels that contain different connexins. It does not affect other cell biological processes in addition to gap junction channels (Guan *et al.*, 1997).

5.2.METHODS

The effect of gap junction blockers on tissues used in this part of the experimental study (mouse and rat spinal cord and frontal cortex) was examined using dissociated cell cultures grown on MEAs. The tissues were dissociated following the protocol described in chapter 2.2. The cells were seeded on the MEAs at a density of 4.5 x 10^5 cells and the cultures were then left to mature for a minimum of three weeks prior to the electrophysiological examination. During this period, the culture medium was changed twice a week.

Immunohistochemical studies were performed on glass coverslips rather than on the MEAs due to the high cost of the MEAs. The cells were seeded on the coverslips at the same density as the MEAs, 3 x 10^5 per coverslip = 4.5 x 10^5 per MEA.

5.2.1. Drugs

The following drugs, classified as gap junction blockers, were used in the gap junction blocker study. They were all obtained from Sigma (USA). The mechanism of action of these drugs has been described in the introduction to this chapter.

3b-Hydroxy-11-oxoolean-12-en-30-0ic acid 3-hemisuccinate (carbenoxolone disodium salt; CBX) and 2-Bromo-2-chloro-1,1,1-trifluoroethane (Halothane) were diluted in sterile water.

1-octanol and oleamide were solubilised in dimethyl sulfoxide (DMSO; Sigma, USA) and diluted to final concentration in culture medium.

The volume of medium in the recording chamber was maintained at 2ml. Drugs were added directly to the chamber. Drugs were removed from the chamber by medium changes.

5.2.2. Data Analysis

Spike and burst rate histograms and burst amplitude, burst rate and neurone count histograms were constructed using NACTAN and I-burst (CNNS, University of North Texas, USA). The histograms were used to study the effects of the drugs on the neuronal cultures. The spike train rasters were constructed within the Nex programme (Plexon Inc., USA) and used to represent the firing of the neurones over time. In a spike train display, a single vertical black tick represents a single action potential (spike) and a cluster of these ticks two or more spikes close together, bursts. The red and yellow bands of ticks represent increasingly higher firing frequencies (Fig. 5.4 and 5.7).

5.2.3. Connexin Protein 36 (Cx36) Immunohistochemistry

It was necessary for the gap junction blocker study to show that the dissociated neuronal cultures grown on MEAs expressed Cx36, the most commonly expressed connexin in the central nervous system (see introduction to this chapter). This was done by staining dissociated cell cultures grown on coverslips for a minimum of three weeks for the Cx36.

The dissociated cell cultures of mouse spinal cord (32DIV) and frontal cortex (22DIV) were fixed for 2 minutes with 1:1 acetone and methanol. The fixative was removed and the samples were rinsed with PBS. After the rinses, Tris buffered saline (TBS; 0.005M Tris/HCl, pH 7.6) supplemented with 0.3% Triton X-100 was added for 30 minutes. After the incubation, the samples were washed with distilled water and 1ml of H_2O_2 in 59ml of methanol was added for 30 minutes and the samples were washed twice in TBS. DAKO protein block (Dako, UK) was added for 10 minutes prior to the addition of the primary antibody, anti-Cx36 (Zymed, USA, 1:50). The primary antibody was diluted with TBS containing 0.05% Triton X-100 and left on over night at 4°C. The control samples were incubated without the primary antibody.

The samples were washed twice with TBS and the secondary antibody from the DAKO kit was added for 30 minutes. Again, the samples were washed twice with TBS and the tertiary antibody from the DAKO kit was added for 30 minutes. The samples were washed twice in TBS and transferred into a fume

hood. 200µl of 3,3'-diaminobenzidine (DAB, Dako, UK) substrate was added on all samples for maximum of 5 minutes. The samples were the washed for 3x5 minutes in SUPW and counterstained with Harris' haematoxylin. The samples were washed with SUPW and a drop of Vectashield mounting medium was placed on each sample and the samples were mounted on microscope slides.

5.3. RESULTS

5.3.1. Immunohistochemistry

The connexins of the gap junctions exhibit a characteristic structure which consists of cytoplasmic amino and carboxyl termini, a cytoplasmic loop, four transmembrane domains and two extracellular loops. The antibodies that selectively recognise specific connexins are based on the differences in the carboxyl termini of various connexins (Bannerman *et al.*, 2000). This enabled not only the use of immunohistochemistry in visualising the gap junctions but, in theory, also the separation of different gap junction types. Unfortunately, due to the lack of time and funds available, the immunohistochemistry was performed only for Cx36, the most common neuronal connexion, to identify that the dissociated cell cultures used in these experiments expressed the gap junction forming protein.

The immunohistochemistry for Cx36 was performed on glass coverslips rather than MEAs due to the high cost of the MEAs. Both frontal cortex (Fig. 5.2.) and spinal cord (Fig. 5.3.) cultures were found to express Cx36 protein.

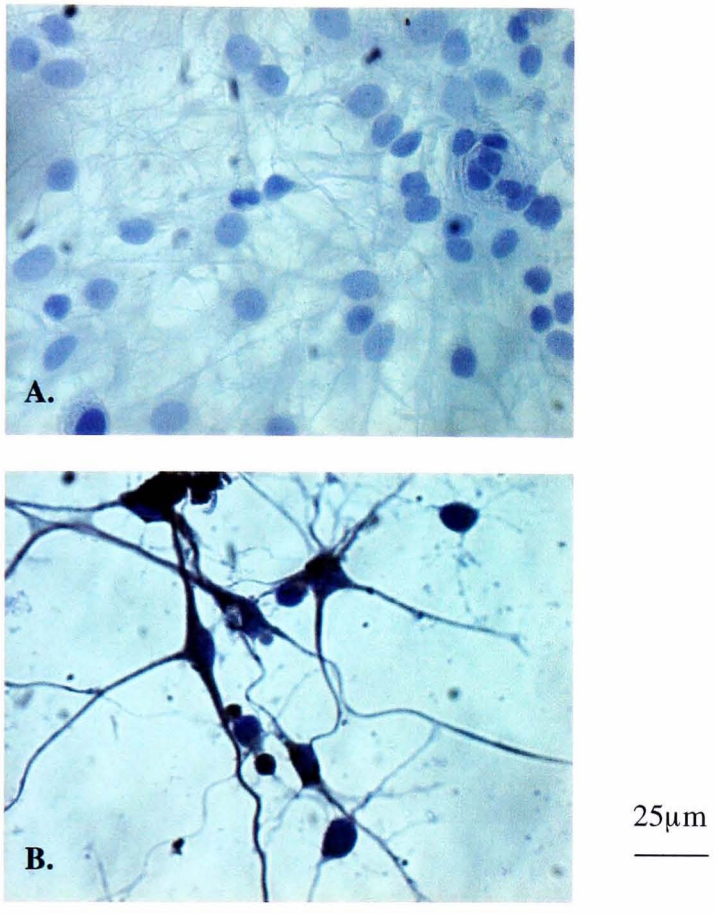

Figure 5.2. Photomicrograph of Cx36 stained rat frontal cortex culture, 22DIV (x40), n = 3. **(A)** Negative control, showing no evidence of Cx36. **(B)** The Cx36 protein (brown stain) is visible around the cell bodies and protein transporting dendrites.

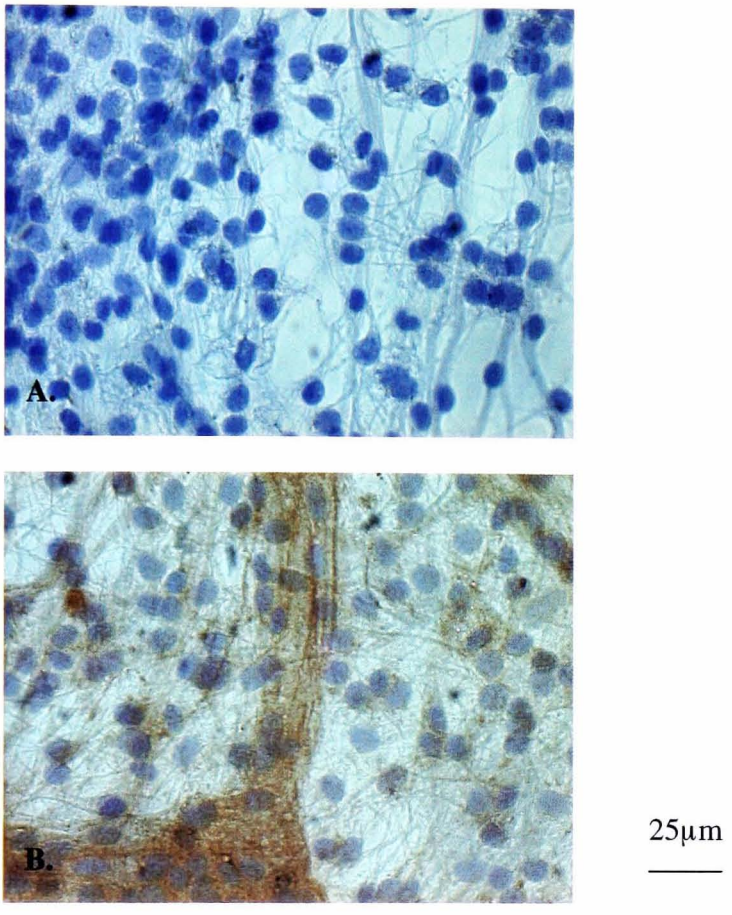

Figure 5.3. Photomicrograph of Cx36 stained rat spinal cord culture, 32DIV (x40), n = 3. **(A)** Negative control, showing no evidence of Cx36. **(B)** The Cx36 protein (brown stain) is visible around the cell bodies and protein transporting dendrites.

5.3.2. Electrophysiology

The effects of four gap junction blockers (carbenoxolone (CBX), halothane, 1-octanol and oleamide) were compared on the spontaneous activity of mouse frontal cortex and spinal cord cultures grown on MEAs. In general the

spontaneous neuronal firing in frontal cortex cultures was inhibited in a dose-dependent manner, which included total cessation of activity by halothane, CBX, 1-octanol, or oleamide at concentrations 250μM, 100μM, 20μM and 20μM respectively. All cultures showed spontaneous recovery at lower concentrations and, at higher concentrations, reversibility after culture medium changes. Measurements of network burst rates and coefficients of variation of burst period indicated that burst co-ordination was reduced by these compounds. These responses were generally mirrored in the spinal cord, except for CBX, which produced a paradoxical transient intense increase in network spike and burst production. The glycine receptor antagonist strychnine (1μM) prevented this transient activity increase in spinal cord, implying that CBX may temporarily block glycine inhibition.

5.3.3. Responses of Mouse Frontal Cortex Cultures to Gap Junction Blockers

5.3.3.1. *Carbenoxolone*

The typical effect, decrease of activity and synchrony levels, of increasing concentrations (50-125μM) of CBX on spontaneous spike and burst production in mouse frontal cortex cultures is shown in Fig. 5.4.

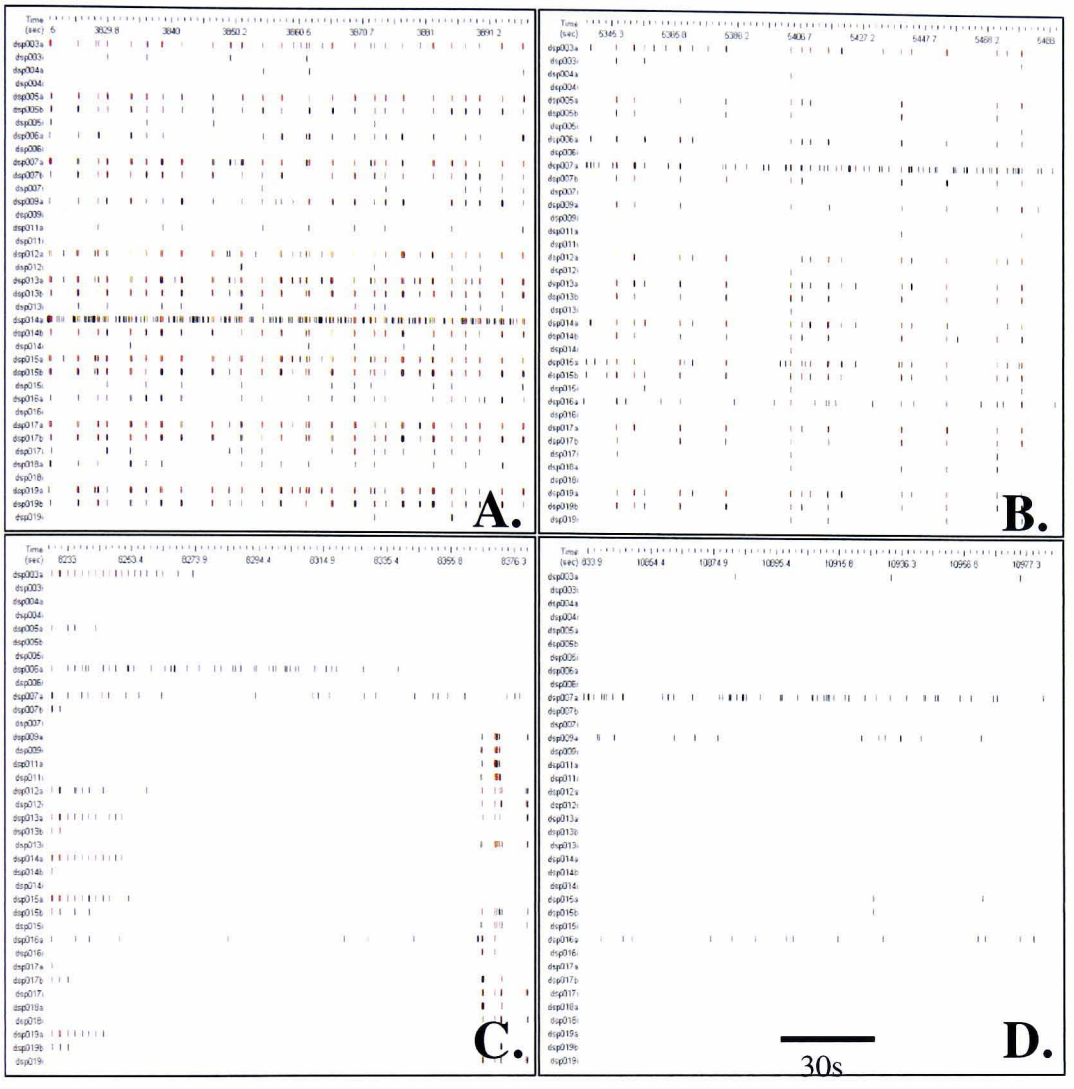

Figure 5.4. Multichannel raster plots, where single vertical lines represent individual action potentials and thicker lines more than one action potential, of 2.5min duration showing general changes in spike and burst activity with increasing concentrations of CBX on a representative frontal cortex culture (n=37 cells). (A) reference activity, (B) under 50μM CBX, (C) under 100μM CBX, and (D) under 125μM CBX. Note the simultaneous spiking and bursting, typical for frontal cortex cultures, throughout the network and the decrease of the synchrony and activity levels with increasing concentrations of CBX.

The change of averaged network activity per minute for spike and burst production under the influence of CBX is shown in Fig. 5.5. After a 5 to 8 min delay, the drug applications were followed by rapid decreases in spike and burst rates. The maximum decreases were concentration dependent and were followed by a spontaneous recovery to near reference levels. The data represented in Fig. 5.5., as well as the data from other subsequent experiments, suggests that the level of spontaneous recovery may also be concentration dependent. This is indeed the case as only a partial recovery (to 50% of reference) was observed at 200µM and no recovery at 250µM (Table 5.2.). EC_{50} value for CBX on mouse frontal cortex was calculated to be between 25 and 50µM (Fig. 5.6.)

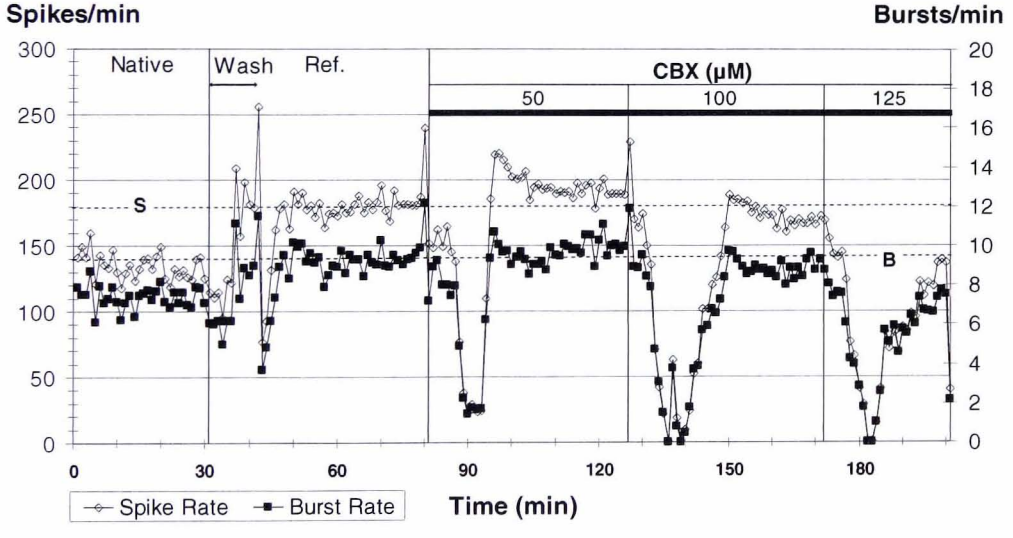

Figure 5.5. Temporal evolution of mean network activity of mouse frontal cortex culture (28DIV) under the influence of different concentrations of CBX. Data points represent average values for spike and burst production per minute obtained from all selected active units (n=35). The first addition of CBX (50

μM) was made at 81 min after 35 min of stable reference activity (Ref). Responses to CBX appear after a delay of ~6 min and take the form of transient decreases followed by relatively stable plateaus around reference levels. Dashed horizontal lines represent the reference levels for spiking (S) and bursting (B). Native: spontaneous activity prior to change to the recording medium, wash: full medium changes.

Concentration	% Decrease (spikes/min)	Delay (min)	Length of the effect at 50% max (min)
25μM	12 ±6	0	0
50μM	29 ±31	1.6	3
100μM	40 ±41	2.8	14
150μM	34 ±19	1.6	5
200μM	51 ±11	10	6/No recovery
250μM	90 ±6	8	No recovery

Table 5.2. Summary of the effect of CBX on mouse frontal cortex cultures (n[cells]=255, n[cultures]=5). The relatively large standard deviations are due to great differences in the reference activity of the cultures (122-575 spikes/min).

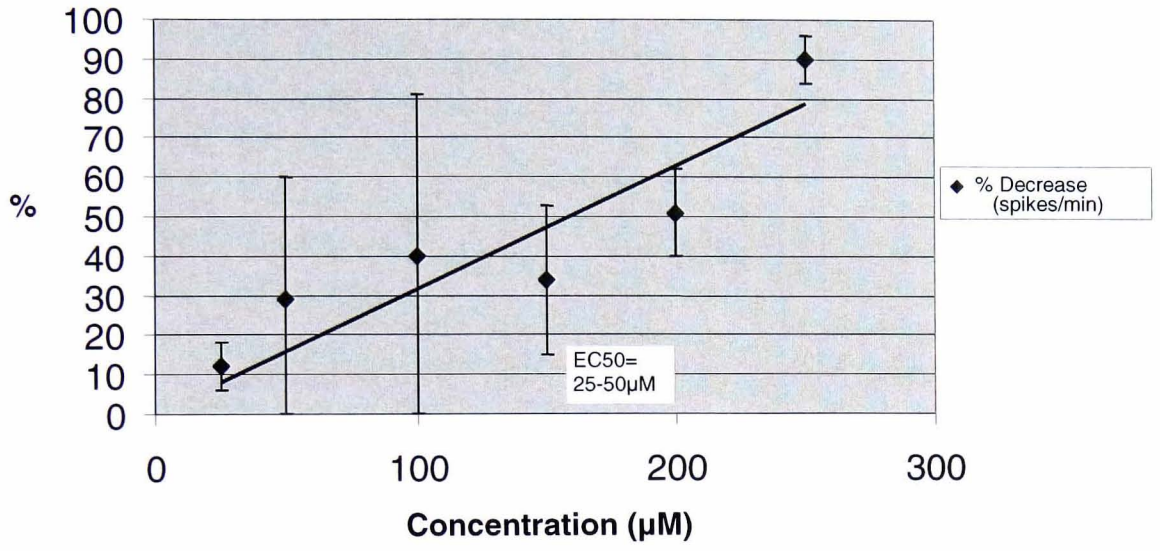

Figure 5.6. Concentration response curve for CBX on mouse frontal cortex. The maximum effect is estimated to be 45% and the EC_{50} value is between 25 and 50µM.

5.3.3.2. *1-Octanol*

The typical effect of 1-Octanol on spike and burst rates of mouse frontal cortex cultures is shown in Fig. 5.7. The effects of 1-Octanol are summarised in Table 5.3. The EC_{50} value for 1-Octanol on mouse frontal cortex was estimated to be less than the lowest concentration tested i.e. <1µM (Fig. 5.8.).

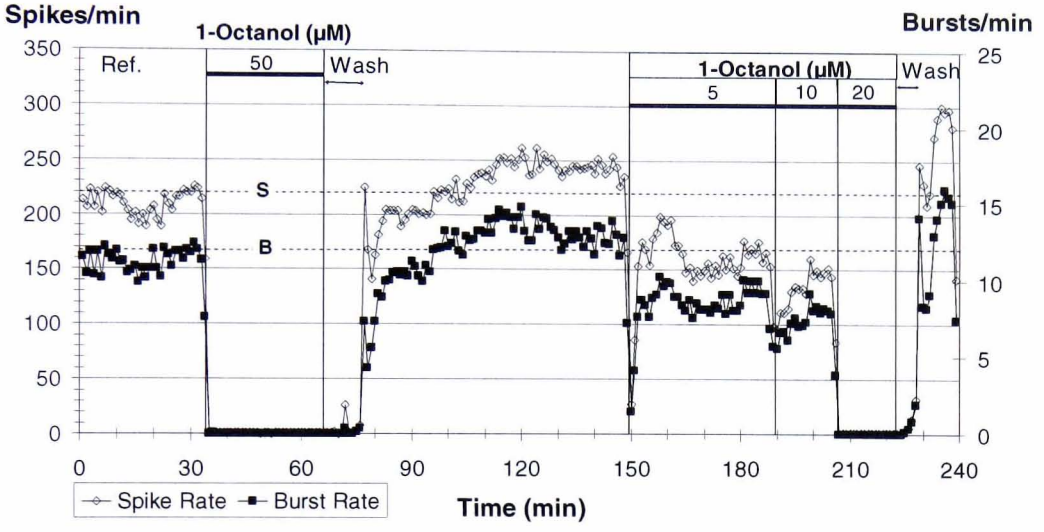

Figure 5.7. Temporal evolution of mean network activity of mouse frontal cortex dissociated cell culture (28DIV) under the influence of different concentrations of 1-Octanol. Data points represent average values for spike and burst production per minute obtained from all selected active units (n=35). Responses to 1-Octanol are almost immediate and take the form of decreases followed by relatively stable plateaus around reference levels after full medium changes. Dashed horizontal lines represent the reference levels for spiking (S) and bursting (B). Wash: full medium changes. Spontaneous recovery without medium changes was not observed.

Concentration	% Decrease (spikes/min)	Delay (min)	Length of the effect at 50% max (min)
1μM	57 ±44	0	35
5μM	95 ±6	0	3/No recovery
10μM	61 ±0	0	8
20μM	100 ±0	0	No recovery

Table 5.3. Summary of the effect of 1-Octanol on mouse frontal cortex cultures (n[cells]=156, n[cultures]=3).

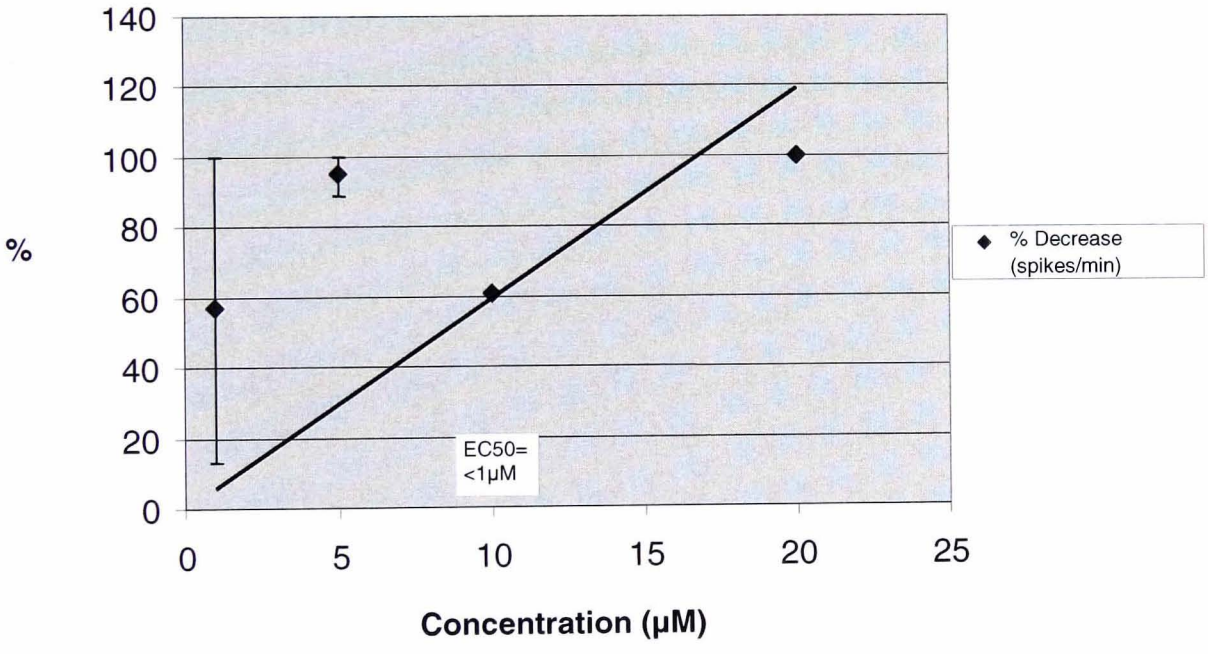

Figure 5.8. Concentration response curve for 1-Octanol on mouse frontal cortex. The maximum effect is estimated to be 80% and the EC_{50} value is less than the lowest tested concentration, 1μM.

5.3.4. Responses of Mouse Spinal Cultures to Gap Junction Blockers

5.3.4.1. *Carbenoxolone*

The typical effect, increase of activity levels, of increasing concentrations (50-150μM) of CBX on spike and burst rates in mouse spinal cord cultures is shown in Fig. 5.9. Note the totally opposite effect to that on frontal cortex cultures.

Figure 5.9. Multichannel raster plots of 2 min duration showing general changes in spike and burst activity with increasing concentrations of CBX on a representative spinal cord culture (n=37cells). (A) reference activity, (B) under 50µM CBX, (C) under 100µM CBX, and (D) under 150µM CBX. Note the lower level of synchrony compared to the frontal cortex cultures.

The change of averaged network activity per minute for spike and burst production under the influence of CBX is shown in Fig. 5.10. After a ~8 min delay, the drug applications were followed by rapid increases in spike and burst rates. The maximum increases were concentration dependent and were followed

by spontaneous recovery to near reference levels. The effect of CBX on spinal cord cultures (Fig 5.10.) is the opposite of that observed on frontal cortex (Fig. 5.5.). The effects of CBX on mouse spinal cord cultures are summarised in Table 5.4. The EC_{50} value for CBX on mouse spinal cord was estimated to be between 25 and 50μM (Fig. 5.11.).

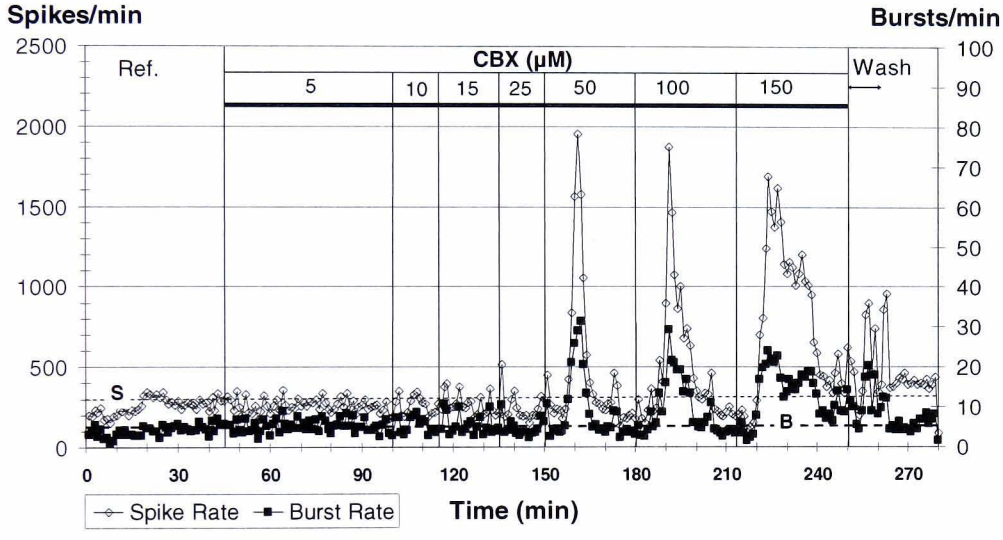

Figure 5.10. Temporal evolution of mean network activity of a representative mouse spinal cord dissociated cell culture (35DIV) under the influence of different concentrations of CBX. Data points represent average values for spike and burst production per minute obtained from all selected active units (n=59). The first addition of CBX (5 μM) was made at 45 min after 26 min of stable reference activity (Ref). Responses to CBX (at concentrations 50 μM and above) appear after a delay of ~10 min and take the form of transient increases followed by relatively stable plateaus around reference levels. Dashed horizontal lines represent the reference levels for spiking (S) and bursting (B). Wash: full medium changes.

Concentration	%Increase (spikes/min)	Delay (min)	Length of the effect at 50% max (min)
25μM	23 ±7	0	0
50μM	194 ±186	8.3	4
100μM	253 ±162	7.7	8
150μM	267 ±143	4.8	8
200μM	182 ±17	10	8

Table 5.4. Summary of the effect of CBX on mouse spinal cord cultures (n[cells]=338, n[cultures]=5). The large standard deviations are due to great differences in the reference activity of the cultures (192-420 spikes/min).

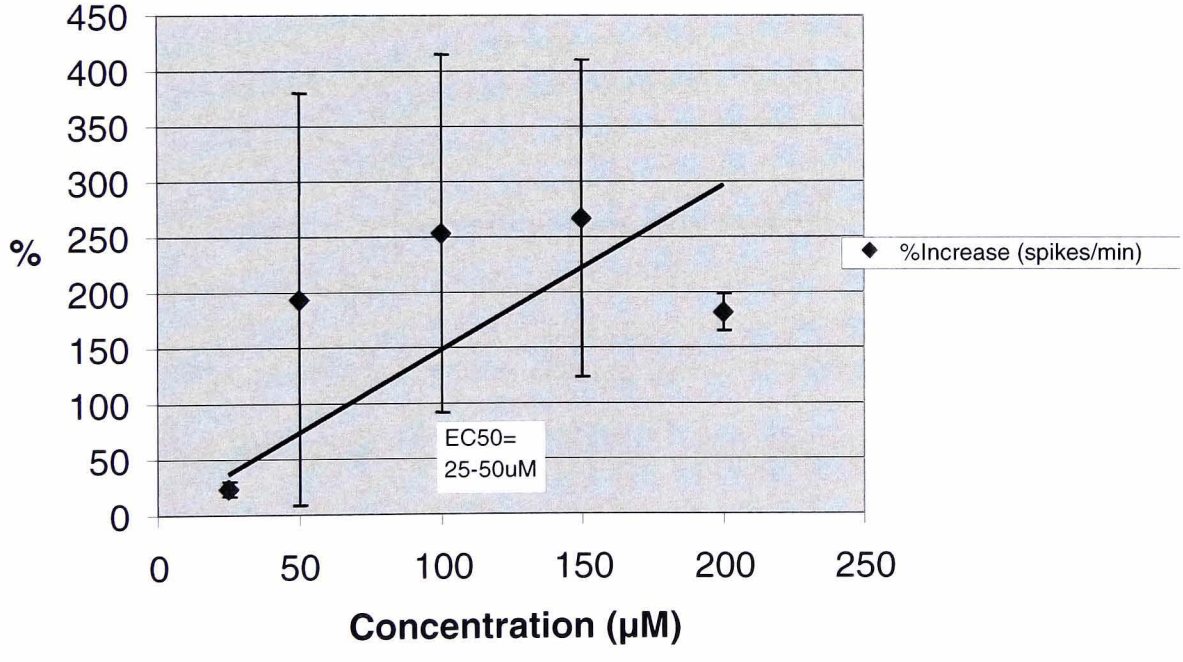

Figure 5.11. Concentration response curve for CBX on mouse spinal cord. The maximum effect is estimated to be 250% and the EC_{50} value is between 25-50 μM.

5.3.4.2. *1-Octanol*

The typical effect, a rapid decrease in activity, of 1-Octanol on spike and burst rates of mouse spinal cord cultures is shown in Fig. 5.9 and Table 5.5. The EC_{50} value for 1-Octanol on mouse spinal was estimated to be less than 1µM (Fig. 5.13.)

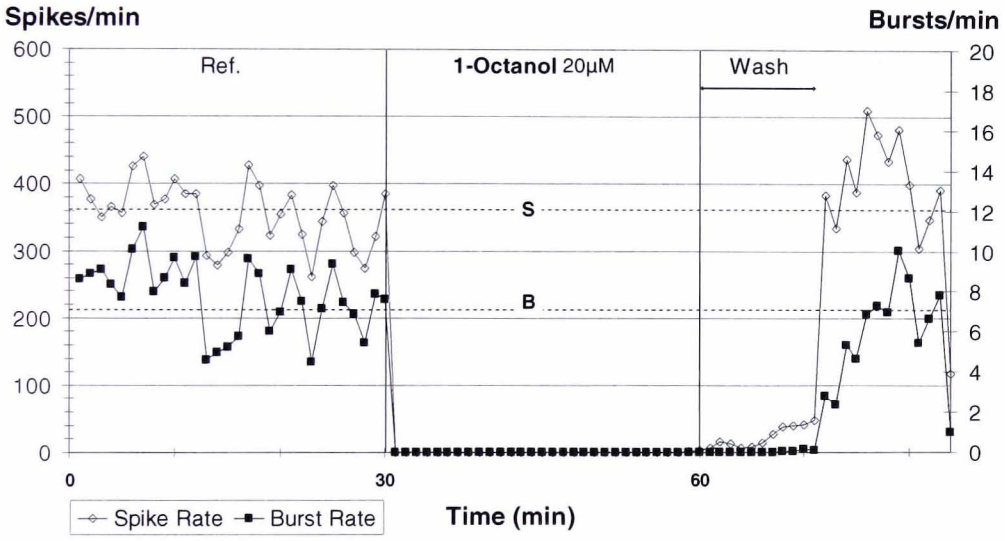

Figure 5.12. Temporal evolution of mean network activity of a representative mouse spinal cord dissociated cell culture (35DIV) under the influence of different concentrations of 1-Octanol. Data points represent average values for spike and burst production per minute obtained from all selected active units (n=59). The first addition of 1-Octanol (50 µM) was made after 30 min of reference activity (Ref). Responses to 1-Octanol are almost immediate and take the form of decreases followed by relatively stable plateaus around reference

levels after full medium changes. Dashed horizontal lines represent the reference levels for spiking (S) and bursting (B). Wash: full medium changes.

Concentration	% Decrease (spikes/min)	Delay (min)	Length of the effect at 50% max (min)
1μM	64 ±0	1	4
5μM	84 ±4	0.6	6
10μM	87 ±16	0.6	8
20μM	99 ±1	1	No recovery
25μM	100 ±0	0	No recovery

Table 5.5. Summary of the effect of 1-Octanol on mouse spinal cord cultures (n[cells]=146, n[cultures]=3).

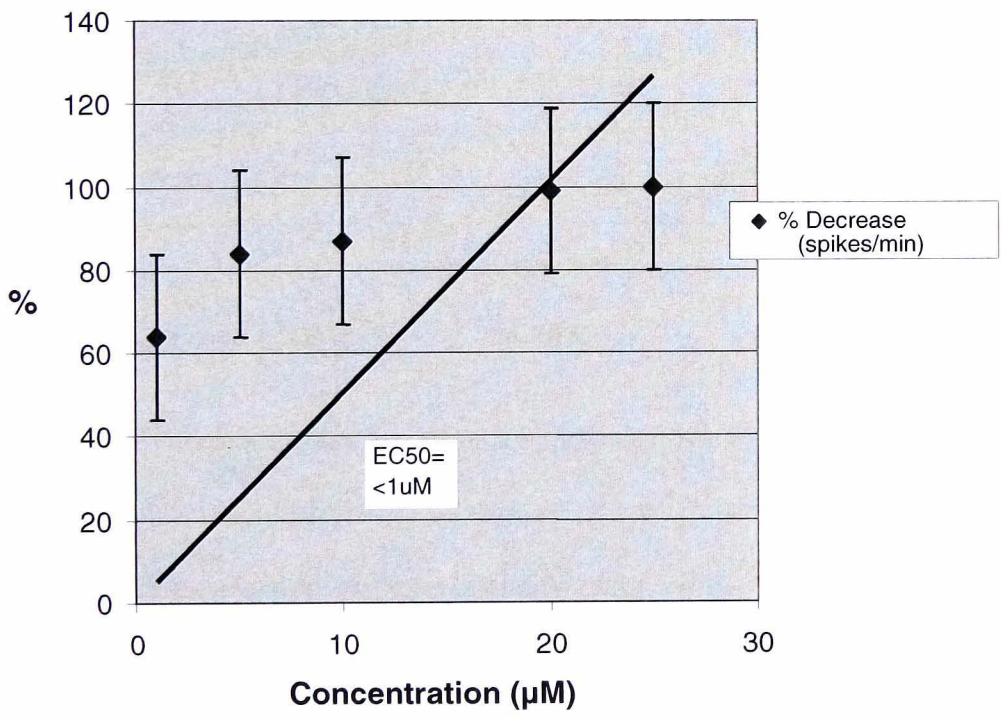

Figure 5.13. Concentration response curve for 1-Octanol on mouse spinal cord. The maximum effect is estimated to be 100% and a the EC50 below the lowest tested concentration, 1μM.

5.3.5. Other Gap Junction Blockers

In addition to CBX and 1-Octanol, the gap junction blockers halothane and oleamide were used. Generally the response of both frontal cortex and spinal cord cultures to halothane and oleamide was similar to the response to 1-Octanol, a rapid decrease of activity. The activity decreasing effect of halothane (500µM) on spike and burst rates of mouse frontal cortex culture, 28 days *in vitro* is shown in Fig. 5.14.

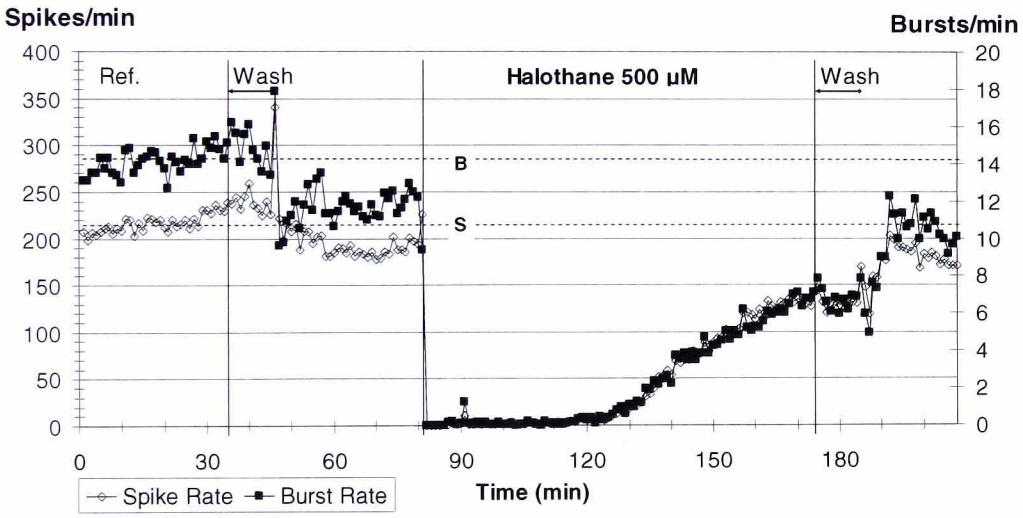

Figure 5.14. Temporal evolution of mean network activity of a representative mouse frontal cortex dissociated cell culture (28DIV) under the influence of 500µM of halothane. Data points represent average values for spike and burst production per minute obtained from all selected active units (n=35). A medium change was performed after 35 min of reference activity (Ref) and the addition of halothane was performed 25 min later. Responses to halothane are almost immediate and take the form of decreases followed by relatively stable

plateaus around reference levels after full medium changes. Dashed horizontal lines represent the reference levels for spiking (S) and bursting (B). Wash: full medium changes. Total number of experiments = 4.

The activity decreasing effect of halothane (250µM) on spike and burst rates of mouse spinal cord culture, 40 days *in vitro* is shown in Fig. 5.15.

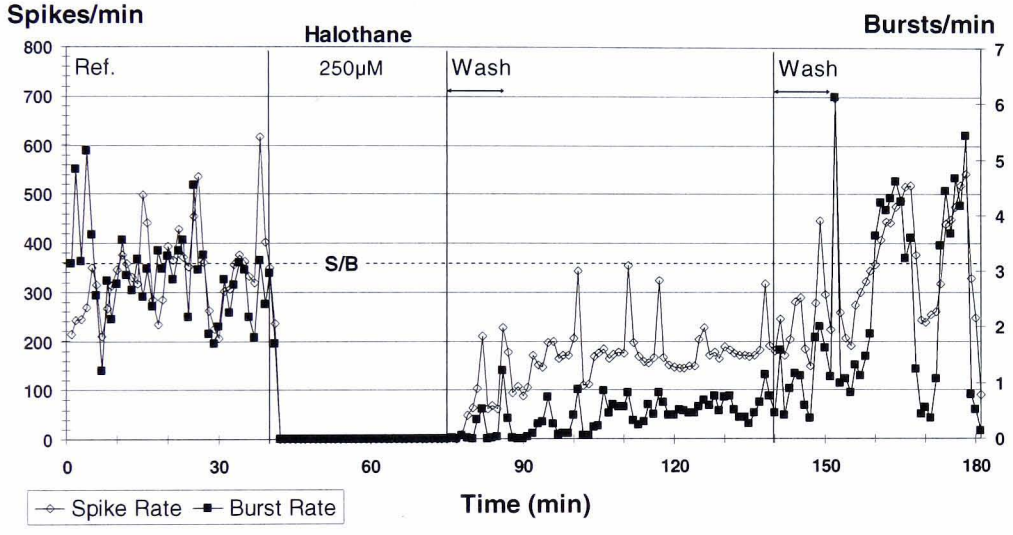

Figure 5.15. Temporal evolution of mean network activity of a representative mouse spinal cord dissociated cell culture (40DIV) under the influence of 250µM of halothane. Data points represent average values for spike and burst production per minute obtained from all selected active units (n=61). The addition of halothane was performed of reference activity (Ref). Responses to halothane are almost immediate and take the form of decreases followed by relatively stable plateaus around reference levels after full medium changes. Dashed horizontal lines represent the reference levels for spiking (S) and bursting (B). Wash: full medium changes. Total number of experiments = 3.

The effect of oleamide (with and without thapsigargin (Sigma, USA), rapid decrease in activity, on spike and burst rates of mouse frontal cortex culture, 41 days *in vitro* is shown in Fig. 5.16.. Thapsigargin is a compound that blocks the intracellular movement of Ca^{2+} (Thastrup, 1990) and was thought to be partially responsible on the rapid decrease in activity in the mouse spinal cord cultures after the application of gap junction blockers. Thapsigargin was used in concentrations from 1μM to 5μM and it did not affect the effects of the gap junction blockers used.

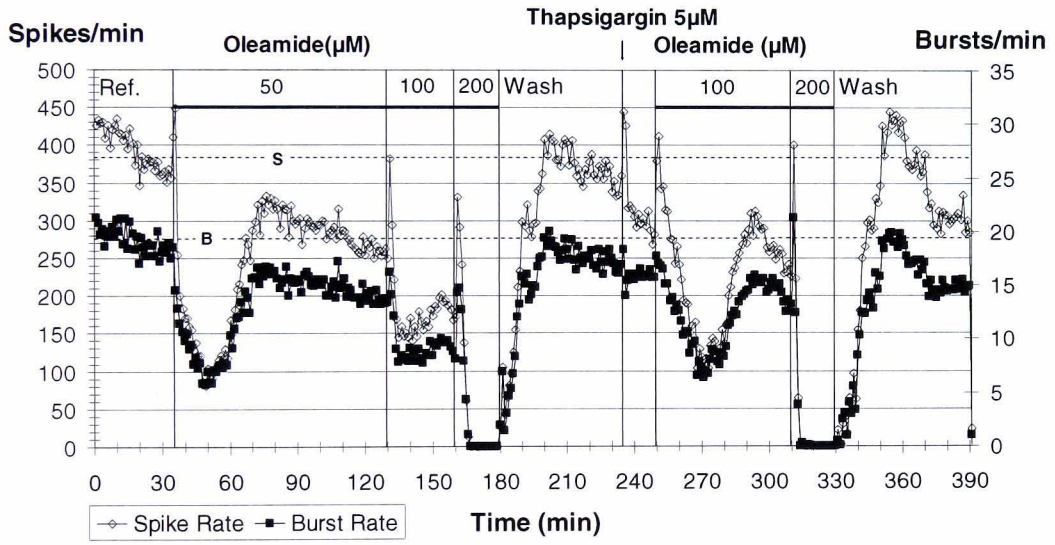

Figure 5.16. Temporal evolution of mean network activity of a reperesentative mouse frontal cortex dissociated cell culture (41DIV) under the influence of oleamide (and thapsigargin). Data points represent average values for spike and burst production per minute obtained from all selected active units (n=72). The first addition of oleamide (50μM) was performed after 35 min of reference activity (Ref). Responses to oleamide are almost immediate and take the form of decreases followed by relatively stable plateaus around reference levels after full medium changes. Dashed horizontal lines represent the reference levels for

spiking (S) and bursting (B). Wash: full medium changes. Total number of experiments with thapsigargin = 2, without thapsigargin = 4.

The activity decreasing effect of oleamide on spike and burst rates of mouse spinal cord culture, 29 days *in vitro* is shown in Fig. 5.17.

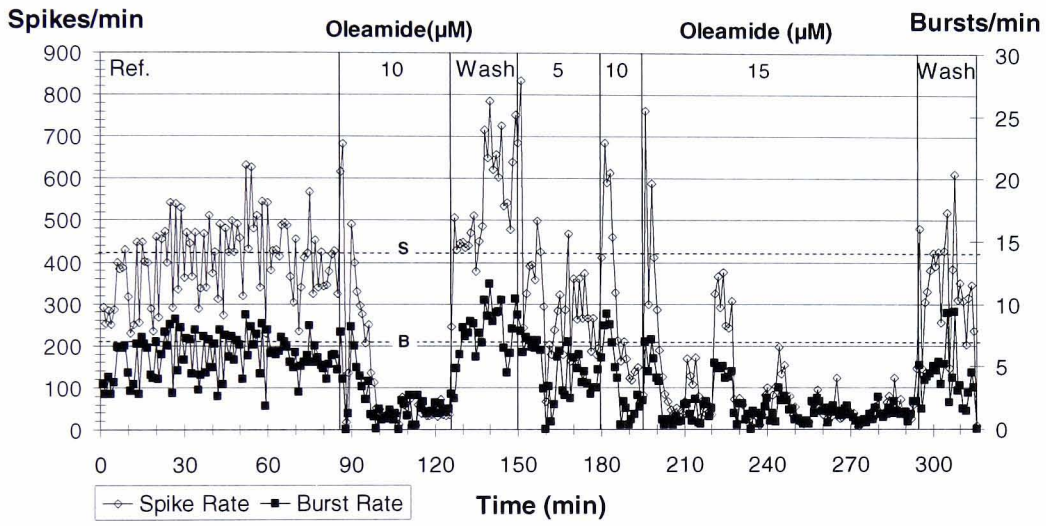

Figure 5.17. Temporal evolution of mean network activity of a represntative mouse spinal cord dissociated cell culture (29DIV) under the influence of oleamide. Data points represent average values for spike and burst production per minute obtained from all selected active units (n=39). The first addition of oleamide (10µM) was performed after 85 min of reference activity (Ref). Responses to oleamide are almost immediate and take the form of decreases followed by relatively stable plateaus around reference levels after full medium changes. Dashed horizontal lines represent the reference levels for spiking (S) and bursting (B). Wash: full medium changes. Total number of experiments = 5.

5.3.6. Responses of Rat Frontal Cortex and Spinal Cord Cultures to Gap Junction Blockers

Due to the lack of time on MAP data acquisition system available, this part of the study was carried out using Recorder, a less sophisticated data acquisition system originally designed for *in vivo* electrophysiological recordings. We established that Recorder is usable with MEAs but the quality of data acquired may not be as high as when using MAP as a result of number of factors:

(1) Recorder allowed only one channel to be displayed at any one time and therefore made it impossible to qualify on-line the effect of the drugs and the stability of the network during the experimental procedure.

(2) Recorder only enabled recording from one single unit per electrode restricting the amount of collectable data to a maximum of 64 cells per experiment. It is, however, possible to separate units off-line.

The effect of two gap junction blockers, CBX and 1-octanol, were studied on dissociated rat frontal cortex and spinal cord cultures grown on MEAs. Both of these gap junction blockers were found to rapidly decrease the spontaneous activity in both culture types (Tables 5.6., 5.7., 5.8. and 5.9.) suggesting that the activity increasing effect of CBX on mouse spinal cord might be, not only tissue specific, but also species specific. The concentrations used and the effects of CBX (Tables 5.6. and 5.7.) and 1-octanol (Tables 5.8. and 5.9.) on rat frontal cortex and spinal cord cultures are summarised below. The degree of the decrease in the spontaneous activity on both, frontal cortex and spinal cord, was

found to be concentration dependent. The EC_{50} value for CBX on rat frontal cortex was estimated to be 25μM (Fig. 5.18.). The concentration response (increase/decrease) of rat spinal cord to CBX is shown in Fig. 5.19. The EC_{50} value for 1-Octanol on rat frontal cortex and spinal cortex was not calculated due to the small number of experiments. The estimated EC_{50} values suggest that the agents used have similar potencies in the both mouse and rat tissues.

Concentration	% Decrease (spikes/min)	Delay (min)	Length of the effect at 50% max (min)
25μM	54±25	12	8.5
50μM	90±9	15	No recovery

Table 5.6. Summary of the effect of CBX on rat frontal cortex cultures (n[cells]=31, n[cultures]=2).

Concentration	% Increase/ Decrease(spikes/min)	Delay (min)	Length of the effect at 50% max (min)
25μM	191 (increase)	8	2
50μM	1244 (increase)	5	1
75μM	222 (increase)	5	13
100μM	164 (increase)	13	5
200μM	67	Steady decrease	No recovery
300μM	49	Steady decrease	No recovery
400μM	54	Steady decrease	No recovery

Table 5.7. Summary of the effect of CBX on rat spinal cord cultures (n[cells]=72, n[cultures]=6).

Concentration	% Decrease (spikes/min)	Delay (min)	Length of the effect at 50% max (min)
100nM	96	0	27
200nM	97	0	35

Table 5.8. Summary of the effect of 1-Octanol on rat frontal cortex cultures (n[cells]=14, n[cultures]=2).

Concentration	% Decrease (spikes/min)	Delay (min)	Length of the effect at 50% max (min)
100nM	98 ±2	5	16.5
200nM	100	0	14
300nM	100	0	32

Table 5.9. Summary of the effect of 1-Octanol on rat spinal cord cultures (n[cells]=110, n[cultures]=5).

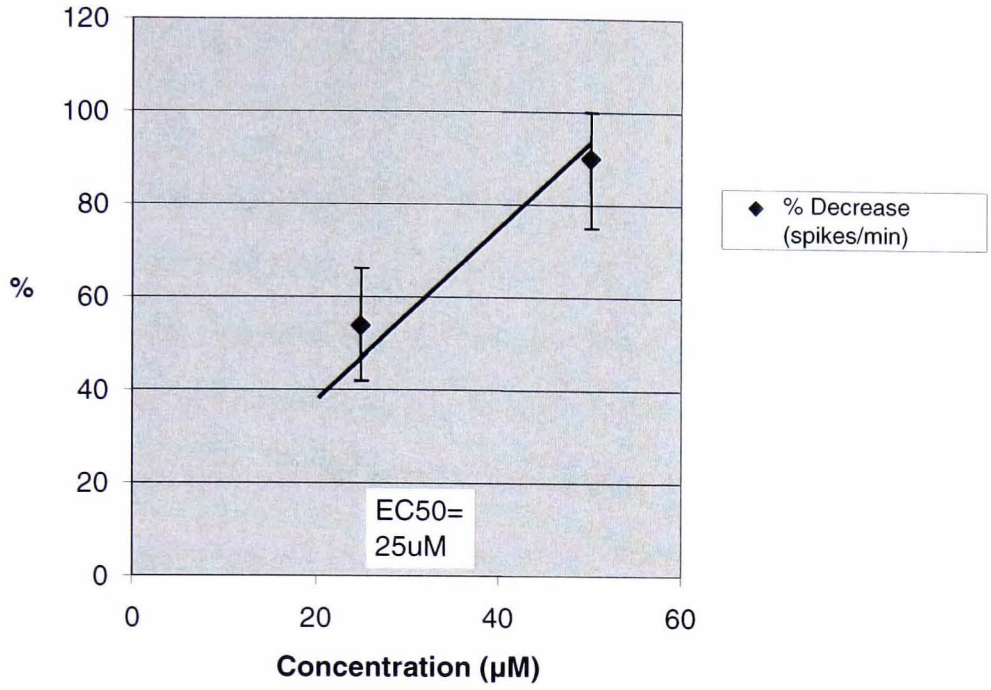

Figure 5.18. Concentration response curve for CBX on rat frontal cortex. The EC_{50} value is 25µM.

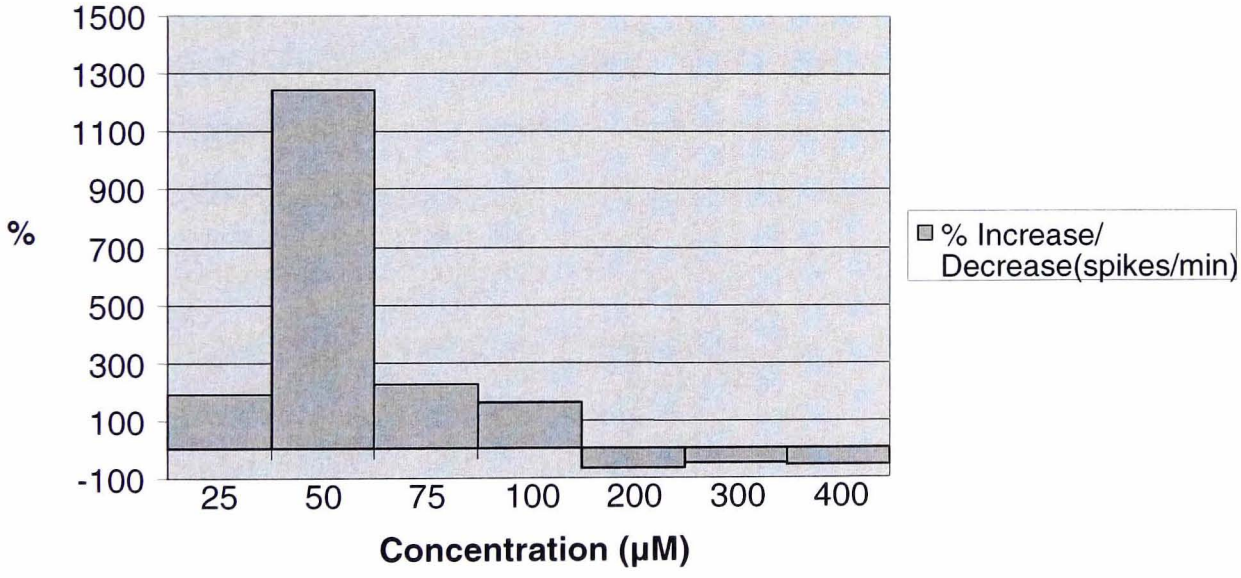

Figure 5.19. The concentration response (increase/decrease) of rat spinal cord to CBX.

5.4. DISCUSSION

Synapse is a specialised structure mediating a functional interaction between two neurones or between a neurone and another cell type. It is formed by the pre-synaptic terminal and the post-synaptic target site separated by a cleft (Bennett, 1999). Two types of synaptic transmission co-exist: chemical and electrical. In case of the chemical transmission, a chemical substance, liberated from the pre-synaptic neurone upon arrival of an action potential, interacts with the post-synaptic cell and propagates the stimulus. In electrical transmission, the action potential in the pre-synaptic neurone induces a passive current flow into the post-synaptic cell.

Chemical and electrical synapses differ not only in their molecular mechanisms of information transfer, but also in their morphological organisation. When at chemical synapses, there is no continuity between the cytoplasm of the two connected cells and the synaptic cleft varies between 20-40nm, the electrical synapses are characterised by an area of very close apposition, 2-4nm between the pre- and post-synaptic membranes. Within this area, the two connected cells communicate through gap junctions, cell-to-cell pores serving as conduits between the cytoplasm of the two cells (see Hormuzdi *et al.*, 2004).

Gap junctions are involved in the transmission of metabolic signals between cells by permitting the passage of second messengers, such as the inositol trisphosphate (IP3; Saez *et al.*, 1989). Electrical synapses also function as low

pass filters. They preferentially, but not exclusively, transmit low-frequency stimuli that allow the rapid transfer of a pre-synaptic impulse into an electrical excitatory post-synaptic potential in the post-junctional cell (Bennett, 2000). If the transmitted current is sufficient to depolarise the membrane above a certain threshold, activation of voltage-gated ion channels will lead to generation of action potentials. And, since the ionic current flow is free between the two cells, the electrical transmission via intercellular channels can be two directional. This two way transfer of the stimulus is supported by the electrical, but not chemical, neurotransmission. It is thought to be, together with the transfer of sub-threshold potentials favouring synchronous activity, one of the advantages of electrical synapses. It needs to be remembered, however, that the cell-to-cell communication via the gap junctions does not mean mutual excitation. If a cell is coupled to another cell that is less depolarised, it can excite the less depolarised cell but the less depolarised cell tends to inhibit its' more depolarised partner (Hormuzdi *et al.*, 2004).

The ability of gap junctions to promote electrical coupling and two directional current flow makes them extremely suitable candidates to synchronise the discharges of interconnected cells. Several studies of gap junctions between neurones throughout the brain suggest that gap junctions indeed are at least partly responsible for the synchrony in neuronal networks (e.g. Traub & Bibbig, 2000). It has also been shown that synchrony and oscillations of specific neuronal populations are altered in Cx36 knockout animals (e.g. Long *et al*, 2002). The current literature, supported by the results of this experimental study, suggests that the transmission of electrical signals directly through gap

junctions decrease the heterogeneity of discharges enhancing synchrony in neuronal networks.

The initial main hypothesis at the beginning of this study was that gap junctions might be involved in synchrony and bursting of neural networks. In the frontal cortex, the inhibition of the neural discharge activity following the application of gap junction blockers, coupled with reduced probability of simultaneous firing between neurones, strongly implied the participation of gap junctions in synchrony and bursting of these networks. Other preliminary data involving culture density led to the view that cell clusters have a higher probability of forming gap junctions which may in turn lead to enhanced burst synchronisation.

According to Tamas *et al.* (2000) the rhythmic electrical activity in central neural networks arises from interneuronal electrical coupling via the gap junctions together with the chemical synaptic inhibitory transmission. The results of this study strongly agree with gap junctions playing an important role in the synchronisation of the neural networks. But it is important to realise that even though gap junctional coupling clearly can result as synchronised in-phase firing, the strength of the coupling and the characteristics of the neuronal model matter have an effect on the network output (Carlen *et al.*, 2000).

According to Margineanu & Klitgaard (2001), the decrease in the number of bursts caused by gap junction blockers occurs via a gradual decrease in the burst amplitude which, in turn, suggests a decrease in the number of synchronised

neurones. Figure 5.5. shows a simultaneous decrease in both burst number and burst amplitude in mouse frontal cortex culture after addition of a gap junction blocker, CBX. This could suggest that the synchrony of the neurones in the culture also decreased by blocking the gap junctions in the culture. This agrees with results from other brain areas (e.g. Yang & Michelson, 2001). Similar results have been obtained also using 1-octanol (e.g. Yang & Michelson, 2001). Interestingly, the response to CBX in the spinal cord cultures (Fig. 5.8.) is a mirror image of that in frontal cortex cultures, showing simultaneous increase in burst number and burst amplitude. The reason for this behaviour is still unclear.

The definitions of spikes and bursts in this study followed those of Streit *et al.* (2001). A local spike was defined as one/ a few spikes seen at one or more microelectrodes, whereas a burst consisted of an episode of activity lasting from 50ms up to several seconds and occurring simultaneously in many channels over the network. However, the definitions of burst vary widely across the literature.

One of the key features of developing neuronal networks is the generation of spontaneous activity typically organised into synchronous bursts or episodes (O'Donovan, 1999). Rhythmical synchronised activities in the CNS are important for the integration of distributed neuronal information (Gray, 1994). The capability of the networks to generate these spontaneous recurrent bursts progressively declines during the maturation of the neuronal circuits. Immature circuits of spinal cord, as well as other parts of the CNS, spontaneously generate synchronous patterns of rhythmic activity for a short time during their development. In the case of spinal cord it is still unknown, how strongly these

synchronised bursts generated by interneurones are associated with motoneurones and whether the decrease in spontaneous bursting during the circuit maturation proceeds parallel in motoneurone and intreneurone networks (Rosato-Siri *et al.*, 2004). There is, however, some evidence that the motor network development regulates the bursting inputs from interneurones to motoneurones *in vitro* and that the stable spontaneous bursting of the spinal cord networks switches into random patterns after the first week in culture (Rosato-Siri *et al.*, 2004). This theory is supported by the random spiking and bursting patterns seen in the spinal cultures of this experimental study thought to be caused by the progressive emergence of multiple functional subnetworks from originally synchronous network (Yvert *et al.*, 2004).

The considerable degree of electrical coupling via gap junctions has been one of the major problems in electrophysiological studies of confluent monolayer culture in recent years. Electrical coupling of cells leads to significant errors in voltage-clamp and whole cell patch-clamp experiments because most of the current injected into a single cell to achieve changes in its membrane voltage flows into the neighbouring cell. This causes the actual clamp current to appear enlarged (Böhmer *et al.*, 2001). The method used in this study overcomes this problem and provides accurate results of single cell activity and allows electrophysiological recording from these cultures providing useful information about the gap junctions themselves.

The uncoupling by gap junction blockers reduces junctional conductance by decreasing the channel mean open time and increasing the channel mean closed

time. All the gap junction channels formed by different connexins have distinct unitary conductances as well as gating properties and permeability characteristics. When using gap junction blockers which effect some connexin formations more than others (e.g. halothane, homotypic vs. hetrotypic channels), the profile of single-channel events may not be quantitatively representative of the population of the channels contributing to the conducatnce in cells expressing more than one connexin (He & Burt, 2000). The MEAs allow simultaneous multichannel recording of neural activity and are therefore likely to be more reliable than other electrophysiological methods when studying the effects of gap junction blockers on networks.

Interestingly, there is a continual argument, whether or not the gap junctions exist between neurones and glial cells. For example Alvarez-Maubecin *et al.* (2000) demonstrated the presence of gap junctions between neurones and glia in a brainstem noradrenergic nucleus, whereas Rash *et al.* (2001) claimed that the neurones only have gap junctions with other neurones but not with glia. There still seems to be uncertainty about the neuronal gap junctions, this clearly is an area where more research is needed.

However, the presence of glial cells in the cell cultures is critical. Even though too large population of glial cells may result as a glial 'carpet' causing the cell to detach from the MEA surface, the glial networks have been shown to provide functional support to neuronal activity and may constitute dynamic pathways. For example, in forebrain cultures, the gap junction-mediated unidirectional propagation of calcium waves from astrocytes to neurones has been suggested

to account for glial-neuronal signalling (Nedergaard, 1994). In the cultures used in this study, the presence of glial cells was not controlled as such. However, the glial growth enhancing serum was removed from the culture medium after the initial 30 days *in vitro* to avoid the formation of the glial 'carpet'. The glial cells were, however, still present at the time of the electrophysiological recordings and are clearly visible in the cell cultures grown on MEAs (see figure 2.3.).

Unfortunately, due to the lack of selective gap junction blockers, it is impossible to distinguish, whether the gap junction blockers target the neurones or astrocytes. The currently used gap junction blockers also affect other ion channels and receptors as well as gap junctions themselves. Rouach *et al.* (2003) showed that CBX inhibits spontaneous and bicuculline-induced neuronal network activity and neuronal excitability. They also concluded that the CBX induced changes in neuronal network activity are not caused by blockade of astrocytic gap junctions and are also unlikely to be related to neuronal gap junctions. There is a strong possibility the CBX indeed works through a direct mechanism other that the gap junctions.

When using volatile alcohol (octanol) as a gap junction blocker, we found relatively small doses (μM range) to be effective in our spinal cord and frontal cortex cultures. Bannerman *et al.* (2000) claimed the smallest effective dose to be at mM range and therefore it can be suggested that the mature *in vitro* cell cultures on MEAs to be much more sensitive to volatile alcohol gap junction blockers than the freshly removed embryos used by Bannerman *et al.* (2000). Generally, a wide range of concentrations of the gap junction blockers was

found to be effective. This might be due to the type of tissue, type of culture and also to the electrophysiological methods used by different research groups.

When using the gap junction blockers on *in vitro* cell cultures, control cultures should be treated with compounds that are structurally related to the gap junction blockers used, but do not close gap junctions. The control compounds such as hexanol and palmitoylethanolamide, for octanol and oleamide respectively, could be used. This is important because the gap junction blockers often have other actions besides closing the gap junctions. It is also important to remember that the known gap junction blockers are not specific for any one particular connexin type (Carlen *et al.*, 2000), even though some connexins and connexin formations (in heterotypic channels) are more easily uncoupled than others (He & Burt, 2000). It is near impossible to say whether the decrease in activity as a response to an addition of gap junction blockers seen in this experimental study is due to a general depression of activity or a direct result of changes in electrical coupling.

If no inhibition in gap junctions is observed when the gap junction blockers are applied, it may be possible that the drug diffuses out/is not absorbed before reaching the binding site. Therefore, the higher concentrations should cause at least a moderate amount of blockade (DeCoursey, 1995).

The application of halothane leads to an increase in slow transitions between the open and closed stages of the gap junction channel, although also fast transitions have been occasionally observed. This pattern has also been detected in

response to certain other gap junction blockers, such as heptanol and quinine (Srinivas & Spray, 2003). These slow transitions have been attributed to a distinct form of channel closure that presumably involves conformational rearrangements of the channel (Trexler *et al.*, 1996). If so, the question remains if these rearrangements return to normal by medium changes allowing the recovery of the gap junction functions.

The presence of the gap junctional coupling between the spinal motoneurones could have been expected to cause relatively lower input resistance in the recorded neurones compared to that in non-coupled neurones. However, the present studies have not shown significant difference in the input resistance of the electrically coupled and non-coupled cells. Mentis *et al.* (2002) in fact showed that the input resistance was highest in neonatal animals (P0 – P2), when the electrical coupling is most pronounced. They suggested that in order for the electrical coupling to have an effect on the input resistance, the most sites of the coupling would need to be located close to the cell body rather than be dendro-dendritic at distal sites. In addition, the resistance is not only dependent on the gap junctional conductance between the coupled cell pair, but also the non-junctional conductance caused by the ion channels in the cell membranes as well as the active synaptic inputs on the recorded neurones. This experimental study did not measure input resistance of the cells – use of alternative methods, such as patch-clamp, would be required.

The electrical coupling between the spinal motoneurones is functionally specific and developmentally regulated (e.g. Chang *et al.*, 1999) suggesting that the gap

junctional communication plays an important role in synchronising the electrical activity as well as in the metabolic signals during the early development (Kiehn & Tresch, 2002).

CHAPTER 6

GENERAL DISCUSSION

6.1. DISCUSSION OF METHODS

Immunohistochemistry proved to be one of the initial problems in this experimental study. Several different types of immunohistochemistry were used (see chapters 3.2. and 5.2.) on olfactory tissue, spinal cord and frontal cortex. The first step in the preparation of tissue for light and electron microscopy is fixation. The purpose of fixation is to preserve the tissue so that it will survive in its natural state (or as near to that as possible) during storage, experiments and examination in the light and/or electron microscopes. Fixation stabilises the cellular organisation of the tissue so that:

1. The tissue does not undergo further degradation in response to endogenously released enzymes or the micro-organic action.
2. The steps of the processing of the tissue do not extract tissue components.
3. The tissue is hardened to aid sectioning.

It is also important that the fixative used penetrates the tissue rapidly, acts fast and is irreversible.

For light microscopy the most commonly used fixative is an aldehyde fixative. The aldehydes react primarily with proteins and stabilise the tissue by cross-linkage. Aldehyde fixatives are generally prepared in a buffered solution at physiological or slightly higher pH (Bolam, 1992). The most commonly used fixative for immunohistochemical studies is 4% formalin in a phosphate buffer (Naukkarinen & Von Boguslawsky, 1998). However, the fixative used for all light microscopy in this study is Bouins fixative, which contains 75% of saturated picric acid, 20% of methanol and 5% of glacial acetic acid. Picric acid is a fast fixative and preserves well the micro-organisation of the tissue and is therefore considered to be better for the purposes of this study than more commonly used aldehyde fixatives. Another advantage of the picric acid is its yellow colour, which enables the progress of the fixation being followed.

The fixative used for electron microscopy in this study is 1% paraformaldehyde. The fixative used also contained 3% of glutarldehyde and was prepared in 0.1M cacodylate buffer. The initial fixative is followed by osmium tetroxide as a post-fixative. This is very commonly used protocol in electron microscopy (Bolam, 1992). Alternatively aldehyde fixative could have been used.

All the stainings used in this study for light microscopy are well established and relatively simple. Haematoxylin and eosin staining is used to differentiate nucleus from other cell components and therefore to see the basic morphology of the tissue in question. It stains the nuclei of cells blue and the other components shades of red and pink.

The periodic acid Schiff's staining (PAS) is a histochemical reaction in which the periodic acid oxidises the carbon present to carbon forming aldehydes that react to the fuchsin-sulfurous acid and form the purple colour. Therefore the PAS staining can be used for demonstration of the glycogen and other periodate-reactive carbohydrates present in tissue. In this study salivary gland is used as a control tissue.

Immunohistochemistry is used to indicate the antigen of the tissue with a specific antibody. The antibodies used in this study are commercial and well characterised and tested. The negative controls are used to prevent possible wrongly positive stains. It is important in immunohistochemistry, as in all the work done in this study, to use clean equipment to prevent non-specific reactions.

The aim of the immunohistochemical double stainings is to indicate two different antibodies in one tissue section. The most important considerations when planning an immunohistochemical double staining are:

1. To prevent cross-reactions between the different reagents used, and
2. To be able to distinguish the two different stains form each other (Naukkarinen & Boguslawsky, 1998).

The primary antibodies used in this study are raised in different species (rabbit and mouse) and therefore the secondary antibodies are able to bind themselves only to the antibodies specific to them. The stains are clearly distinguishable when using confocal microscope.

The problems encountered in this study were initially with cross-reaction in the immunohistochemical double staining for neurofilament and glia. These problems were overcome via small alterations of the protocols used. Unfortunately, this proved not to be the only problem in olfactory tissue immunohistochemistry. The discovered autofluorescency of the olfactory tissue meant that the immunofluorescent protocols had to be abandoned and immunoperoxidase protocols had to be used instead.

The techniques used for organotypic slice cultures and dissociated cell cultures in this study are well established but have, however, undergone major changes and developments during the last 25 years due the introduction of plastic labware, more advanced slicing techniques and the development of chemically defined culture medium (Gähwiler *et al.*, 1991). However, the techniques used in this study are reliable when performed properly and the results of these experiments are dependable.

6.2. GENERAL DISCUSSION

A great amount of time was used in studying the olfactory system. The aim of the study, to develop an *in vitro* sensory processing system and to be able to monitor sensory input, such as electrophysiological effects of chemicals/agents, using the system developed, required a spontaneously electrically active tissue/cell preparation. After a thorough experimental evaluation of this system, it was decided that the olfactory system was unsuitable for the purposes of this experimental study as the olfactory cultures rarely exhibited spontaneous electrical activity. Therefore, the rest of this study focused on spinal cord and frontal cortex cultures, especially their responses to compounds known as gap junction blockers.

But why is blocking gap junctions so important? Gap junctional coupling has been shown to interfere with isopotentiality, which is one of the essential requirements for successful voltage-clamp experiments. More importantly, successful blockade of gap junctions is a key to the ongoing research of the role of gap junctions both *in vivo* and *in vitro*. Gap junction blockers are thought to provide a possible cure or treatment for pathological conditions in which gap junction over expression is an underlying cause or, alternatively, a consequence causing further problems (Rozental *et al.*, 2001).

The main problem in ongoing gap junction studies is the lack of a specific gap junction blocker. All the gap junction blockers currently used have side effect

and/or do not provide total gap junctional blockade but only partially impair the conductance of the gap junctions. Total blockade of gap junctions is unreachable because of several physiological reasons, such as the inaccessibility of gap junction proteins to the extarcellular space and the lumen of gap junctions being continuous between connexons contributed by each cell forming the gap junction and thus being inaccessible to extracellular agents (Rozental *et al.*, 2001).

The currently used gap junction blockers were generally discovered as side effects of drugs that also affect other ion channels. Recently the use of antibodies directed toward the extracellular loop domains required for the formation of gap junction channels as gap junction blockers has become more and more popular together with connexion-mimetic peptides (Rozental *et al.*, 2001). These methods provide the possibility to block gap junction channels under long-term *in situ* conditions combining high specificity, potency as well as efficacy and should be considered in future studies.

So why were the gap junction blockers CBX, 1-octanol, halothane and oleamide chosen for this study? All four blockers are relatively well known and their effects are well described in the current literature (see chapter 5.1.). Furthermore, they are easily accessible and easy to use. Only CBX and 1-octanol were used in further studies on rat cell cultures following personal communication with several leading scientists in the area, including Dr R. Llinas, NYU Medical Center, New York, USA. All the gap junction blockers currently in use can be described as 'dirty drugs', implying that they have other

pharmacological effects in addition to their capability to block gap junctions. Dr R. Llinas has a longstanding research experience in gap junction blockade and, in his experience, CBX and 1-octanol have a more specific interaction with gap junctions than the other known gap junction blockers. They are also known to decrease spontaneous neuronal synchrony (Margineanu & Klitgaard, 2001), primarily caused by gap junctions.

All the gap junction blockers used in this experimental study proved to have problematic side effects: CBX has been found to interact with mineralocorticoid receptors and to inhibit the enzyme 11-dehydrogenase (e.g. Sewell *et al.*, 1998) and also to shift the reversal potential of the opioid current in the locus coeruleus neurones to the K^+ equilibrium potential (Rozental *et al.*, 2001). Octanol, like ethanol, has been found to unspecifically perturb the function of ion channels and other proteins and, oleamide potentiates the function of serotoniergic receptors and benzodiazepine-sensitive GABA systems (e.g. Boger *et al.*, 1998; Yost *et al.*, 1998).

Out of the four gap junction blockers used in this study, halothane is the most problematic. In general, it depresses neuronal firing and excitatory synaptic transmission by suppressing Ca^{2+} influx into presynaptic terminals (Krnjevic, 1992) and potentiates synaptic inhibition. It also has been shown to alter inhibitory postsynaptic currents in hippocampus (Jones & Harrison, 1993), to block both TTX-resistant and TTX-sensitive Na^+ channels (e.g. Sirois *et al.*, 1998), to inhibit the function of certain glutamate receptor subtypes (e.g. GluR3) but enhance kainate (GluR6) receptor function (Dildy-Mayfield *et al.*,

1996; Minami *et al.*, 1998), to antagonize glutamatergic neurotransmission at the NMDA receptor (Beirne *et al.*, 1998), to inhibit the muscarinic receptor function through protein kinase C activation (e.g. Minami *et al.*, 1997), to activate K^+ channels (e.g. Patel *et al.*, 1999), to inhibit thromboxane A_2 signalling at the membrane receptor (Honemann *et al.*, 1998) and to modulate $GABA_A$ receptor function (e.g. Jones & Harrison, 1993).

The strength of the functional gap junctional coupling varies between different cell types and can be measured in several ways. Commonly used methods are the measurement of the junctional conductance using patch clamping and the measurement of diffusion of a tracer (e.g. Lucifer Yellow) after intracellular injection (Rozental *et al.*, 2001). None of these methods were used in this experimental study and, therefore, it is impossible to determine the strength and number of the gap junctions present in the cultures prior to the addition of the gap junction blockers, though we can be certain that the gap junctions existed in the cultures used due to the positive immunohistochemical staining against Cx36 (see chapter 5.3.). The decrease seen in the activity levels after the addition of gap junction blockers can be due to the decrease in gap junctional communication as well as the side effects of gap junction blockers used. If indeed the gap junctional coupling was reduced in the cultures, the decrease would have been due to alteration of the effective size or charge selectivity of the channels forming the gap junctions, decreased percentage of time the channels are open, and/or, decrease in the number of the gap junction channels functioning. It also has to be taken into consideration, that the pore diameters vary among homotypic gap junction channels and that the pore diameter has a

direct effect on the ionic permeability depending on the relative strength of the electrostatic surface charge of the pore and the distance to the permeant ion or molecule (Rozental *et al.*, 2001).

The neuronal synchrony is maintained by two separate systems – chemical transmission mechanisms and electrical coupling. Interneurones are connected via gap junctions in several brain regions (e.g. Koos & Tepper, 1999) promoting synchronous activity in differentially connected networks of interneurones. By using gap junction blockers, we were hoping to disturb this natural synchrony of electrical activity found also in the cultures used in this experimental study. The level of synchrony was seen to decrease to some degree prior to the reduction of the level of activity. Unfortunately, due to the rapid loss of activity, it was near impossible to determine the true level of the decrease in synchrony.

The results of this study show the effect of the gap junction blockers to be not only tissue specific, but also in some cases to differ from species to species (see tables 5.4. and 5.7). Generally, the gap junction blockers used in this study caused a decrease in the burst rates when applied on frontal cortex and spinal cord cultures. This was true in both mouse and rat cultures. Interestingly, the mouse, but not rat, spinal cord cultures showed a simultaneous increase in burst number and amplitude as a response to an application of CBX. Further experimentation would be required to know whether these differences seen really are through the blockade of gap junctions, or due to the secondary effects of the blockers used. Some preliminary experiments were performed to determine if, for example, the intense increase in spike and burst production in

mouse spinal cord cultures after addition of CBX, were due to the movement of intracellular calcium rather than the gap junctions. Blocking intracellular calcium mobility with thapsigargin (up to 5μM) did not affect the effects of gap junction blockers used. Further studies also showed that strychnine (1μM) prevented this transient activity increase in spinal cord networks, implying that CBX may temporarily block glycine inhibition. However, the amount of data collected is not sufficient to be able to draw conclusions. The question of the importance of gap junctions for the synchronisation of cell cultures and the further questions of the methods of experimentation arisen during this experimental study need further examination.

The suitability of the system used for studying the electronic coupling of neurones (and astrocytes) also remains questionable. It has been reported (Fróes *et al.*, 1999) that the electronic coupling of neurones is most frequent between 48h and 72h *in vitro* and declines over the next four days accompanied by progressive neuronal maturation. The presence of heterocellular electrical coupling and coupling between neurones and astrocytes has been confirmed in these cultures. The gap junctions provide metabolic and electrotonic interconnections between neuronal and astrocytic network at the early stages of neuronal development. These connections are weakened as the cell differentiation progresses. Therefore, it has to be questioned whether the dissociated cell cultures grown on MEAs are the right method for studying the role of gap junctions. These neuronal cell cultures require a minimum of 21 days *in vitro* to mature, by which time the number of gap junctions has decreased. The relative proportions and expression patterns of connexins also

change during the central nervous system development (e.g. Willecke *et al.*, 1991) and it is possible that cellular compartments distinct in terms of metabolic and electrical co-operation could play a role in the functional sculpting of the brain.

The main neuronal connexion, Cx36, is most strongly expressed in neurones of the inferior olive, olfactory bulb, CA3/CA4 hippocampal subfields and several brain stem nuclei. It is also strongly expressed in pineal gland and retinal ganglion cell and inner nuclear layers (Condorelli *et al.*, 1998). Astrocytes express mainly Cx43 and oligodedrocytes Cx32 (e.g. Dermietzel *et al.*, 1989). There are also less known connexins that participate in the formation of gap junctions throughout the central nervous system. Cx36, as mentioned before, is expressed predominantly, or even only, in neuronal cells and is differentially expressed in various regions of the central nervous system. Spinal cord and frontal cortex, however, express only moderate levels of Cx36. Therefore, the suitability of these preparations for studying the properties of gap junctions is questionable. It is unlucky that the original choice of tissue type, the olfactory bulbs, did not express spontaneous electrical activity, as it would have formed a better model for the gap junction study in basis of its Cx36 expression! The role of gap junctions in physiology and pathology of mammalian neurones still remains unclear and needs further experimentation. It is important to remember that the widespread and complex pattern of connexins, especially Cx36, suggests that the connexins may play an elaborated functional role in the mammalian neuronal physiology.

The importance of the methods developed during this experimental study can not be underestimated. A great part of this study was focused on the growth of dissociated cell cultures on MEAs – a technique of great importance for studying the interactions between neurones in culture. The range of pharmaceutical and physiological evaluations enabled by these cultures is immense and the possibilities should be further investigated.

The advantages of using cell cultures grown on MEAs in studying the pharmacological and physiological properties of neuronal cell cultures are numerous. MEAs are non-invasive and permit long term monitoring of the electrophysiological activity. They also allow multisite recording. This leads to high-throughput screening and to reduction in the number of animals needed (Chiappalone *et al.*, 2003). Neuronal networks grown on MEAs can be kept alive *in vitro* for a significant amount of time providing stable systems and, therefore, good models for studying the electrophysiological properties of the neuronal tissues and the effect of pharmaceutical compounds. *In vitro* cultures, especially spinal cord tissue, show repeatable, stable activity providing exceptionally good models.

The ultimate goal of this experimental study was to use the neuronal cell cultures grown on MEAs as biosensors. This would enable the evaluation of pharmacological substances in industrial research, e.g. exposure studies. At the current stage, the collective electrophysiological activity of the cultures can be modulated by pharmacological compounds giving useful information on the primary mechanisms involved in the network responses. There have been

several previous reports in the literature (e.g. Keefer *et al.*, 2001; Gramowski *et al.*, 2004) of the suitability of the neuronal networks grown on MEAs for pharmaceutical investigation. These systems show high sensitivity to neuroactive-toxic compounds and the results gained are reproducible. Moreover, the subtle changes induced by the pharmacological agents (e.g. membrane receptor subsets) can be studied looking at the dynamics of the networks in terms of correlation between the different channels, spiking and bursting activity and, electrically modulated activity.

On-field application of these neuronal networks grown on MEAs can be foreseen. The current models, however, need further development, for example, as suggested by Potter & DeMarse (2001), the use of miniaturised microsystems and microincubators providing stand-alone embedded systems based on microelectronic devices coupled with living neuronal cell population.

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