PROPERTIES OF AN IDENTIFIED DOPAMINE CONTAINING NEURONE FROM THE SNAIL HELISOMA TRIVOLVIS

Stuart Julian Haris

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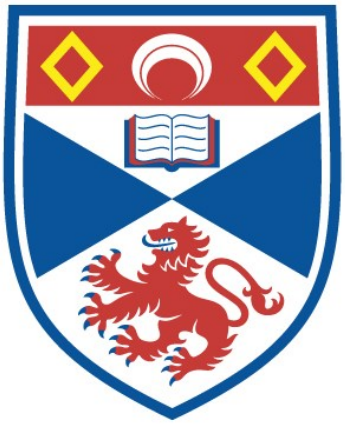

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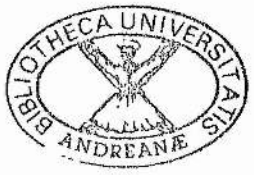
Stuart Julian Harris

Submitted September 1995



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ABSTRACT

1. The giant neurone in the left pedal ganglion of *Helisoma trivolvis* is homologous with the giant dopamine containing neurone of *Planorbis corneus*. The neurones have a similar morphology, and both react with glyoxylic acid to produce fluorescence indicative of dopamine. The neurone is referred to as the giant dopaminergic neurone (GDN).

2. Conditions for the extension of neurites and the formation of chemical junctions in culture have been determined for the *H. trivolvis* GDN.

3. Some electrical properties of the GDN were altered when it was maintained in culture. The peak spike amplitude was increased, action potential half width was decreased and the firing pattern changed.

4. In culture, the GDN formed chemical connections only with neurones with which it was known to form chemical connections in-situ. The chemical connections were of the same sign as those observed in-situ. They formed rapidly within 18 hours, but were not stable and were lost within 48 hours to be replaced by electrical connections.

5. Chemical junctions formed in both directions between the GDN and the large serotonergic neurone (LSN). The direction in which junctions formed could be influenced by plating each neurone out at different times.

6. Local application of dopamine to the axon or axon hillock, but not the soma of the isolated GDN, evoked a fast strongly desensitising, depolarising response.

7. Intracellular perfusion of the GTP analogue GDP-ß-S abolished the hyperpolarising effect of dopamine but left the fast depolarising effect intact.

8. Dopamine evoked small unitary outward currents, in outside-out patches prepared from the axon and axon hillock of the isolated GDN.

9. The results suggest that the fast depolarising response to dopamine of the GDN and its follower neurones is directly ligand gated. This is the first evidence of an ion channel that is directly gated by dopamine.

I, Stuart Harris, hereby certify that this thesis, which is approximately 35,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

1-9-95

I was admitted as a research student in September 1990 and as a candidate for the degree of PhD. in September 1990: the higher study for which this is a record was carried out in the University of St. Andrews between 1990 and 1995.

1-9-95

I Hereby certify that the candidate has fulfilled the conditions of the Resolution and regulations appropriate for the degree of PhD. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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ABBREVIATIONS

ABS	Antibiotic saline
ACh	Acetylcholine
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
^{o}C	Degrees Celsius
Con A	Concanavalin A
CM	Conditioned medium
CNS	Central nervous system
DM	Defined medium
FMRFamide	Phenylalanine-methionine-arginine-phenyalanine-NH_2
GDN	Giant Dopamine Neurone
GDP	Guanosine diphosphate
G-protein	GTP binding protein
GTP	Guanosine triphosphate
5-HT	5-hydroxytryptamine
Hz	Hertz
LSN	Large Serotonin Neurone
mg	Milligramme
ml	Millilitre
mm	Millimetre
mM	Millimole
µM	Micromole
µm	Micrometre
ms	Millisecond
mV	Millivolt
MW	Molecular weight
MΩ	Megaohm
nA	Nanoamp
nm	Nanometre
pA	Picoamp
pS	Picosiemens
psi	Pounds/square inch
s	Second
VD4	Visceral Dorsal 4 neurone

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CHAPTER I

INTRODUCTION

I.1. THE IMPORTANCE OF DOPAMINE AS A NEUROTRANSMITTER

Dopamine is a catecholamine neurotransmitter which plays an important role in the function of both vertebrates and invertebrates. In mammals dopaminergic neurones project from a series of nuclei in the brain stem, located primarily in the ventral tegmentum and the substantia nigra. From these nuclei the neurones have widespread projections to areas including the basal ganglia, the hypothalamus and the limbic system. Dopamine and dopaminergic neurones are an area of intensive research in all areas of neurobiology from molecular biology to psychology. The widespread interest in dopamine lies primarily in its association with certain neuropathological disorders.

It was the observation of a consistent deficiency of dopamine in postmortem brains from patients with a history of Parkinson's disease that provided the first example of a brain disease that was linked to the deficiency of a specific transmitter substance. It is now well established that Parkinson's disease results from the loss of midbrain dopaminergic neurones (Kish, Shannak & Hornykiewikz 1988; German, Manaye, Smith, Woodward & Saper 1989). The link between schizophrenia and dopaminergic neurones is less clear, but it was thought that a link may exist when it was observed that the major side effects of antipsychotic drugs produced a syndrome resembling parkinsonism. The potency of antischizophrenic drugs is also related to their relative abilities to inhibit dopamine release and bind to dopamine receptors (Seeman & Lee, 1975; Seeman, Lee, Chau-Wong & Wong, 1976; Seeman 1987). As well as Parkinson's disease and schizophrenia, dysfunction of dopaminergic neurotransmission has been implicated in Tourettes syndrome, hyperprolactinaemia, tarditive dyskinesia and Huntington's chorea. The range of different pathologies associated with dopamine reflect its

widespread actions within the CNS, including roles in motor control, cognition, neuroendocrine regulation, positive reinforcement and cardiovascular regulation.

It is thought that dopaminergic neurones do not perform specific integrative roles in the CNS, but act to regulate the activity of other brain regions (Le Moal & Simon, 1991). Selective lesions of dopaminergic neurones result in a variety of different behavioural syndromes which characterise the different regions to which the dopaminergic neurones project. It appears that certain regions of the brain which receive dopaminergic input have the intrinsic ability to produce neurophysiological functions, but they remain in a quiescent state unless certain minute amounts of dopamine are present. This widespread regulatory role for dopamine helps to explain why the systemic administration of L-dopa can relieve the specific symptoms of Parkinson's disease.

It has been suggested that the deficit of dopaminergic nigrostriatal neurones in Parkinson's disease may be overcome by the injection of dissociated dopamine-releasing neurones into the appropriate brain location (Dunnett & Bjorklund, 1987). It is therefore important to obtain information on the properties of dopaminergic neurones in isolation and factors that influence extension of their neurites and synapse formation.

I.2. THE USE OF INVERTEBRATES AS MODELS

Invertebrates have been extensively used in the study of neuroscience. Often, the relatively simple nervous systems of these animals has allowed experiments to be performed which would have been impossible in vertebrate preparations. The gastropod molluscs including *Aplysia californica, H. trivolvis,*

H. aspersa and *P. corneus* are commonly used and offer certain technical advantages. Intact, isolated central ganglia are easily maintained at room temperature in an appropriate saline solution for periods of several hours. The central nervous systems of these animals contain a relatively small number of neurones (e.g. 10^5 in *A. californica*). The somata of the neurones are large, up to 1mm in diameter, which facilitates electrophysiological recording. It is possible to identify individual neurones, allowing experiments to be performed on an identical cell in different animals. The neurones are easily accessible, being peripherally arranged around a central neuropile.

I.2.1. *Helisoma trivolvis*

H. trivolvis is a freshwater snail, a basommatophoran pulmonate mollusc, closely related to *L. stagnalis* and *P. corneus*. Of these three species, *L. stagnalis* has been studied in most detail, but more recently the central ring ganglia of both *H. trivolvis* and *P. corneus* have begun to be mapped (Sonetti et al. 1988). The central nervous system of *H. trivolvis* consists of nine central ring ganglia, arranged above and below the oesophagus. The suboesophageal ganglia are two parietal, two pleural, two pedal and a single visceral ganglion. Above the oesophagus is a pair of cerebral ganglia. These ganglia are all linked by connectives.

Most work using *H. trivolvis* has centred on neurones from the buccal ganglia, which are involved in the control of feeding behaviour. In particular two identified buccal neurones, B5 and B19, have been used extensively to study aspects of synapse formation, synaptic plasticity and neurite outgrowth (e.g. Bulloch & Jones 1988, Bulloch & Ridgway 1989, Haydon 1988, Kater & Mills 1991). However until relatively recently little work had focused on the neurones of the central ring ganglia of *H. trivolvis*.

I.2.2. The Giant Dopaminergic Neurone

The initial aim of this study was to examine the properties of dopaminergic neurones, in particular neurite outgrowth and synapse formation. In *P. corneus* there is a well characterised giant dopamine containing neurone. This neurone was first described by Marsden & Kerkut in 1970 when they observed a large primary amine containing neurone in the left pedal ganglion of *P. corneus* using amine histofluorescence techniques. It was suggested that the green fluorescence observed in the cell was probably due to the presence of dopamine.

The presence of dopamine in the neurone was confirmed by spectrofluorometric analysis of extracts of isolated cells which showed that the neurone contained approximately 5.5 ng of dopamine and no detectable noradrenaline (Powell & Cottrell 1974). A detailed morphological description of the giant dopamine neurone was achieved autoradiographically, following injection of tritiated dopamine (Pentreath & Berry 1975). The neurone was found to give rise to an axon which passed through the left pleural ganglion and into the left parietal and visceral ganglion where it formed many fine branches. Electronmicroscopic analysis showed many terminal processes which contained dopamine containing dense cored vesicles ranging in size from 50-160 μm.

The giant dopamine neurone was shown to form many synaptic connections with follower cells in the visceral and left parietal gangliona. The connections included fast and slow excitatory, slow inhibitory, and biphasic connections. These synaptic effects were mimicked by iontophoretic application of dopamine (Berry & Cottrell 1975). The ease of identification of this neurone

and its multiple postsynaptic actions make this an ideal system for the study of dopaminergic neurotransmission.

Although the giant dopamine neurone of *P. corneus* is advantageous in many respects, *H. trivolvis* was chosen as the experimental animal. The arrangement of neurones in the central ganglia of the two animals appeared similar, with a giant neurone in the left pedal ganglion of *H. trivolvis* identical in location to the GDN of *P. corneus*. Homology of the two neurones was confirmed morphologically, histochemically and electrophysiologically. *H. trivolvis* had been used extensively in other laboratories to study synapse formation and protocols for the primary culture of *H. trivolvis* neurones were already established (e.g. Wong et al. 1981). Also *H. trivolvis* is more easily bred in the laboratory. These advantages combined with the homology of the GDN in the two species led to the decision to use *H. trivolvis*.

I.3. INVERTEBRATE NEURONE CULTURES AND SYNAPSE FORMATION

Primary cell culture of neurones from many invertebrate species have been used extensively to study neuronal function, in particular growth cone development and synapse formation. There are many advantages of primary cell culture over more intact preparations to study the way in which cells form connections.

I.3.1. Advantages of neurone culture

Advantages which apply equally to both mammalian and invertebrate cell culture include the ability to maintain neurones in exactly defined conditions including the elimination of external synaptic and hormonal input. This is

important in the study of the effects of signalling or substrate molecules on the ability of a neurone to extend neurites and form synapses.

From a practical point of view drugs can be easily applied to cultured neurones in known concentrations because problems of diffusion through layers of tissue and metabolism are eliminated. Electrophysiology is facilitated because cultured neurones can be directly visualised during recording, and the full range of patch clamp recording techniques can be used, including excised patches. It is possible to make recordings under direct visualisation from all parts of the neurone. The ability to see individual living cells enables the use of vital dyes such as calcium indicators to monitor cell function.

Particular advantages of cultured invertebrate neurones relate to the general advantages of invertebrate neurones, including their large size and the ability to use identified neurones. The use of identified neurones allows direct comparison of neurone properties *in situ* , with in culture making clear any effect that the act of culturing a neurone has on its properties. The use of identified neurone pairs which are known to form, or not to form, a specific synaptic connection when *in situ* have been important in the study of the specificity of synapse formation. There are very few preparations in which electrophysiological recordings can be made simultaneously both pre and post synaptically. The presynaptic terminal is usually too small or inaccessible to allow microelectrode penetration. One such preparation was devised by Haydon 1988 in which synapses were formed between neuronal somata which were cultured in such a way that they did not extend neurites.

I.3.2. Uses of invertebrate neurone culture to study synapse formation

Synaptic specificity, or the mechanisms by which neurones determine their appropriate targets have been extensively studied using molluscan neurone culture. It has been shown that free from any environmental cues, certain neurones retain the ability to form appropriate synaptic connections in culture. A striking example of this ability was the reconstruction in culture of the respiratory central pattern generator from *L. stagnalis* (Syed et al. 1990).

Certain signals affecting synapse formation and some of the transduction mechanisms effecting changes have been elucidated. For example, the formation of either an electrical or chemical synapse between certain neurones is dependent on whether there is active outgrowth in both cells at the time that contact takes place (Hadley et al. 1986). In turn it has been shown that certain neurotransmitter substances are able to modulate the outgrowth of neurites (e.g. Haydon et al. 1987). In these cells it is thought that the effect of transmitter on neurite outgrowth is mediated by changes in intracellular calcium concentration (Kater & Mills 1991). Local environmental cues including substrate molecules, which regions of the neurones make contact and fasiculation with other neurones have also been shown to have an effect on outgrowth and synaptic specificity (Lin & Levitan 1987; Nicholls et al. 1990; Hawver & Schacher 1993).

The neuronal growth cone, the motile tip of an extending neurite, plays an important role in target selection and synapse formation, and the large growth cones of cultured invertebrate neurones are ideally suited to study their function. Living growth cones in culture are easily observed and the effect on their morphology or activity of any environmental change, is readily

recorded. For example transmitter substances have been shown to evoke both growth cone collapse and more rapid neurite extension, depending on the type of transmitter and the neurone involved (Haydon et al. 1987). The ability to visualise living growth cones has also made possible the investigation of certain growth cone signal transduction mechanisms. For example fluorescent calcium indicators have been used to monitor changes in growth cone calcium levels (Cohan et al. 1987), and cyclic AMP has been shown to regulate organelle transport within the growth cone (Forscher et al. 1987).

The large size and accessibility of the growth cones has also made electrophysiological study possible, and many of the ionic currents and channels present in certain growth cones have been characterised (e.g. Berladetti et al. 1986; Green & Cottrell 1990). Differences in single channel activity have also been observed between growing and non-growing growth cones (Cohan et al. 1985). The ability of growth cones to release transmitter almost immediately after contact with a follower neurone has also been shown using cultured *H. trivolvis* neurones (Haydon & Zoran 1989), hinting at the growth cones' similarities with a synaptic terminal.

I.4. NEURONAL CELL SURFACE RECEPTORS

Neurotransmitters exert their effect by acting on cell surface receptors of their target cell, whether it be muscle, neuronal of any other target cell type. Cell surface receptor proteins fall into three distinct classes: Ion channel linked, G-protein linked and enzyme linked. Neurotransmitters only act on two of these three types of receptor, these are the G-protein linked and ion channel linked.

I.4.1. G-protein linked receptors

Trimeric GTP-binding protein (G-protein) linked receptors constitute an extremely large family of cell surface receptors, with over 100 different types already defined in mammals. Although there is a great diversity of receptor types and in the signalling molecules that bind to them, all of the known G-protein linked receptors have a similar structure. They consist of a single polypeptide chain which has a series of seven transmembrane domains.

The cell surface receptors are functionally coupled to their target enzyme or ion channel via a G-protein. A G-protein consists of three subunits, called α, β and γ. The G-protein exists in an inactive state in which the α subunit is bound to a molecule of GDP. Activation of the cell surface receptor by the signalling molecule results in a conformational change in the receptor which reveals a binding site for its associated G-protein. The G-protein is then able to diffuse in the plasma membrane and bind to the receptor. Binding of the G-protein to the receptor molecule results in a conformational change of the α subunit, reducing its affinity for GDP. GDP dissociates, allowing GTP to bind to the α subunit. This causes the α subunit to dissociate from the $\beta\gamma$ subunit. In this dissociated form the G-protein is activated and is able to exert its effect on its effector enzyme or ion channel. Activation of the G-protein is switched off when the GTP is hydrolysed to GDP by the α subunit, allowing it to reassemble with the $\beta\gamma$ subunit which is released when the signalling molecule dissociates from the cell surface receptor.

Different G-protein linked receptors can have entirely different effects when activated. Although all G-proteins have the same basic trimeric structure, there are several different G-proteins which have been identified. One

possible action is for a G-protein to directly activate an ion channel, for example muscarinic ACh receptors in the heart stimulate the inhibitory G-protein G_i to directly activate potassium channels. However, G-proteins usually act to alter the concentration of intracellular signalling molecules called second messengers such as calcium or cyclic AMP. For example activation of G_i can inhibit adenylate cyclase and thus reduce the levels of intracellular cyclic AMP, whereas G_s can stimulate adenylate cyclase, having an opposite effect. Gq activates the enzyme phospholipase C which triggers calcium release from the endoplasmic reticulum via IP3 and protein phosphorylation via C-kinase.

The multi-step cascade which is initiated by the activation of a G-protein linked receptor provides a method for the amplification of the initial signal e.g. The binding of one signal molecule to the receptor can result in the production of many active G-protein subunits as long as the ligand is bound, each of these subunits may bind an adenylate cyclase molecule. Each of these will in turn catalyse the hydrolysis of many molecules of ATP to cAMP for as long as the G-protein α subunit remains bound. A second feature of the enzymatic cascade is that the timecourse of the response produced is relatively slow compared with that of a an ion channel linked receptor.

I.4.2. Ion channel linked receptors

Ligand gated ion channel receptors are specialised for the fast transmission of signals from one cell to another at a synapse. The receptor and ion channel are all part of a single complex, and binding of the transmitter to the receptor results in direct activation of the ion channel. Transmitter substances which have been shown to activate this type of receptor include:ACh, 5-HT, GABA, glycine, histamine, glutamate and ATP. Of the receptors activated by these

transmitters, the nicotinic ACh, glycine, $GABA_A$, 5-HT_3 and NMDA receptors have been cloned and have been shown to belong to a superfamily of ligand gated ion channels (Noda et al. 1982; Grenningloh et al. 1987; Schofield et al. 1987; Maricq et al. 1991; Moriyoshi et al. 1991.)

I.4.2.1. A superfamily of ion channel linked receptors

It is thought that the ion channels which are members of the superfamily, each consist of five subunits arranged around a central pore. Each subunit has four transmembrane domains, with a long extracellular N-terminus. There is a large intracellular loop between the third and fourth membrane spanning regions which contains the phosphorylation sites for various protein kinases which may result in desensitisation of the receptor (Swope et al. 1992).

Although members of the ligand gated superfamily are structurally similar and have some sequence homology of their cDNA the different receptors do have different properties. For example the glycine and $GABA_A$ receptors have chloride selective ion channels whereas the nicotinic ACh, 5-HT3 and NMDA receptors have cation selective channels. Also NMDA and certain cloned 5-HT3 ion channels are blocked in a voltage dependent manner by magnesium ions whereas the others are not.

I.4.2.2. Other ion channel linked receptors

Ligand gated ion channels which have been identified but are not members of the superfamily of receptors which have subunits with four membrane spanning domains are the Histamine, ATP, and FMRFamide receptors. The receptor at which ATP directly gates an ion channel is the P_{2X} purinoceptor. ATP activates a cation selective ion channel which has a relatively high

calcium permeability. This receptor has now been cloned (Valera et al. 1994), and structural predictions suggest that it has only two membrane spanning domains and a pore forming region which resembles that of potassium channels.

The Histamine and FMRFamide receptors have not been cloned so it is not possible to determine whether they belong to the superfamily of directly gated ion channels, the P_{2X}-like channel or whether they belong to an entirely different class of receptors. The FMRFamide gated channel is the first directly gated ion channel to be activated by a peptide neurotransmitter, and it is the only directly gated channel which is sodium selective. These two differences suggest that this receptor belongs to neither of the above two receptor types.

I.4.3. Evidence that an ion channel is directly ligand gated

The first indication that a response evoked by an applied drug or transmitter substance is directly ligand activated, comes from the relatively rapid onset of the response. Responses evoked through a second messenger system are slower in onset and usually longer in duration due to the kinetics of the enzyme cascade involved. Ligand gated ion channels also often show desensitisation. It is possible to eliminate the involvement of a G-protein in the response by intracellular perfusion of either G-protein activators or inactivators (e.g. GTPγS or GDPßS respectively). The use of calcium blockers can help to eliminate a role of extracellular calcium.

The best evidence that an ion channel is ligand gated comes from the use of the patch clamp technique. In the cell attached patch configuration, application of the agonist will not activate ligand gated ion channels within the patch because the agonist is unable to reach the receptor/ion channel

complex. However a second messenger activated channel may be activated because activation of receptors outside the patch may result in the generation of a diffusible intracellular messenger which activates channels in the patch.

A ligand gated ion channel may be activated in an excised patch if the agonist is applied to the outside membrane surface. In excised patches the environment on both sides of the membrane may be exactly controlled. The activation of a second messenger mediated ion channel requires energy released from the hydrolysis of nucleotide triphosphates, and as a result can not be activated in excised patches where they are omitted. Calcium is an important second messenger, therefore indifference of an evoked current to changes in calcium concentration on the intracellular side of a patch, adds further support to a channel being ligand gated.

Certain ligand gated ion channels have now been cloned, and distinct structural homology has been observed between some of these receptors, giving rise to a receptor super family (Maricq, Peterson, Brake, Myers & Julius 1991). Ultimate confirmation that an ion channel is directly ligand gated may come through cloning and its addition to the ligand gated ion channel superfamily.

I.5. DOPAMINE RECEPTORS

I.5.1. Molecular biology of the dopamine receptor family

Until relatively recently it was thought that dopamine exerted its action through only two types of receptors, the D_1 and D_2 dopamine receptors (Kebabian & Kalne 1979). Activation of the D_1 receptor resulted in the stimulation of adenylyl cyclase whereas the D_2 receptor inhibited adenylyl

cyclase. However as far back as 1979 Kebabian and Kalne suggested that the simple D_1, D_2 classification was insufficient and that further subcategories would be described. The application of molecular biology techniques has led to the cloning of a whole family of dopamine receptor subtypes (Grandy & Civelli 1992; Gingrich & Caron 1993).

An important breakthrough came with the cloning of the rat D_{2S} receptor cDNA (Bunzow et al. 1988), as a result molecular probes for the dopamine receptor family became available. Shortly afterwards the human D_2 dopamine receptor gene was cloned and also a second form of the receptor D_{2l} which resulted from alternative splicing of the primary transcript. The D_1 receptor was cloned simultaneously in several laboratories (Dearry et al. 1990; Monsma et al. 1990; Sunhara et al. 1990; Zhou et al. 1990). Homology screening techniques based on the D_1 and D_2 receptor clones have led to the discovery of three new dopamine receptor subtypes. In total there are now five distinct dopamine receptor subtypes, D_1-D_5.

D_3 and D_4 were isolated using probes based on the D_2 receptor. The first of the new receptor subtypes to be cloned, the D_3 receptor, was isolated by Sokoloff et al. 1990. Soon afterwards Van Tol et al. 1991 using a probe based on the D_{2S} receptor identified a clone from which the human D_4 dopamine receptor was determined. The D_2, D_3 and D_4 receptors belong to the D_2-like family. The third, and to date final, dopamine receptor subtype to be cloned was the D_5 receptor. This receptor has also been referred to as the D_{1B} and $D_{1ß}$. (Grandy et al. 1991; Sunahara et al. 1991; Tiberi et al. 1991; Weinshank et al. 1991). All of the dopamine receptor molecules have seven membrane spanning regions in common with other G-protein linked receptors.

I.5.2. Second messenger coupling of the dopamine receptor subtypes

All of the dopamine receptor subtypes are thought to exert their actions through the activation of G-proteins, but the second messenger systems affected vary depending on the receptor subtype. The D_1-like receptors (D_1 and D_5) are both coupled to the stimulation of adenylate cyclase, resulting in increased levels of cAMP.

Second messenger coupling of the D_2-like receptors is more varied reflecting the greater differences in the structure of the receptor molecules. The D2 receptor inhibits adenylate cyclase (Albert et al. 1990), and is also coupled to the activation of potassium channels (Einhorn et al. 1990). These two pathways are pertussis toxin sensitive, suggesting that the G-protein G_i is involved (Bates et al. 1991). The D_2 receptor is also thought to inhibit calcium channel opening through activation of a G-protein. The D_4 receptor has also been shown to mediate inhibition of adenylate cyclase (Cohen et al. 1992). Study of the functional activation of the D_3 receptor has proven difficult, and it has been reported to only couple weakly to G-proteins (Sokolof 1992). However, recently activation of the D_3 receptor has been shown to result in inhibition of cAMP (Potenza et al. 1994)

I.5.3. Anatomical localisation of dopamine receptor subtypes

Projections from the dopaminergic neurones in substantia nigra and ventral tegmentum are widespread, and this is reflected in distribution of dopamine receptors in many areas of the brain. Overall D_1-type receptors are more abundant than D_2-type. D_1 receptors are located postsynaptically in both the neocortex and the striatum. Also present post synaptically in the striatum are

D_2, D_3 and D_5 receptors. The globus pallidus contains both postsynaptic D_3 and D_5 receptors.

There is also evidence that in some areas of the brain dopamine receptors are located on presynaptic terminals. These include D2 receptors thought to reside on the terminals of cortico-striatal neurones, and D1 receptors on the terminals of striatal and pallido-nigral neurones (Review De Keyser 1993)

I.5.4. Mammalian and molluscan dopamine receptors: a comparison

CNS preparations from molluscs are used as convenient model systems to study many aspects of neuroscience. In many ways the structure and function of the CNS of molluscs and mammals are similar, but there is a considerable phylogenetic distance separating them. It is likely therefore, that although there are many similarities, there will also be significant differences. Although the CNS of several molluscan species have been shown to contain dopaminergic neurones, it is unclear to what extent their dopamine receptors resemble the known mammalian receptor subtypes.

I.5.4.1. Pharmacology

One system which has been studied in relative detail is that of the growth hormone producing cells of *L. stagnalis* These cells receive a dopaminergic input, and it was initially thought that they were regulated by both D_1 like and D_2 like dopamine receptors. The first studies of these cells showed that dopamine and selective D_2 agonists induced hyperpolarisation which was blocked by selective D_2 antagonists, although certain selective D_2 agonists and antagonists were ineffective (Stoof, De Vlieger & Lodder 1985). These neurones were also depolarised by selective D_1 receptor agonists, an effect

which was blocked by D_1 antagonists. These results suggested that *L. stagnalis* possessed mammalian type D_1 and D_2 receptors. However subsequent studies by the same group have revealed considerable differences in the pharmacology of mammalian and *L. stagnalis* dopamine receptors (Werkman, Lodder, De Vlieger & Stoof 1987).

Other groups have compared the pharmacology of molluscan and mammalian dopamine receptor subtypes. Audesirk (1989) studied the effects of specific dopamine agonists and antagonists on the identified neurones B2 and RPeD1 of *L. stagnalis*. From the results of this work it was concluded that there was no match between the pharmacological profiles of mammalian and the *L. stagnalis* dopamine receptors studied. Differences were also observed compared with the receptors described on the growth hormone producing cells. Pharmacological studies have also been made on inhibitory dopamine receptors of *H. aspersa* (Holden-Dye & Walker 1989). The rigid dopamine analogue 6,7-dihydroxy-3,4,5,6-tetrahydronapthalene (6,7ADTN) was a potent agonist, but the 5,6 isomer had no effect. This is a property of almost all dopamine recognition sites studied. Specific D_1, but not D_2 receptor agonists mimic the effect of dopamine at this receptor, suggesting that it is D_1-like. However the response is blocked by both D_1 and D_2 selective antagonists.

I.5.4.2. Mechanism of action

The mechanism of action of molluscan dopamine receptors is less confused and appears to bear a closer similarity to mammalian receptors than the receptor pharmacology. The proposed D_2-like receptors on the growth hormone producing cells of *L. stagnalis* exert their inhibitory effect by opening potassium channels. This effect is mediated via a reduction in the levels of

adenylate cyclase and cyclic AMP. This is similar to the effect on mammalian D_2 receptors. The effect of the D_1-like receptors in *L. stagnalis* and *H. aspersa* are mimicked by the experimental manipulations which increase the levels of intracellular cyclic AMP. This again resembles the action of the mammalian D_1 receptor.

It is clear that although many similarities exist between molluscan and mammalian dopamine receptors, the differences are considerable. It is possible that much of the structure of the dopamine receptor recognition site is conserved between molluscs and mammals, but the conservation is not perfect resulting in the pharmacological differences observed particularly with drugs which are very selective for a specific mammalian receptor subtype. Although the receptors differ it appears that the second messengers and ion channels activated by the receptors are highly conserved.

CHAPTER II

METHODS

II.1. ANIMALS

The animals used for all experiments were laboratory reared adult freshwater snails *Helisoma trivolvis* (shell diameter 10-17 mm). They were maintained in aquaria filled with $^1/_3$ tap water $^2/_3$ distilled water at 20-23 ^oC, and fed on fresh-water fish food and lettuce.

II.2. NEURONE CULTURE

II.2.1. Dissection

II.2.1.1.Deshelling animals

The animals were deshelled by making a radial incision around the shell splitting it into two halves. The retractor muscle was cut allowing the animal to be freed from the shell (Fig. II.1). The deshelled animals were immersed in a solution of antibiotic saline (ABS) (see solutions) for 15 minutes before the central ganglia were dissected out.

All of the following dissections were performed in a room kept as sterile and dust free as practical, with the dissecting microscope enclosed in a Perspex fronted cabinet to reduce the risk of fungal or bacterial contamination. Dissection was performed using a Nikon SMZ-U microscope with a range of magnification between 7.5-75 times (Fig. II.2).

II.2.1.2. Removal of Central Ganglia

The animals were pinned dorsal surface uppermost in a Sylgard lined sterile dissecting dish filled with ABS. A longitudinal incision was made along the

Figure II.1

a. Dorsal view of *H. trivolvis*, the dashed line indicates the radial incision of the shell.

b. Dorsal view of a deshelled *H. trivolvis*, the dashed line indicates the dorsal incision along the head.

c. Dorsal view of *H. trivolvis* following the dorsal incision, showing the position of the central ganglia in relation to other organs.

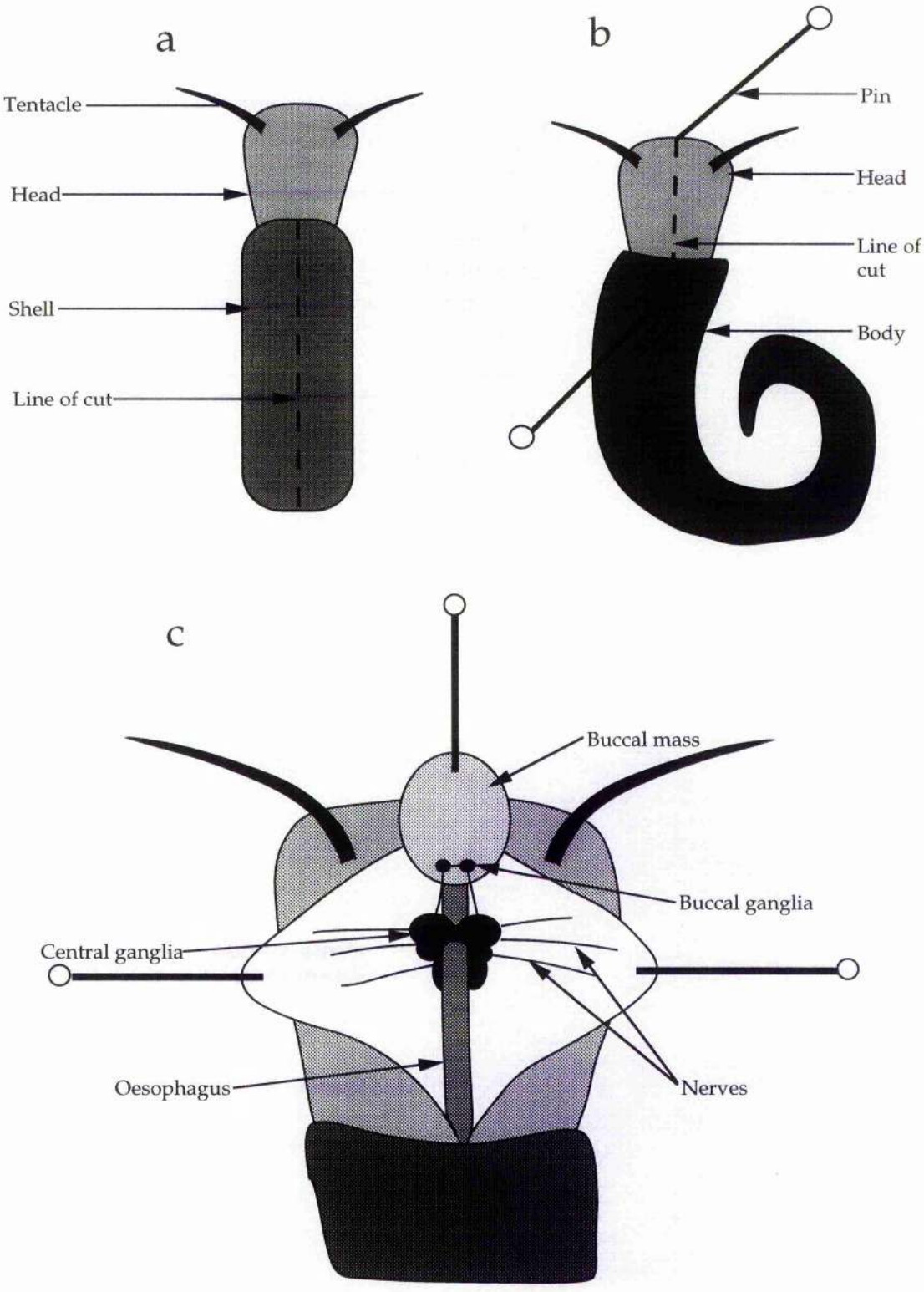
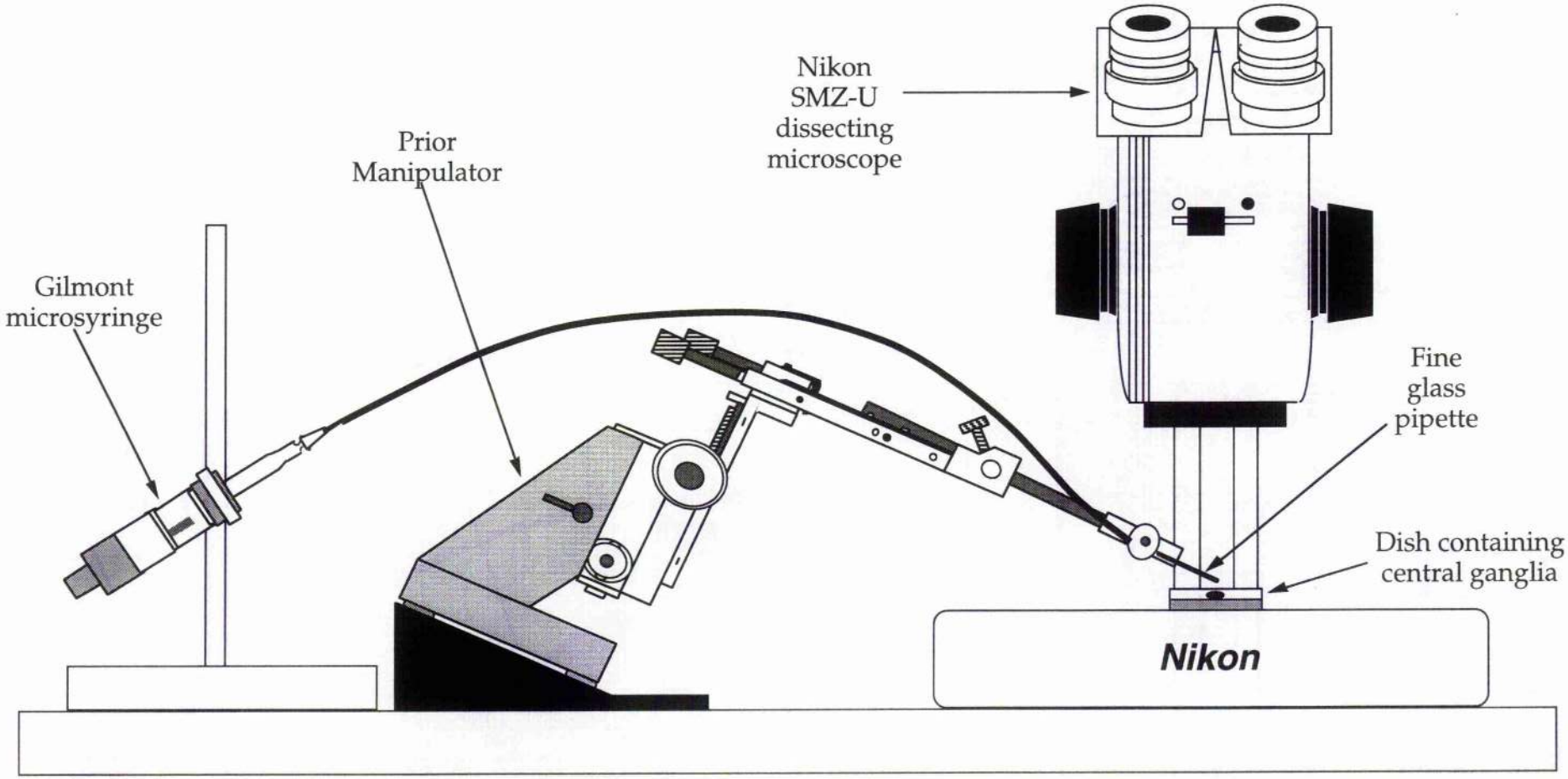

a
Tentacle
Head
Shell
Line of cut
b
Pin
Head
Line of cut
Body
c
Buccal mass
Buccal ganglia
Central ganglia
Oesophagus
Nerves

Figure II.2

The arrangement of apparatus used for sterile dissection and the isolation of individual neurones. The apparatus shown in this figure, was enclosed within a Perspex fronted cabinet. This helped to reduce contamination from airborne particles.


Nikon
SMZ-U
dissecting
microscope
Prior
Manipulator
Gilmont
microsyringe
Fine
glass
pipette
Dish containing
central ganglia
Nikon

dorsal surface of the animal's head, revealing the buccal mass. The buccal mass was pinned forward exposing the central ganglia. The oesophagus running through the central ganglia was cut near to its distal end and pulled clear. Nerves from the ganglia were cut leaving them as long as possible and the ganglia removed and placed in a vial containing Defined Medium (DM)(see solutions).

II.2.1.3. Isolation of individual neurones

The central ganglia were exposed for 25 minutes to 0.2% trypsin (Sigma type III) dissolved in DM to soften connective tissue overlying the ganglia. Isolation of individual neurones was carried out in a sterile Sylgard-lined dish containing DM. The cerebral commisure was cut, and the cerebral ganglia pinned out to reveal the dorsal surface of the suboesophageal ganglia. Loose outer connective tissue was removed using sharpened Dumont No. 5 forceps. The pedal ganglia were then rotated so that the GDN and LSN were visible in the centre of the dorsal surface of the left and right pedal ganglia respectively (Fig. II.3). To view the neurone VD4 in the visceral ganglion, the entire central ganglionic ring was stretched by pulling on the visceral nerves. The VD4 could then be seen as a pale oval neurone on an imaginary line adjacent to the visceral-right parietal connective (Fig II.3).

Removal of neurones with a section of axon attached was aided by either crushing or cutting the connectives between the ganglion containing the desired neurone, and its adjacent ganglia. This resulted in the severing of any inter-ganglionic axonal processes which may otherwise have anchored the neurone within the ganglion. The soma of the neurone to be dissected was exposed by making a slit in the connective tissue adjacent to the soma using a tungsten microknife, which had been sharpened by immersion in

Figure II.3

Dorsal view of isolated central ganglia from *H. trivolvis*, arranged to show the positions of the Giant Dopamine Neurone (D), Large Serotonin Neurone (S), and the visceral neurone VD4 (V).

Scale bar 250μm


D
S
I
V

molten sodium nitrate. Once a slit had been made in the connective, gentle pressure was applied to the surface of the ganglion in order to ease the desired soma through the slit.

The soma and attached axon of the desired neurone were gently sucked, using a Gilmont microsyringe, into a fire polished glass pipette (see manufacture of glass suction pipettes). The neurone was then transferred to a culture dish containing appropriate medium (see preparation of culture dishes). The culture dishes containing the neurones were left undisturbed for at least 4 hours at room temperature (15-22°C) to facilitate adhesion of the neurones to the substrate. They were then transferred to a humidified incubator maintained at 22°C.

II.2. 2. Solutions

Normal saline. mM: NaCl, 51.3; KCl, 1.7; $CaCl_2$, 4.1; $MgCl_2$, 1.5; HEPES, 10; pH 7.3 with NaOH.

Antibiotic saline (ABS). mM: NaCl, 51.3; KCl, 1.7; $CaCl_2$, 4.1; $MgCl_2$, 1.5; HEPES, 10; gentamicin sulphate 150μg/ml; pH 7.3 with NaOH. Prior to addition of the gentamicin, the ABS was autoclaved to ensure sterility.

4X *Helisoma* Saline. mM: NaCl, 159.9; KCl, 6.8; $CaCl_2$, 16.4; $MgCl_2$, 6.0; HEPES, 40.0; pH to 7.3 with NaOH. 4X saline was a concentrated stock designed to provide an identical concentration of inorganic salts when used to make up Defined Medium as found in normal *Helisoma* saline. This was achieved by lowering the proportion of NaCl to allow for the contribution of sodium salts contained within Leibowitz-L15.

Leibowitz L-15. This was supplied as a 2X concentrate from Gibco without inorganic salts, and contained;

Amino Acids. μg/ml: DL-Alanine, 900.0; L-Arginine (free base), 1000.0; L-Asparagine, 500.0; L-Cysteine (free base), 240.0; L-Glutamine, 600.0; Glycine, 400.0; L-Histidine (free base), 500.0; DL-Isoleucine, 500.0; L-Leucine, 250.0; L-Lysine (free base), 150.0; DL-Methionine, 300.0; DL-Phenylalanine, 500.0; L-Serine, 400.0; DL-Threonine,1200.0; L-Tryptophan, 40.0; L-Tyrosine, 600.0; DL-Valine, 400.0

Vitamins. mg/ml; DL-Ca pantothenate, 2.0; Choline chloride, 2.0; Folic Acid, 2.0; i-inositol, 4.0; Nicotinamide, 2.0; Pyroxidine HCl, 2.0; Riboflavin-5`-phosphate,sodium, 0.2; Thiamine monophosphate, 2.0

Other Components: mg/ml: D (+) Galactose, 1800.0; Phenol red, 20.0; Sodium pyruvate, 1100.0.

Defined Medium. This was the standard culture medium for *H. trivolvis* neurones and was made as follows;

For 100ml;

25 ml 4X saline

25 ml 2X Leibowitz L-15

50 ml Milli Q filtered H_2O (Millipore)

100 μg/ml gentamicin sulphate

950 μg/ml Glutamax (Gibco)

Glutamax (L-alanyl-L-glutamine) was used as a stable substitute for L-glutamine. Defined medium was sterile filtered through a 0.2μm Sartorious filter and stored in 50 ml tissue culture flasks.

II.2.3. Conditioned medium

Conditioned medium (CM) was the medium in which neurones were cultured if neurite outgrowth was required. The preparation of CM was based on the method described by Wong et al. (1981). Central ganglia were dissected from adult *H. trivolvis* under sterile conditions as described, and 2 sets of ganglia/ml placed into DM and incubated at 22°C for 72 hours. The resulting solution was passed through a 0.2μM filter (Gelman HT-200 Tuffryn membrane) to sterilise and remove free cells. CM was measured into 2 ml aliquots and stored frozen for up to a month, until required.

II.2.4. Manufacture of glass suction pipettes

Pipettes used for neurone isolation were fabricated from thin walled borosilicate microelectrode glass (Clark Electromedical Instruments GC 150T-15), which was pulled on a horizontal microelectrode puller (Narishige PN-3) at a high heat setting. The resulting pipette had a long, slowly tapering shank. The tip of the pipette was cut back under a dissecting microscope using a diamond knife until a tip diameter slightly larger than that required was reached. The tip was then polished by briefly passing it through a bunsen flame. The final internal tip diameter was slightly larger than that of the neurone to be dissected.

II.2.5. Preparation of culture dishes

Neurones were plated out onto several different substrates to determine suitable conditions for neurite extension and also for viewing the neurones under both light and electron microscopy. Coverslips washed in either nitric

or chromic acid were used initially. Neurones were also plated directly onto the base of either Cel-Cult 35mm petri dishes or Falcon 3001 dishes.

II.2.5.1.Washing glass coverslips

To ensure that coverslips (BDH 22*22mm #1 borosilicate) used for cell culture were clean and free from grease they were washed in acid. Initially chromic acid was used, but later nitric acid was used, as it was thought that this was less likely to leave a toxic residue.

Procedure for acid washing coverslips:

1. The coverslips were immersed individually into either concentrated nitric or chromic acid and left for several hours.

2. Acid was drained from the coverslips and the dish containing the coverslips was rinsed with running tap water for one hour.

3. Coverslips were then individually washed in Milli-Q filtered water, followed by immersion in 70% ethanol for one hour.

4. Coverslips were then placed on a rack to dry in a sterile flow cabinet.

5. When dry, coverslips were placed into the bottom of a culture dish.

II.2.5.2. Poly-L-lysine coating

During the course of experiments, poly-L-lysines of several different molecular weights were used, and both glass coverslips and the plastic bases of culture dishes were coated. However the procedure for coating with poly-L-lysine was the same in each case;

1. Poly-L-lysine was dissolved in sterile 0.15 M tris buffer pH 8.4 to a concentration of 1 mg/ml.

2. Approximately 1 ml of the poly-L-lysine solution was placed in the bottom of each culture dish, covering the area onto which cells would be plated.

3. The dishes were covered and left to incubate for at least 16 hours.

4. The poly-L-lysine solution was pipetted from each dish and discarded.

5. Each dish was rinsed twice with sterile Milli-Q filtered water, then filled with ABS and left for a further 20 minutes.

6. The ABS was discarded and the dishes were rinsed a further two times with Milli-Q filtered water, then allowed to dry before use.
Poly-L-lysine coated plates were discarded if they had not been used within two weeks.

II.2.5.3. Coating with Concanavalin-A

The plant lectin concanavalin-A (con A) was tested as a substrate for neurone plating. To coat with con A, dishes were incubated with a con A solution (Sigma type IV) 2 mg/ml for 2 hours. The dishes were then rinsed three times with Milli-Q filtered water and allowed to dry before use.

II.2.6. Dispersal of neurones from central ganglia

Experiments were performed to determine the proportion of neurones which extended neurites on different combinations of substrates. For these experiments it was necessary to make counts of a large population of neurones to gain an accurate description of the proportion of neurones extending neurites.

Central ganglia were dissected from *H. trivolvis* as described (see removal of central ganglia). The ganglia were incubated in 0.2% trypsin for 20 minutes, then transferred to a culture dish containing the substrate under test. The connective tissue sheath surrounding the ganglia was gently opened using sharpened number 5 forceps. To disperse the neurones across the culture dish, the ganglia were gently flicked using a steel pin held in forceps.

Neurones from the dispersed ganglia were left undisturbed for 18 hours to adhere to the substrate. The effectiveness of different conditions to stimulate neurite outgrowth was compared by counting neurones with a soma greater than 25μm in diameter at set time intervals. Only those neurones that adhered firmly to the substrate and which were also phase bright and contained the orange pigment characteristic of neurones from *H. trivolvis* were counted. The use of these criteria prevented counting of damaged and non-neuronal cells. A neurone was considered to show outgrowth if, after three days, it had extended one or more neurites to a length greater or equal to that of the diameter of the soma.

II.3. STRUCTURE, ULTRASTRUCTURE AND HISTOCHEMISTRY

II.3.1. Glyoxylic acid

Glyoxylic acid staining was used to identify monoamine containing neurones. This was primarily to help to confirm the identity of both the GDN and LSN in *H. trivolvis*. The technique was based on that of Audesirk (1985). Ganglia were dissected from *H. trivolvis*, and loose outer connective tissue was removed to reduce background fluorescence. Ganglia were immersed for 30 minutes in 220 mM glyoxylic acid with 40 mM HEPES buffer, adjusted to pH 7 with NaOH. They were then mounted on glass slides and dried at

room temperature for 30 minutes before heating in an oven at 100°C for 4 minutes. The ganglia were then covered with a small volume of liquid paraffin and sandwiched with a coverslip. Preparations were viewed with a Nikon inverted Diaphot-TMD microscope using filtering similar to that described by Audesirk (1985). This resulted in bright blue fluorescence indicating the presence of dopamine and yellow/green fluorescence indicating serotonin.

II.3.2. Lucifer Yellow

To further aid identification of the GDN and LSN in *H. trivolvis*, both neurones were filled with the fluorescent dye Lucifer Yellow. This enabled the axon and neurites of these neurones to be traced and compared with the homologous neurones in *Planorbis corneus*. Ganglia were dissected from *H. trivolvis* and the outer layers of connective tissue removed using sharpened number 5 forceps. The ganglia were then exposed to 0.2% trypsin for 40 minutes to facilitate microelectrode penetration.

Microelectrode tips (resistance 2-5 MΩ) were filled with a 5% solution of Lucifer Yellow in Milli-Q filtered water. The remainder of the electrode was filled with 1 M LiCl. The soma of either the GDN or LSN was impaled, and the membrane potential monitored using the bridge balance circuit of a preamplifier. Hyperpolarising current pulses of 500 ms, 10 nA were delivered at a rate of 1 Hz by a stimulator attached to the preamplifier. Hyperpolarising current pulses ejected the negatively charged Lucifer Yellow molecule from the tip of the electrode and into the neurone (see section II.4.6.). Current pulsing was continued for 30 minutes, or until the resting membrane potential of the neurone fell to less than 70% of its original level.

After injecting dye into the neurone, preparations were left overnight in normal saline at 4°C. Ganglia were then fixed in 4% paraformaldehyde for 1 hour then dehydrated in an ethanol series. Whole mount fluorescent ganglionic preparations were immersed in methyl salicylate on indented slides. The Lucifer Yellow was viewed with a Nikon Diaphot-TMD inverted microscope equipped with a confocal scanning laser system (Bio Rad MRC 600). This allowed the preparation to be sectioned optically at 10 μm intervals and a composite image made.

II.3.3. Transmission electron microscopy

Isolated neurones were viewed using an electron microscope to compare their ultrastructure with neurones *in situ*, and to attempt to find some ultrastructural evidence for a chemical synapse. Isolated GDNs were plated out with follower neurones using techniques already described. Neurones were plated onto poly-L-lysine coated coverslips and the culture medium was CM. Plating the neurones on coverslips facilitated transfer from one solution to another and made possible separation of the neurones from the substrate without damage.

A single neurone pair was plated onto each coverslip. They were allowed to extend neurites until they made contact, this was usually within 18 hours. Intracellular current clamp recordings were made simultaneously from the two neurones. If a chemical connection was clearly shown to be present, the neurones were photographed using phase contrast microscopy, then fixed for electron microscopy.

II.3.3.1. Fixation

The CM was drawn off the culture dish using a Pasteur pipette, being careful to leave a small amount on the dish so that the neurones did not pass through the meniscus. The dish was then filled with 2.5% glutaraldehyde in 0.2M cacodylate buffer pH 7.2 for half an hour. This was then replaced with 1% osmium tetroxide (OsO_4) in 0.2M cacodylate buffer pH 7.2 for 25 minutes. The dish was then rinsed with cacodylate buffer. The neurones were dehydrated through an ethanol series (50%, 70%, 80% and 90%. 5 minutes each. 100% 2X 15 minutes).

II.3.3.2. Embedding and sectioning

The coverslips with attached neurones were washed (5 minutes each wash) in propylene oxide. The neurones were then immersed in a solution of 1 part resin 2 parts propylene for 20 minutes followed by 2 parts resin 1 part propylene for 20 minutes. A plastic capsule was then filled with 100% resin and positioned over the neurones on the coverslip. The resin was then baked at 60°C for 24 hours. To remove the coverslip from the resin and embedded neurones, the coverslip/resin assembly was immersed in liquid nitrogen; this caused the glass to fracture away from the resin, leaving the neurones exposed and undamaged. Ultrathin sections were made of the preparations and were stained using lead citrate.

II.4. INTRACELLULAR RECORDING

II.4.1. Perfusion system

A feature common to all of the electrophysiological recording set ups was a perfusion system. This allowed a constant flow of saline to be delivered to the preparation in the recording bath. A multiway tap attached to the inflow of the perfusion system allowed smooth changeover of solutions in the bath making it possible to perfuse with different drug or saline solutions during the course of an experiment.

The design of the perfusion system is shown in (Fig. II.4). Briefly, solution was fed by gravity from a series of reservoirs to a multiway tap. The tap allowed solution in any particular reservoir to perfuse through the bath. Solution flowed from the tap into the bath via a tube whose volume was kept as small as practical to reduce delay in changes from one solution to another. The rate at which solution entered the bath was controlled by adjusting a clamp on the inflow line. The bath was drained and its level kept constant by an outflow pipe attached to a suction system.

II.4.2. Arrangement of apparatus (*in situ* recording)

The general arrangement of recording apparatus for *in situ* recording is shown in (Fig. II.5). Dissected ganglia were pinned in the Sylgard lined Perspex bath. Two Narishige micromanipulators were mounted one on each side of the bath. These could be used either to position two recording electrodes, or one recording electrode and one application pipette. A Nikon dissecting microscope, magnification 9-40X, was used to view the preparation. Efficient illumination of the preparation was critical, and was

Figure II.4

Diagram of the perfusion system used during electrophysiological recording. The multi-way tap could be used to switch between up to five different reservoirs, each of which could contain a different solution. The level of solution in the bath was controlled by varying the position of the bath outflow suction pipe.

(not to scale)

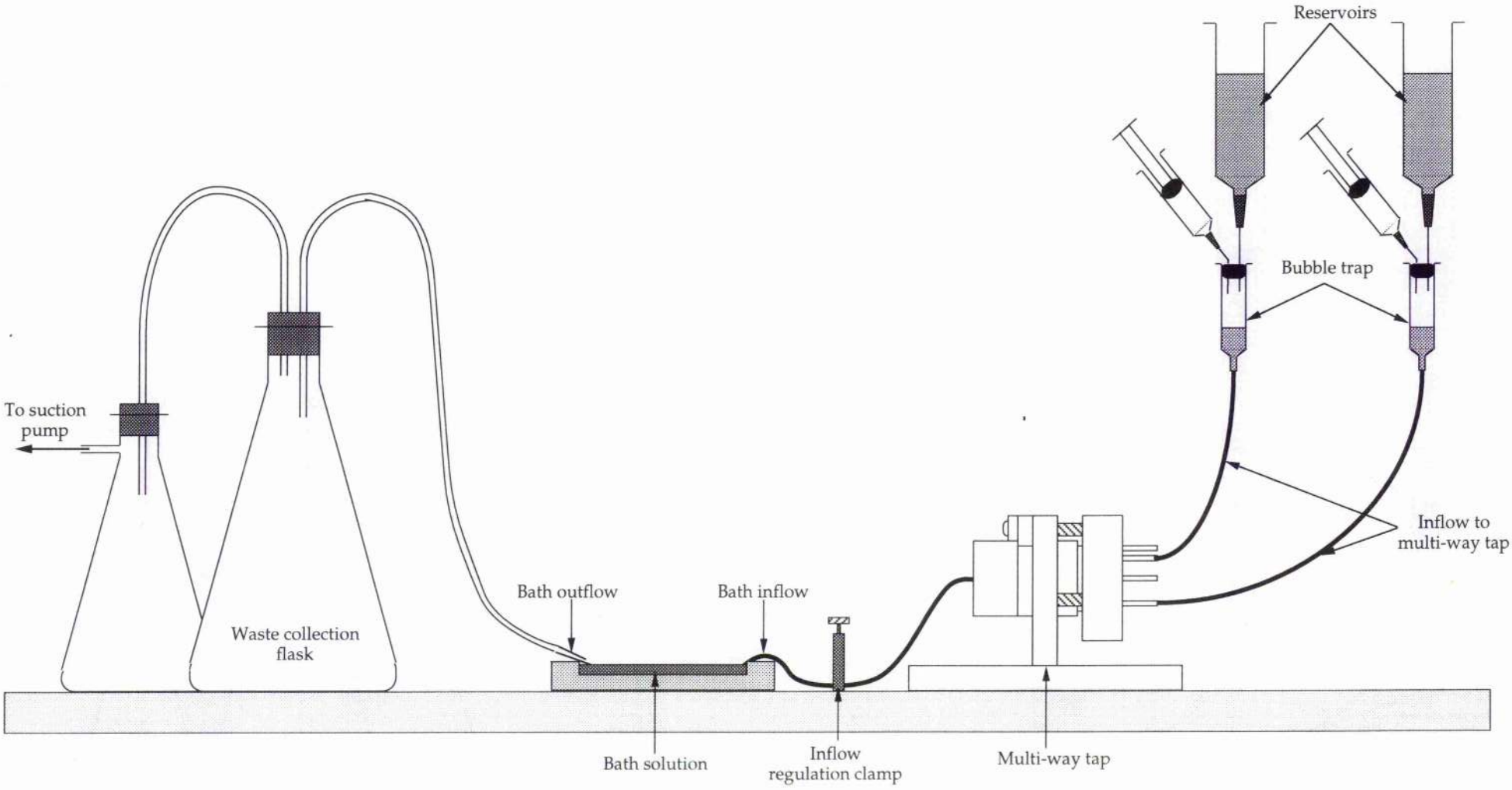

Reservoirs
Bubble trap
To suction pump
Waste collection flask
Bath outflow
Bath inflow
Inflow to multi-way tap
Bath solution
Inflow regulation clamp
Multi-way tap

Figure II.5

Arrangement of apparatus used for intracellular recording from neurones *in situ*. The Faraday cage, baseplate and apparatus were all connected to a common ground. This arrangement could be used for recording from pairs of cells, or for recording from one cell whilst applying drugs from a second micropipette.

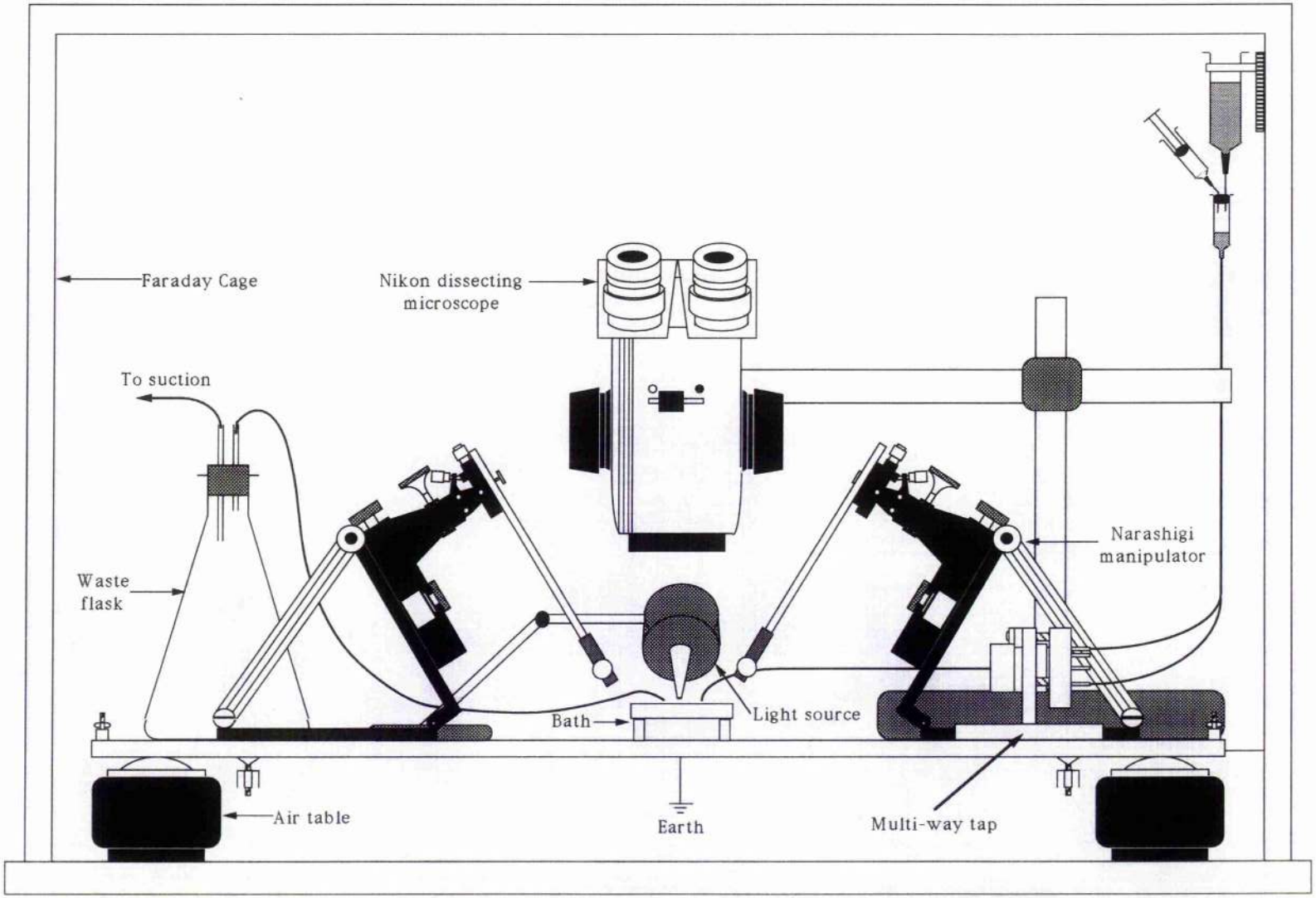
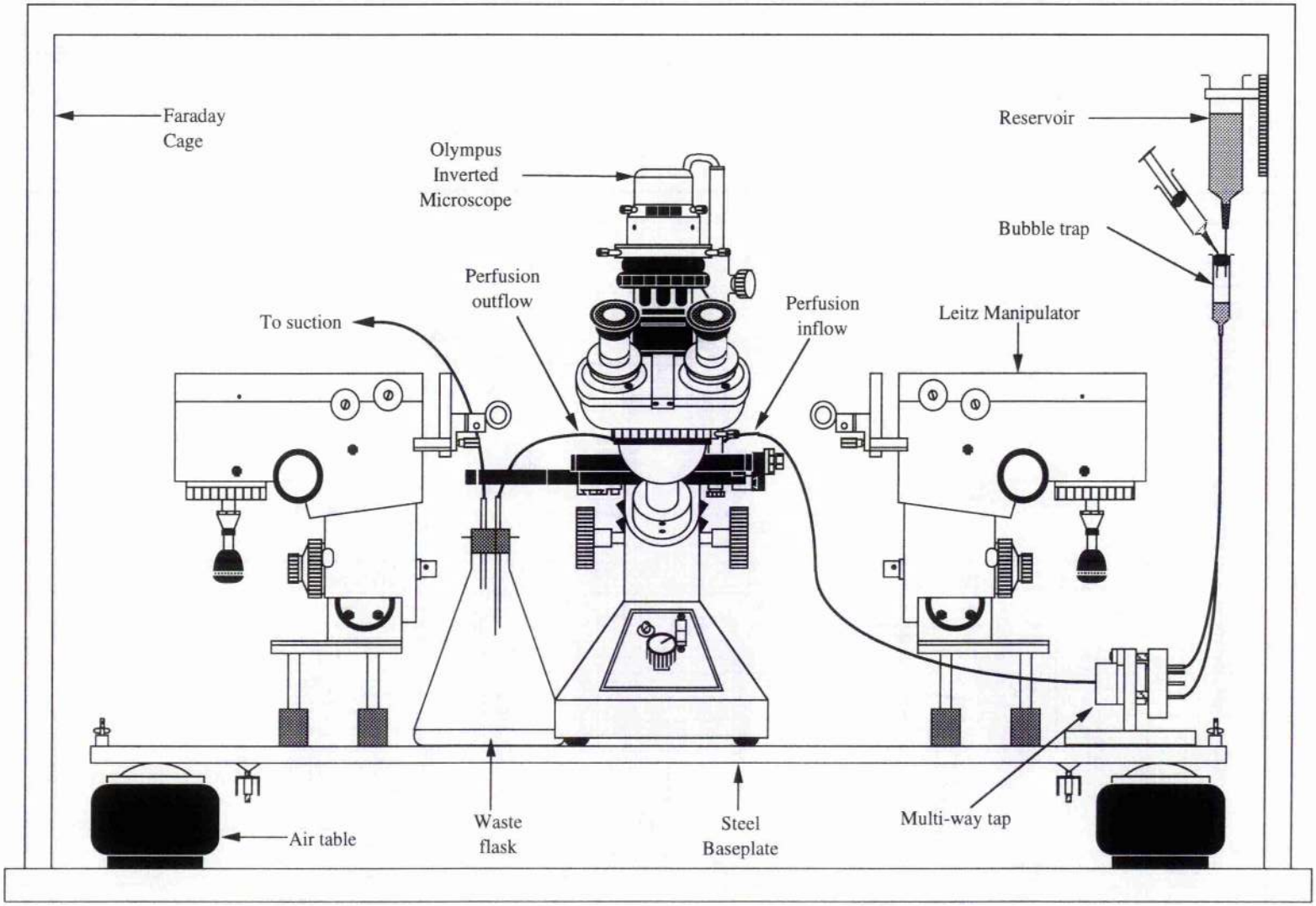

Faraday Cage
Nikon dissecting microscope
To suction
Waste flask
Narashigi manipulator
Bath
Light source
Air table
Earth
Multi-way tap

Figure II.6

Arrangement of apparatus used for intracellular recording from neurones in culture. The Faraday cage, baseplate and apparatus were all connected to a common ground. This arrangement was used predominantly for recording from cell pairs.


Faraday
Cage
Olympus
Inverted
Microscope
Reservoir
Bubble trap
Perfusion
outflow
To suction
Perfusion
inflow
Leitz Manipulator
Air table
Waste
flask
Steel
Baseplate
Multi-way tap

achieved by directing light from a light source onto the ganglion using a tapered glass rod, the end of which was immersed in the bath adjacent to the experimental preparation. The recording apparatus was set on a steel baseplate which was isolated from vibration by foam matting. The apparatus was enclosed in a Faraday cage to reduce pickup of electronic hum.

II.4.3. Arrangement of apparatus (in culture recording)

The apparatus for recording in culture is shown in (Fig. II.6). A pair of Leitz manipulators were used to position either two recording electrodes, or one recording electrode and an application pipette. A third manipulator could be introduced if, for example two recording and an application electrode were required for two electrode voltage clamp experiments. The preparation was viewed using an Olympus phase contrast inverted microscope (final magnification 100X and 160X). Recordings were made with the neurones in the culture dish. However when perfusion was used a Perspex insert was placed in the culture dish to reduce bath volume. The apparatus was placed on a steel baseplate and air table to reduce vibration. A Faraday cage enclosed the recording set-up to reduce pickup of electronic noise.

II.4.4. Preparation of ganglia for *in situ* recording

Ganglia dissected from the animal as described (removal of central ganglia), were pinned out in the recording dish so that the neurones to be recorded from were uppermost. Loose outer connective tissue was dissected away using sharpened no. 5 forceps. The ganglia were then exposed to 0.2% Trypsin for 20 minutes, followed by a saline wash. Neurones were then impaled through the softened connective tissue. If drugs were to be applied

to the preparation, the final layer of connective tissue was dissected away after trypsinisation to allow free access to the neurones.

II.4.5. Manufacture of recording electrodes

Glass microelectrodes were used for intracellular recording, however the specifications of the electrodes varied depending on the type of recordings to be made. All electrodes were manufactured from borosilicate glass with an internal filament to allow backfilling. The open ends of all microelectrodes were fire polished to prevent damage to the silver chloride coating of headstage wires.

II.4.5.1. Single electrode current clamp *in situ* and switched single electrode voltage clamp

Electrodes were manufactured from thin walled glass (Clark Electromedical GC150F-15) pulled on a vertical two stage puller (Narishige PP-83). The first pull on a high heat setting was stopped as the glass started to narrow. The heating element was then moved to the centre of the narrowed area and a second pull which heated the glass to slightly above melting point completed the pull. The electrodes were filled with 200 mM potassium acetate and had a tip resistance of 2-5 MΩ.

II.4.5.2. Single electrode current clamp and two electrode voltage clamp in culture

Electrodes were manufactured from thick walled filamented glass (Clark Electromedical GC150T-F15) pulled on a horizontal puller (Narishige PN-3). Electrodes were filled with 1 M potassium acetate and had a tip resistance of 10-20 M½.

II.4.5.3. Continuous single electrode voltage clamp in culture

Patch clamp micropipettes were used for single electrode voltage clamp recordings of neurones maintained in culture. For details of their manufacture refer to the patch clamping section of the methods.

II.4.6. Single electrode current clamp recording

The method of recording from neurones in culture and *in situ* was similar. The recording electrode was manipulated until it was in contact with the soma of the neurone. Impalement of cultured neurones was usually achieved using the negative capacitance button on the preamplifier. This caused a high frequency oscillating current in the microelectrode and usually resulted in cell penetration. Use of the negative capacitance button was usually not sufficient to impale neurones *in situ*, particularly if the connective tissue was still intact. In this case, a gentle tap on the base of the manipulator usually resulted in penetration.

Electrodes were connected via a headstage to a bridge balance preamplifier (Neurolog NL-102). Correct adjustment of the bridge balance allowed

simultaneous recording and current injection through the same microelectrode. Membrane potential and injected current were monitored using a Tektronix 5103 N storage oscilloscope. Permanent recordings of current and voltage were made on a Racal Store 4DS tape recorder and a Gould 220 chart recorder.

II.4.7. Two electrode voltage clamp recording

The two electrode voltage clamp recording technique was used in some experiments to plot current/voltage relationships. This method has the advantage over single electrode voltage clamp techniques that injection of current and voltage recording are performed by two separate electrodes. This eliminates error in the command voltage caused by current flow across the resistance of the recording electrode. The result is that the command voltage is more accurately controlled than with single electrode techniques (Sherman-Gold 1993)

The neurone was impaled with two microelectrodes; one for recording and one for current injection, filled with 1M potassium acetate, resistance 10-20 mΩ. Electrodes were connected via head stages to an Axoclamp-2B. Signals were filtered at 5 KHz and stored on tape using a Pulse Code Modulator (Sony 701 ES) and video recorder (JVC HR-D 455). Recordings were monitored and measured on a digital storage oscilloscope (Nicolet 3091). Hard copies of results were played out on a Gould 220 chart recorder.

II.4.8. Switched single electrode voltage clamp recording

Switched single electrode voltage clamp is a compromise between two electrode voltage clamp and single electrode voltage clamp. It has the advantage of employing a single electrode for both current injection and voltage recording, and the switching mechanism helps to eliminate error due to current flow across the pipette resistance. The voltage clamp rapidly switches between recording and current injection. If the cycling time of the switch is much shorter than the time constant of the cell membrane, then the membrane potential will not change significantly between injection of a current pulse and sampling of the membrane voltage.

The recording electrode was connected via a headstage to a Dagan 8100 single electrode clamp. A cycling frequency of 3 KHz was used. Recordings were monitored using a Tektronix 5103 N storage oscilloscope. Permanent recordings of current and voltage were made on a Racal Store 4DS tape recorder and Gould 220 chart recorder.

II.5. PATCH CLAMP RECORDING

The patch clamp technique of recording developed by Sakmann and Neher, has proven to be an extremely versatile and powerful electrophysiological tool, with the ability to record the current flowing through an individual ion channel in a precisely defined ionic environment. There are four different configurations in which patch clamp recordings can be made, cell attached, whole cell, inside-out and outside-out. Formation of these patches is described in Figure II.7. During the course of this work three of these configuratons have been used, cell attached, whole cell and outside-out patches.

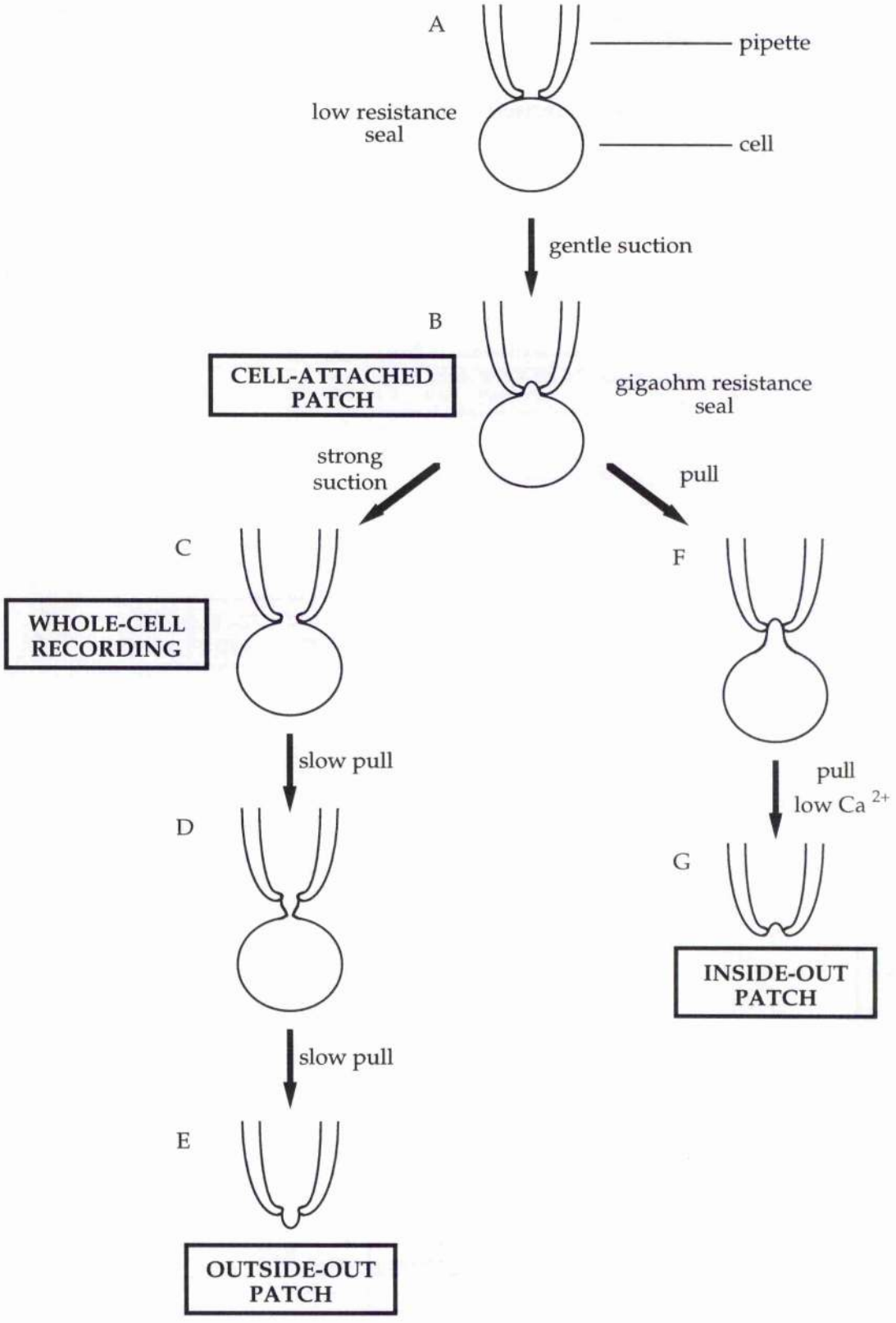
Figure II.7

Representation of different patch clamp configurations.

Contact of the pipette tip with the cell membrane forms a low resistance seal (A). With gentle suction a giga-seal is formed, this is a cell attached patch (B). If the pipette tip is pulled away from the cell membrane in an appropriate low calcium medium, an inside-out patch is formed (G).

From the cell attached configuration application of strong suction ruptures the area of membrane within the pipette resulting in a whole cell recording (C). If the pipette is then pulled away from the cell, a piece of membrane is pulled away and seals over the end of the pipette resulting in an outside-out patch (E).

This figure is adapted from Hamill, Marty, Neher, Sakmann, & Sigworth, 1981.


A
pipette
low resistance seal
cell
gentle suction
B
CELL-ATTACHED PATCH
gigaohm resistance seal
strong suction
pull
C
WHOLE-CELL RECORDING
F
slow pull
pull
low Ca 2+
D
G
INSIDE-OUT PATCH
slow pull
E
OUTSIDE-OUT PATCH

II.5.1. Manufacture of pipettes (patch clamp)

Patch pipettes were manufactured from filamented borosilicate glass (Clark Electromedical GC150F-15). Pipettes were pulled in two stages on a vertical puller (Narishige PP-83). The first pull was stopped just as the glass started to narrow. The heating element was then moved to the centre of the narrowing and a second pull commenced with a heat just sufficient to melt the glass. The pipettes were tested by attaching them to a 10 ml syringe and immersing their tips in ethanol. Pressure inside the pipettes was increased by depressing the plunger of the syringe. When pressure was increased sufficiently bubbles could be seen to emerge from the tip of the pipette. The point at which this occurred was read off from gradutions on the 10ml syringe. Pipettes with a 'bubble value' of 5.5-6.0 were used (smaller bubble value=finer tip).

The tips of the electrodes were fire polished. This was achieved by bringing the electrode tip close to a heated platinum element under direct observation by microscope. The platinum element was glass coated to prevent evaporation of the platinum onto the pipette tip, and a jet of air was directed over the heated element to produce a steep heat gradient to facilitate fire polishing. The heating element was switched off the moment that a change was observed in the pipette tip. The 'bubble test was carried out again and pipettes whose bubble value had decreased by 0.4-0.6 were accepted for use.

II.5.2. Arrangement of apparatus (patch clamp)

Culture dishes containing neurones were placed on the stage of a Zeiss inverted research microscope (magnification 100-400X). The culture dish was attached to a perfusion system as previously described. The recording pipette was attached to the patch clamp headstage by an airtight holder through which suction could be applied using a syringe. Fine manipulation of the headstage and pipette was achieved using a Narishige hydraulic manipulator, and coarse control using a Narishige manual manipulator.

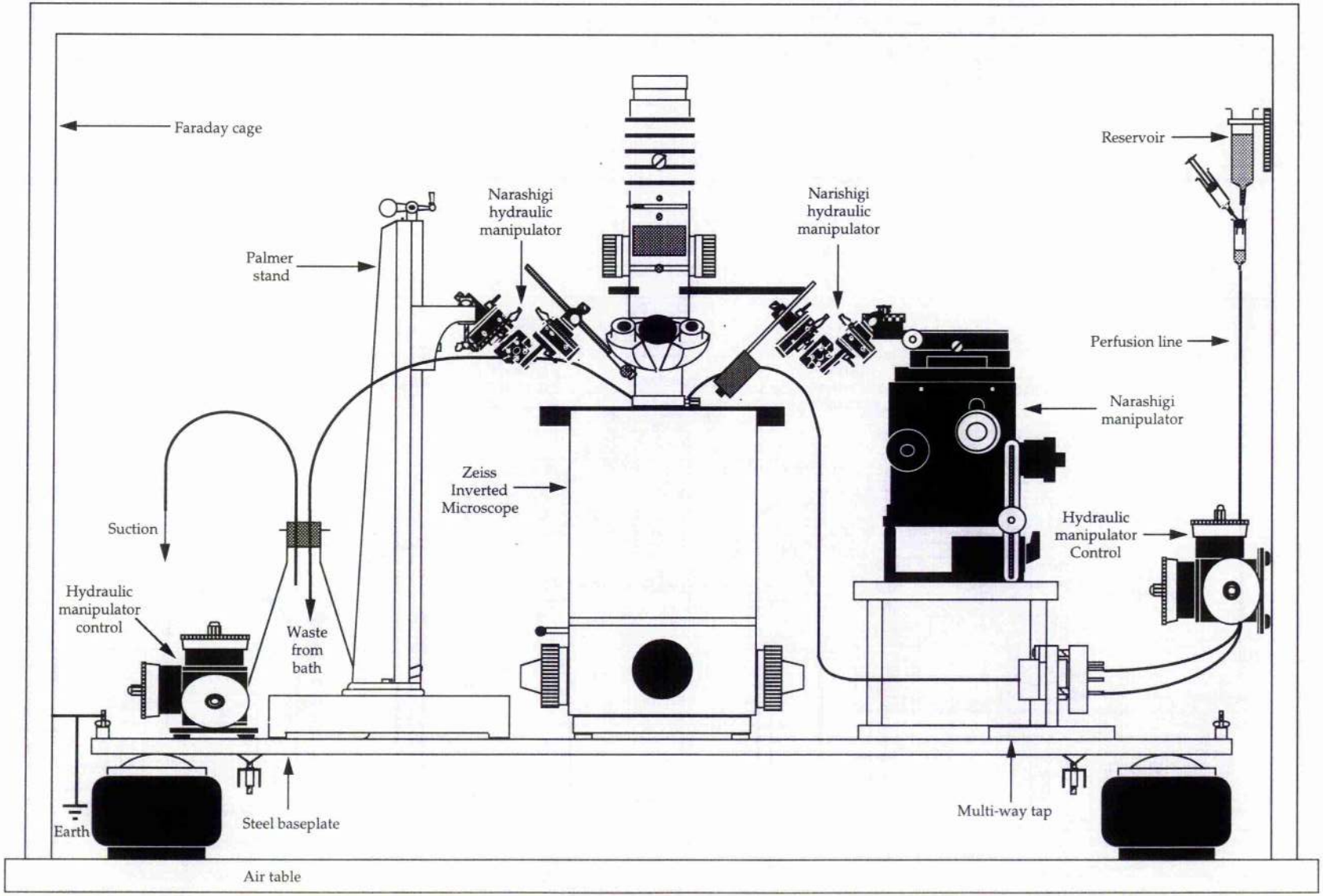
An application pipette could be positioned using a second Narishige hydraulic manipulator attached to a Prior Mass Stand. The apparatus was arranged on a steel baseplate and supported on an airtable to minimise vibration. The apparatus was enclosed in a Faraday Cage to reduce pickup of external electrical noise (Fig. II.8).

II.5.3. Recording (patch clamp)

Recordings were made using a Patch Clamp LM EPC-7 amplifier. Signals were filtered at 5 KHz and stored on tape using a Pulse Code Modulator (Sony 701 ES) and video recorder (JVC HR-D 455). Recordings were monitored on a digital storage oscilloscope (Nicolet 3091). Signals were filtered at 300-800 Hz using a Neurolog Nl-125 filter prior to viewing or playback. Figure traces were filtered, digitised and played out via the oscilloscope on a pen recorder (Gould Brush 220).

Figure II.8

Arrangement of apparatus used for patch clamp recording from neurones in culture. The manipulator on the left was used to position drug application pipettes, whilst the manipulator on the right was used to position the patch recording pipette and headstage. The Faraday cage, baseplate and apparatus were all connected to a common ground.


Faraday cage
Reservoir
Narashigi hydraulic manipulator
Narishigi hydraulic manipulator
Palmer stand
Perfusion line
Narashigi manipulator
Zeiss Inverted Microscope
Suction
Hydraulic manipulator Control
Hydraulic manipulator control
Waste from bath
Earth
Steel baseplate
Multi-way tap
Air table

II.5.3.1.Seal formation (Cell attached patch)

The patch pipette was filled with an appropriate recording solution depending on the experiment being performed, then attached via a silver chlorided wire to the headstage. Prior to immersion in the bath a small positive pressure was applied to the inside of the pipette to reduce the pickup of floating particles by the tip. A constant amplitude current pulse was passed down the pipette via the amplifier and a stimulator (Grass S-44).

The size of the resulting current pulse was monitored, and showed a decrease in size due to an increase in tip access resistance when contact was made with the neuronal membrane. When contact was made with the membrane suction was applied to the inside of the pipette. This resulted in an increase in the resistance of the cell-pipette seal and a further reduction in the size of the current pulses. Usually, a further increase in suction resulted in a sudden large increase in seal resistance, with reduced noise and complete abolition of the current pulses. This was due to formation of a Giga-Seal (Hamill, Marty, Neher, Sakmann & Sigworth 1981) and the subsequent cell attached patch.

In this configuration flow of current through channels in the patch of membrane inside the patch pipette could be recorded. The effective extracellular solution was that in the pipette and the intracellular solution was that of the neurone. The membrane potential across the patch (patch potential) was the command potential set by the patch amplifier minus the membrane potential of the neurone. The membrane potential of the neurone was determined at the end of the experiment by making a whole cell recording.

II.5.3.2.Whole cell patch (continuous single electrode voltage clamp)

After formation of a cell attached patch, the pipette holding potential was adjusted to be similar to the cell membrane potential (approx. -50 mV). The patch of membrane under the pipette was then ruptured by application of further suction (Fig II.7). This resulted in low resistance access to the inside of the neurone. The neuronal cell membrane was then clamped at the command voltage set by the patch amplifier.

This form of single electrode voltage clamp can produce an error between the command voltage and actual membrane potential due to the flow of current across the input resistance. This error was minimised by having low resistance access to the neurone, use of series resistance compensation and the small currents being recorded.

II.5.3.3. Outside-out patch

From the whole cell configuration the recording pipette, filled with a low calcium intracellular saline, was slowly withdrawn from the surface of the neurone (Fig II.7). In successful cases this resulted in a section of membrane being drawn away and sealing over the end of the recording pipette, with the extracellular surface in contact with the bath solution (Hamill et al. 1981). If this operation was successful there was a sudden drop in noise levels and the activity of single channels in the membrane could be recorded. In this configuration the exact ionic composition of the salines on both sides of the membrane are known.

II.5.4. Patch solutions

II.5.4.1.Bath solutions

Normal Saline. mM: NaCl, 51.3; KCl, 1.7; $CaCl_2$, 4.1; $MgCl_2$, 1.5; HEPES, 10; pH 7.3 with NaOH.

Low Potassium: (mM): NaCl, 52.9; KCl, 0.1; $CaCl_2$, 4.1; $MgCl_2$, 1.5; HEPES, 10; pH 7.3 with NaOH.

II.5.4.2. Pipette Solutions

Pipette solutions were filtered through a 0.22 μm millipore filter prior to filling the recording pipette.

Normal saline. mM: NaCl, 51.3; KCl, 1.7; $CaCl_2$, 4.1; $MgCl_2$, 1.5; HEPES, 10; pH 7.3 with NaOH.

High sodium (mM); NaCl 54, KCl 3, $MgCl_2$ 1, EGTA 5, HEPES 10, pH7.3 with NaOH.

High potassium (mM); NaCl 2, KCl 54, $MgCl_2$ 1, EGTA 5, HEPES 10, pH 7.3 with KOH.

High caesium (mM); NaCl 2, CsCl 54, $MgCl_2$ 1, EGTA 5, HEPES 10, pH 7.3 with NaOH.

II.6. APPLICATION OF DRUGS

II.6.1. Perfusion

Drugs could be added at the required bath concentration to the saline in the bath perfusion system. Each reservoir could contain a different solution. The required solution was selected by the multi-way tap (Fig. II.4).

II.6.2. Pressure application

Drugs were applied locally by pressure ejection from a micropipette. The micropipette was filled with the drug dissolved in a saline with the same composition as the bath solution. Drug/saline solutions were filtered through a 0.22 μm Millipore filter prior to filling the application pipette.

II.6.2.1.Manufacture of pressure pipettes

Pressure pipettes were made from borosilicate filamented glass tubing (Clark Electromedical GC150TF-15). Pipettes were pulled using a two stage vertical electrode puller (Narishige PP-83). Pipettes for pressure ejection had a bubble test value of 7.3-7.6 (see manufacture of patch electrodes).

II.6.2.2. Apparatus

The tip of the application pipette was positioned using a micromanipulator. The pipette was attached via tubing to a pressure application device (Picospritzer II). This delivered pulses of controlled pressure and duration to be applied to the pipette. Increasing either the pressure or duration of the pulse resulted in an increased delivery of the drug solution to the neurone.

II.6.3. Iontophoresis

Application of drugs by iontophoresis relies on the drug to be applied having a net charge when in solution. The drug is ejected from the pipette when an electrical gradient is applied from the inside of the pipette to the bath. Depending on the polarity of the gradient and the charge on the drug, the drug is either ejected into the bath or drawn into the pipette.

II.6.3.1. Manufacture of iontophoresis pipettes

Pipettes were pulled from borosilicate glass (Clark Electromedical GC150TF-15) on a horizontal puller (Narashige PN-3). Resulting pipettes had a tip resistance of 5-8 MΩ when filled with 200 mM KCl.

II.6.3.2. Apparatus

The tip of the application pipette was positioned using a micromanipilator. The pipette was filled with the drug solution dissolved in Milli-Q filtered water. A silver chloride wire in the drug solution was connected to the terminal of an iontophoresis programmer (W-P Instruments Model 160). A second wire from the bath connected to earth completed the electrical circuit. A small retaining current was used (20-50 nA) to prevent diffusion of the drug from the pipette. The size of the ejection current was set on the iontophoresis programmer (100-500 nA), and the duration of the pulse controlled by a stimulator (Grass S44).

CHAPTER III

RESULTS I: CELL CULTURE AND JUNCTION FORMATION

III.1. IDENTIFICATION OF THE GIANT DOPAMINE NEURONE

A giant primary catecholamine containing neurone has previously been identified and characterised in the left pedal ganglion of the snail *Planorbis corneus* (Marsden & Kerkut 1970). Powell & Cottrell (1974) confirmed that it contained dopamine. *Helisoma trivolvis* is a closely related species to *P. corneus*, and at first sight the arrangement of the neurones within the central ganglia appears similar. It was important however to confirm that the giant neurone in the left pedal ganglion of *H. trivolvis* was homologous with the GDN of *P. corneus*.

III.1.1. Morphology

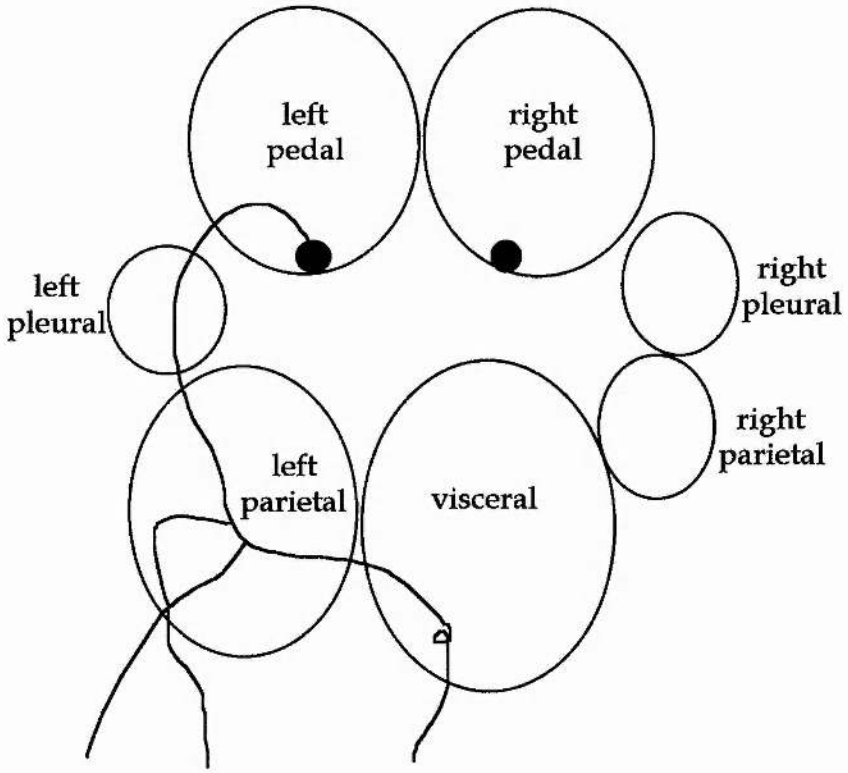
The suspected GDN is easily identified within the left pedal ganglion because of its large size and constant position close to the statocyst. This is the same as the GDN of *P. corneus*. To examine the neurone's morphology in detail, it was filled with the fluorescent dye Lucifer Yellow and viewed using a confocal scanning laser microscope.

Neurones injected with Lucifer Yellow revealed a neurone giving rise to a single thick axon from which arose many fine processes in the pedal ganglion (Fig. III.1.). The axon then passed through the left pleural ganglion and on into the left parietal ganglion. Here it branched into two broad processes one of which left the ganglion via the internal and the other via external parietal nerves. Another, thinner axonal branch passed into the visceral ganglion. There was evidence of many fine axonal branches within both the left parietal and visceral ganglia, these areas correlate with regions in *P. corneus* where the GDN makes connections with many follower neurones (Berry & Cottrell

Figure III.1.

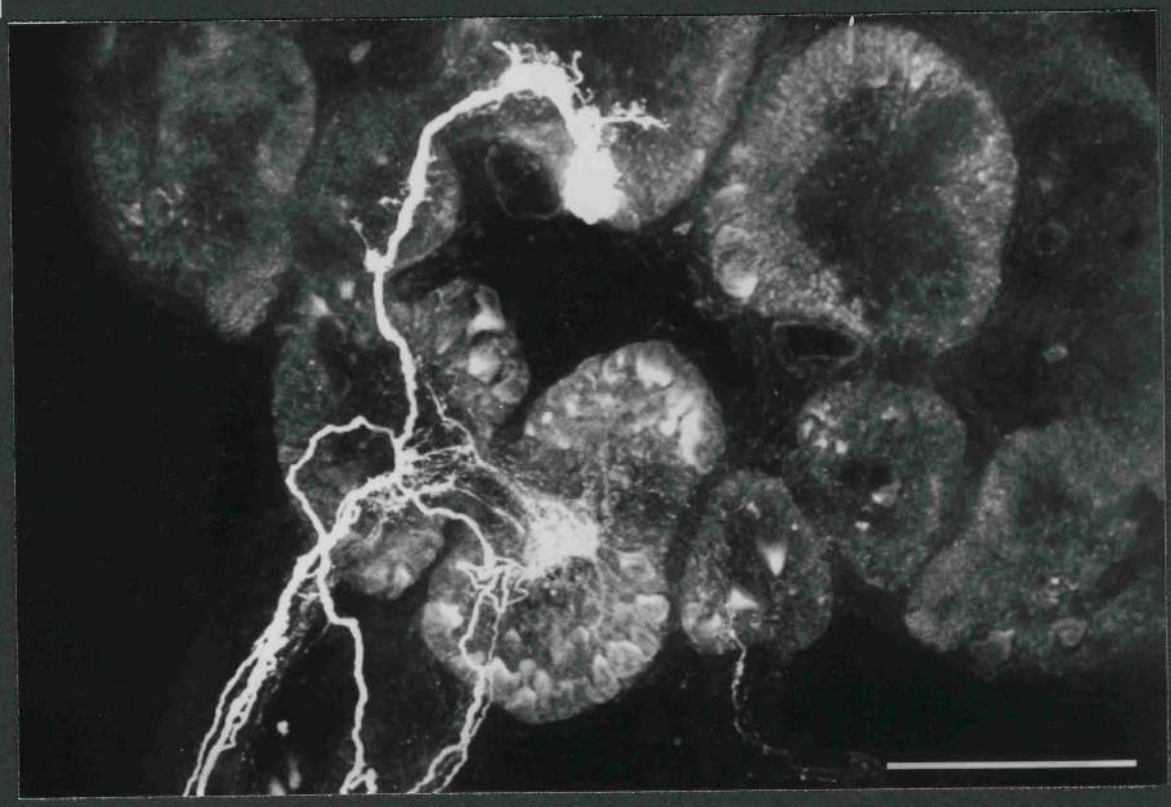
a. Lucifer Yellow fill of the GDN in a whole mount of the central ganglia from *H. trivolvis*. The soma in the left pedal ganglion gives rise to a single axon which passes through the left pleural ganglion and into the left parietal ganglion where it branches. Two branches leave the ganglion through the internal and external parietal nerves. Another branch passes into the visceral ganglion and exits via the anal nerve. Many fine branches can be seen in the left pedal, left parietal and visceral ganglia.

b. Glyoxylic acid stain of a wholemount of *H. trivolvis* central ganglia. The GDN (arrowed D) fluoresced blue in the left pedal ganglion and the LSN fluoresced yellow/green in the right pedal ganglion.

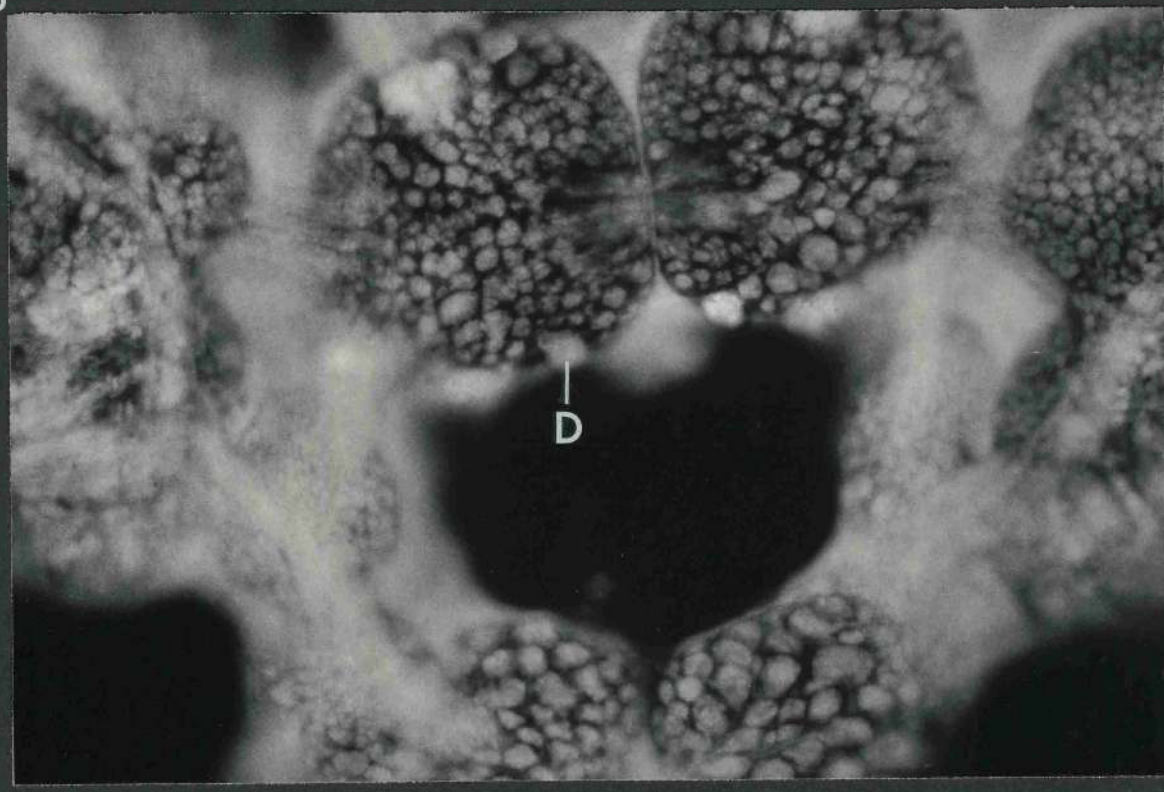


Stylised view of the ganglia in a and b to show the relative positions of the different ganglia. Scale bar 250μm.

a



b



1975). Also during the course of this work follower neurones of the GDN have been found in these areas in *H. trivolvis* (Fig. III.9.).

III.1.2. Transmitter Phenotype

Whole-mounts of the central ganglia from *H. trivolvis* were processed for glyoxylic acid histochemistry to detect the presence of primary catecholamines. The results confirmed the presence of dopamine in the Giant Neurone by the appearance of bright blue fluorescence within its soma indicative of dopamine or noradrenaline (Fig. III.1.). Also apparent in these preparations was another giant neurone directly opposite the GDN within the right pedal ganglion. This neurone showed a yellow green fluorescence indicative of serotonin. Lucifer Yellow fills of this neurone showed it to be morphologically the mirror image of the Giant Dopamine Neurone. To distinguish this neurone, it will be referred to as the Large Serotonergic Neurone (LSN).

Together the results of the Lucifer fills and the glyoxylic acid staining confirm that the identified neurone in *H. trivolvis* is homologous to the GDN of *P. corneus* and may be referred to as the GDN, *H. trivolvis*.

III.2. DEVELOPMENT OF NEURONE CULTURE

Having confirmed the identity of the GDN, the next step was to determine a reliable culture method. The initial requirements for the cultured neurones were:

1. The neurones should remain viable, as determined by a resting membrane potential of greater than -45 mV.

2. The neurones should readily extend neurites to facilitate the study of neuronal growth cones and junction formation.

Techniques for the primary cell culture of *H. trivolvis* neurones have been established in other laboratories and are primarily based on those described by Wong et.al. 1981. In initial experiments, neurones were found to be viable for periods of up to two weeks in culture which was sufficient for the envisaged experiments, but initially very few neurones extended neurites. It therefore became important to determine conditions for neurite outgrowth. Seven different combinations of substrata and media were assessed before satisfactory results were obtained:

1. Glass coverslips were washed with chromic acid and coated with poly-L-lysine WM. 25,000. The culture medium was DM.
2. Glass coverslips were washed with chromic acid, coated with poly-L-lysine MW. 25,000 and then coated with conditioned medium. The culture medium was DM.
3. Glass coverslips were washed with nitric acid, coated with poly-L-lysine MW. 25,000 and then coated with CM. The culture medium was DM.
4. Glass coverslips were washed with nitric acid then coated with Con-A. The culture medium was DM to which 2% haemolymph was added.
5. Glass coverslips were washed with nitric acid and coated with Con-A. The culture medium was DM.
6. Glass coverslips were washed with nitric acid and coated with Con-A, then coated with CM. The culture medium was DM.
7. The bases of Falcon 3001 culture dishes were coated with poly-L-lysine MW. 10,000. CM was used as the culture medium.

The results of these experiments are summarised in figure III.2. Little neurite outgrowth was observed when neurones were plated onto glass coverslips using poly-L-lysine as a substrate, regardless of how the glass was treated. Using Con-A as a substrate increased the proportion of neurones which extended neurites, but the use of Con-A as a substrate has significant disadvantages. The most important disadvantage is that it reduces the likelihood of chemical junctions forming (Lin & Levitan 1987). Of the conditions tested, by far the best outgrowth of neurites was seen when the neurones were plated onto Falcon 3001 culture dishes coated with poly-L-lysine in the presence of CM. This was therefore the combination chosen for experiments requiring neurite outgrowth unless otherwise stated.

Often, neurones which produced no neurite outgrowth were still used for electrophysiological recording. In many cases the physical action of recording from a neurone appeared to stimulate neurite outgrowth almost immediately (Fig. III.3.), but the outgrowth was limited and short-lived. There is evidence that intracellular calcium concentration is important in the regulation of growth cone activity (Kater & Mills 1991; Schwab, Kapfhammer & Bandtlow 1993; Silver, Lamb & Bolsover 1990). It is possible that the mechanical damage to the neuronal membrane and subsequently evoked action potentials would have combined to produce a transient increase in intracellular calcium levels. This may have been sufficient to initiate the growth cone activity.

III.3. ELECTRICAL PROPERTIES OF THE GIANT DOPAMINE NEURONE

With a reliable culture technique established some electrical properties of the GDN were measured to compare with neurones *in situ*. Resting membrane potential, action potential amplitude and duration, and firing pattern at rest

Figure III.2.

The effects of different substrates on neurite outgrowth. The number of neurones which extended neurites with a length equal to or greater than the diameter of the soma expressed as a percentage of the total number of neurones observed in each of the different conditions. The 'n' numbers refer to the total of growing and non-growing neurones counted in each group:

1. Glass coverslips were washed with chromic acid and coated with poly-L-lysine WM. 25,000. The culture medium was DM.

2. Glass coverslips were washed with chromic acid, coated with poly-L-lysine MW. 25,000 and then coated with conditioned medium. The culture medium was DM.

3. Glass coverslips were washed with nitric acid, coated with poly-L-lysine MW. 25,000 and then coated with CM. The culture medium was DM.

4. Glass coverslips were washed with nitric acid then coated with Con-A. The culture medium was DM to which 2% haemolymph was added.

5. Glass coverslips were washed with nitric acid and coated with Con-A. The culture medium was DM.

6. Glass coverslips washed with nitric acid and coated with Con-A, then coated with CM. The culture medium was DM.

7. The bases of Falcon 3001 culture dishes were coated with poly-L-lysine MW. 10,000. CM was used as the culture medium.

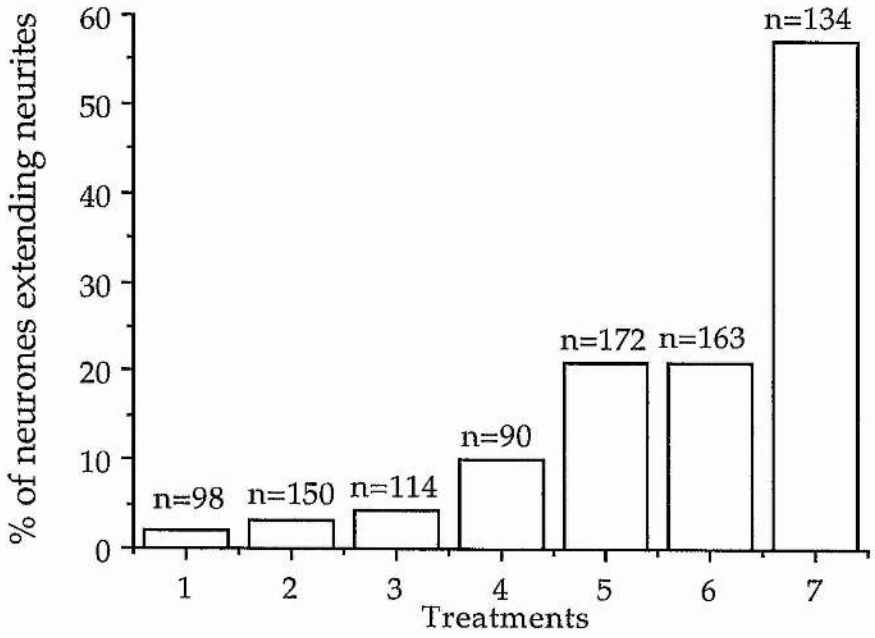

% of neurones extending neurites
60
50
40
30
20
10
0
n=98
n=150
n=114
n=90
n=172
n=163
n=134
1
2
3
4
5
6
7
Treatments

Figure III.3.

Apparent stimulation of neurite outgrowth by making intracellular recordings from a pair of neurones.

a. A neurone pair (LSN and GDN) after 18 hours in culture plated onto poly-L-lysine with CM as the culture medium. Neither of the neurones have produced neurite outgrowth although there is are fine lamellipodia surrounding them.

b. The same neurone pair 10 minutes later following intracellular recording from both neurones. The lamellipodia have become much more prominent and appear to be starting active outgrowth. This was a transient phenomenon and within hours the lamellipodia had returned to their initial pre-recording state.

Scale bar 100μm


a
b

Table III.1.

Comparison of the electrical properties of the GDN in-culture and *in-situ*

	Em (mV)	Peak amplitude (mV)	Half width (ms)
GDNs in-culture	-56±4.2 (n=9)	+33±9.4 (n=8)	4.6±1.0 (n=9)
GDNs *in-situ*	-57±3.7 (n=9)	+20±11.0 (n=8)	8.4±1.1 (n=9)

Students t test was used to compare values in-culture and *in-situ* .

Peak amplitude shows a significant increase when in-culture, and half width shows a significant decrease when in-culture $p<0.05$

were measured. Results, excluding firing pattern are displayed in Table III.1. Current-voltage relationships for GDNs were also recorded. These are shown in Fig. III.4.

The results showed no significant difference in the resting membrane potential between neurones maintained in culture and those *in situ*. However neurones in culture did exhibit a statistically significant increase in peak amplitude and reduction in half width of the action potential. This suggests some change in the membrane properties of the cells in isolation. The current voltage relationships for the neurones in culture and *in situ* do not differ, although a difference might have been expected due to changes in the input resistance of the cells depending on their different morphology in culture.

The firing pattern of the neurones was changed. The GDN *in situ* was usually silent, interspersed with bursts of activity, which appeared to originate from an external excitatory synaptic input (Fig. III.5.). This input was initially thought to be similar to neurone IP3I observed in *L. stagnalis* (Benjamin & Winlow 1981), but persisted when the pedal ganglia were isolated from the other sub-oesophageal ganglia. The excitatory input to the GDN was wide acting and was also found to affect many of the GDNs follower neurones in the visceral ganglion. The bursting was not intrinsic to the GDN (Fig.III.5c). In culture, the GDN showed a regular spiking activity of 1-2 Hz in almost all cases, although the resting membrane potential was not different to that observed *in situ*.. This suggests a reduction in the firing threshold or possibly the development of some pacemaker activity in a region of the neurone too distant from the site of recording for any slow underlying potential changes to be detected.

Figure III.4.

A comparison of mean current/voltage curves recorded from GDNs in culture (n=9), and *in situ* (n=5). Recordings in culture were made using double electrode voltage clamp. Recordings *in situ* were made using discontinuous single electrode voltage clamp. Data points show mean±SEM.

Comparison of mean current voltage relationships from GDNs in-culture and in-situ

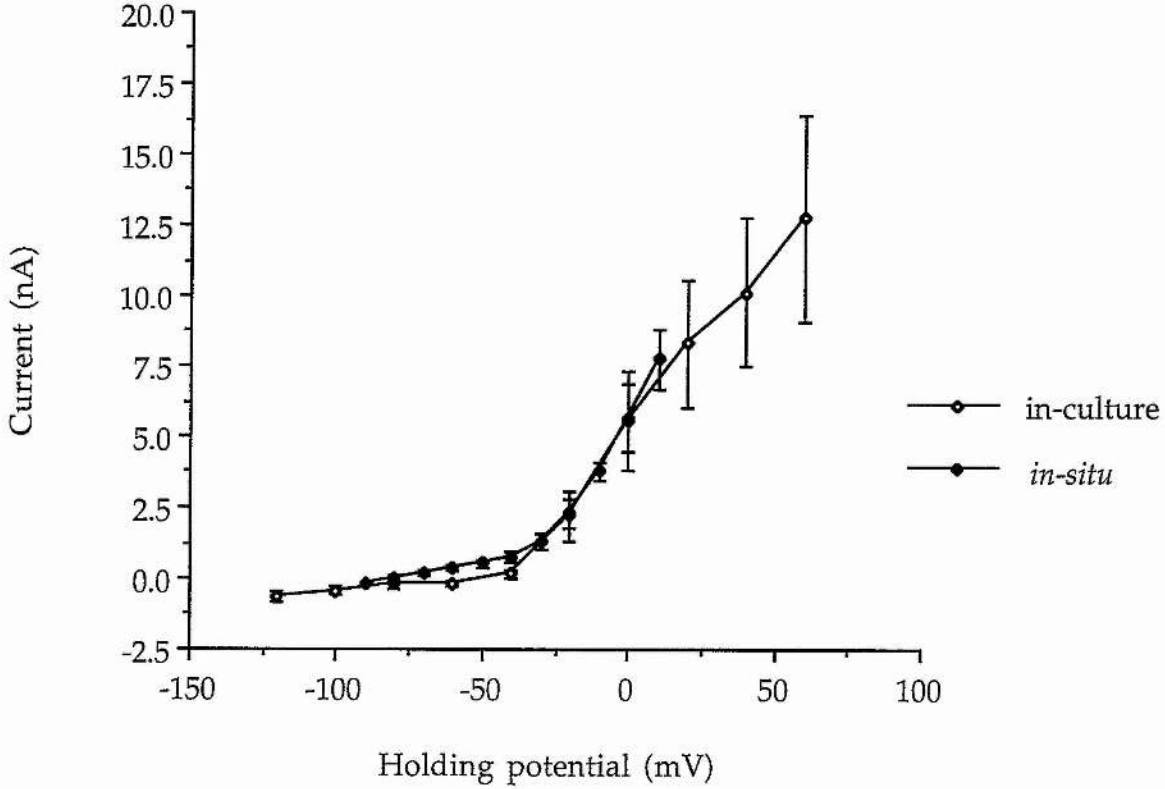


Figure III.5.

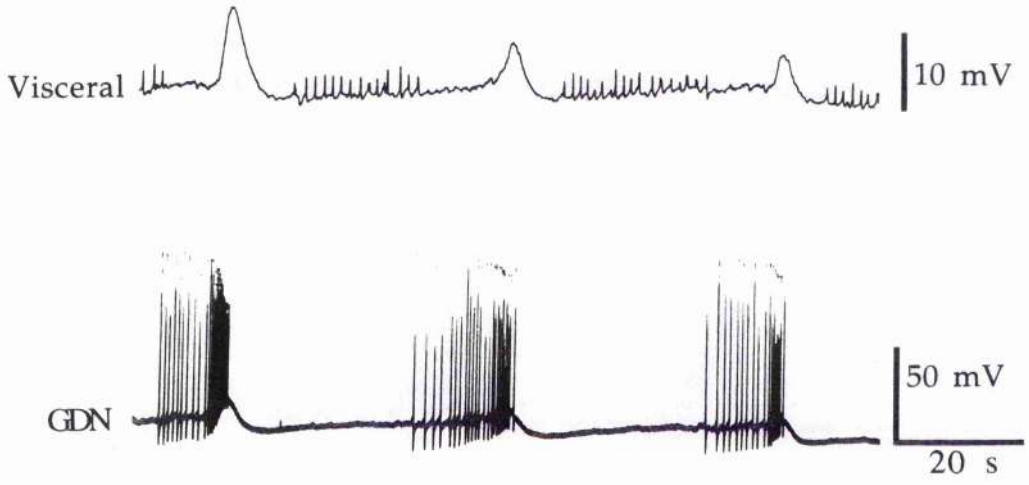
Activity of the GDN *in situ* and in culture.

a. The bottom trace from part 'a' shows typical activity recorded from a GDN *in situ*, with periods of silence interspersed with regular bursts of activity. The periodic depolarisations observed in the GDN are also observed in many of its follower neurones in the visceral ganglion, one of which is shown in the upper trace.

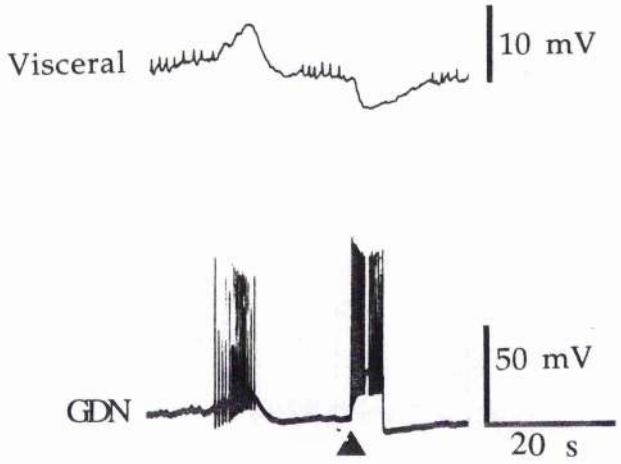
b. The depolarisation observed in the visceral neurone is not brought about by the GDN. Stimulation of the GDN by injection of a depolarising current (arrow) results in hyperpolarisation of the visceral neurone.

c. Typical activity from a GDN in culture, showing a continuous train of regular spikes when at its resting membrane potential.

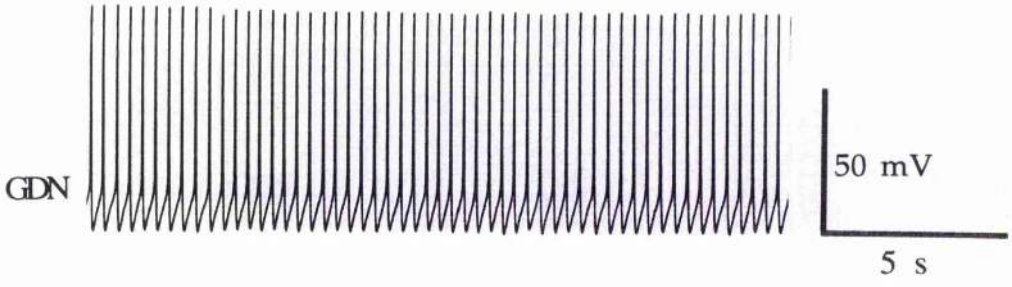
a


Visceral
10 mV
GDN
50 mV
20 s

b


Visceral
10 mV
GDN
50 mV
20 s

c


GDN
50 mV
5 s

III.4. MORPHOLOGY OF THE GIANT DOPAMINE NEURONE IN CULTURE

III.4.1. Can the Giant Dopamine Neurone be identified by its neuritic pattern?

Experiments were performed to investigate whether the GDN shows any distinctive or unique patterns of neurite outgrowth or growth cone shape when plated out in culture. The LSN from the right pedal ganglion was used as another amine-containing neurone with which to make comparisons.

When plated out under conditions described on poly-L-lysine, the GDN extended neurites with large growth cones up to 70μm in diameter, within the first 18 hours (Fig. III.6.). At 48 hours, there was an extensively branching network of neurites and the growth cones started to become smaller. By 72 hours, no further neurite outgrowth occurred and the growth cones had become small, phase-bright terminals (Fig. III.7.). The LSN followed a very similar pattern of growth and it was not possible to distinguish between the two neurone types when cultured in this way. Both the GDN and the LSN produce unusually large growth cones which distinguish them from most other *H. trivolvis* neurones maintained in culture.

Figure III.6.

Development of neurite processes of the GDN and growth-cones with time in culture.

a. A GDN 22 hours after being plated out onto a Falcon 3001 dish coated with poly-L-lysine MW. 20,100. The culture medium was CM. At this stage there are numerous large active growth cones.

2. The same neurone after 4 days in culture. It has reached the limit of its neurite extension and the growth cones have become rounded phase bright terminals.

Scale bar 100μm

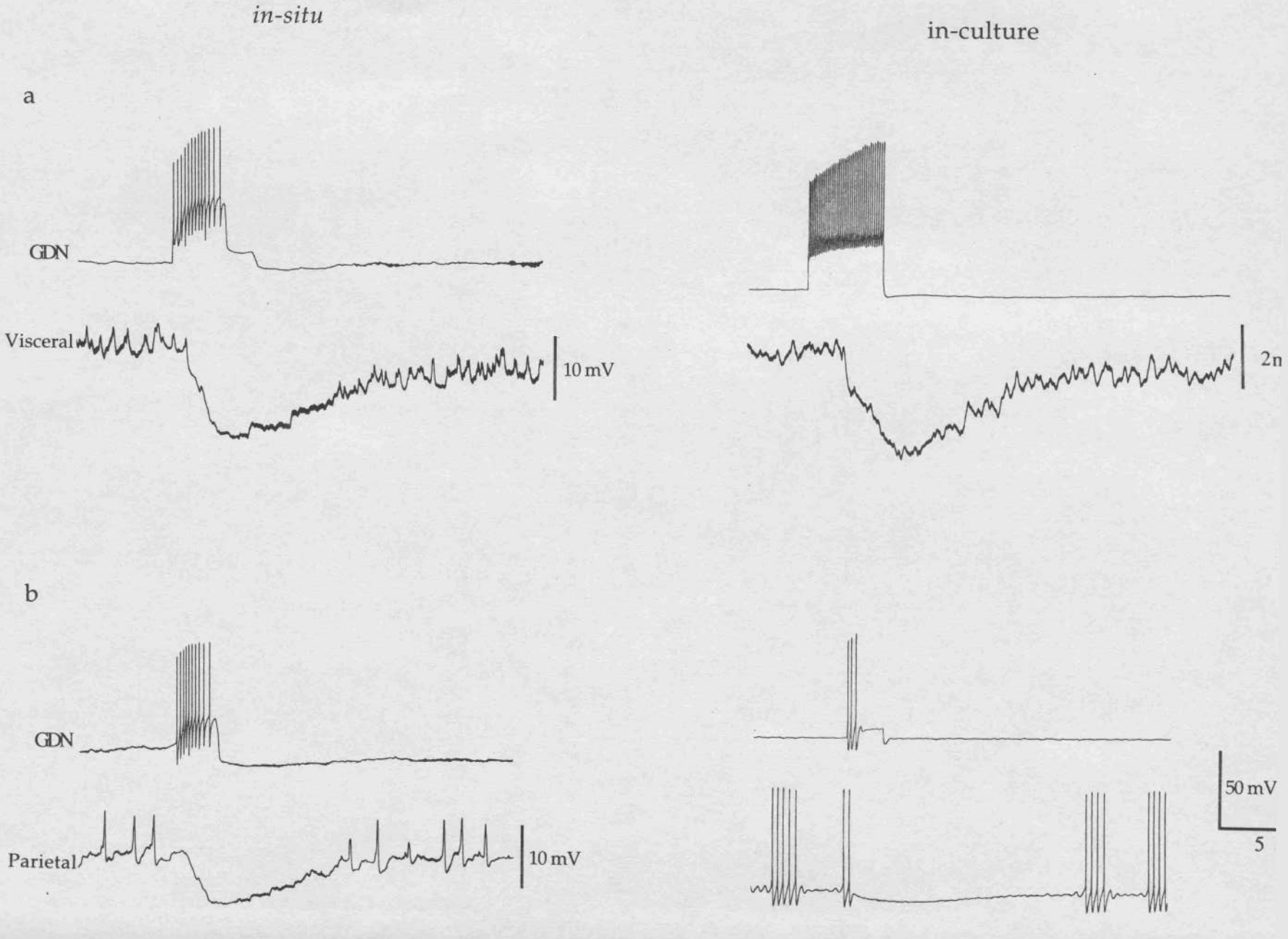

in-situ
in-culture
a
GDN
Visceral
10 mV
2n
b
GDN
Parietal
10 mV
50 mV
5

Figure III.10.

Outgrowth from one neurone avoiding another neurone in culture.

A. Appearance of a GDN with a large section of its axon attached and an unidentified parietal neurone after 24 hours in culture. Both neurones have started to extend neurites, but those from the parietal neurone appear to be avoiding the soma of the GDN. Scale bar 100μm.

B. The same pair of neurones shown at a higher magnification. None of the processes from the parietal neurone have made contact with the soma of the GDN. At this stage, it appears that the repulsion between the neurones is not mutual, because a GDN growth cone can just be made out extending from the GDN soma, directly opposite the axon, and making contact with the parietal neurone. Scale bar 50μm.

This pair of neurones did not form a chemical or an electrical connection.


A
B

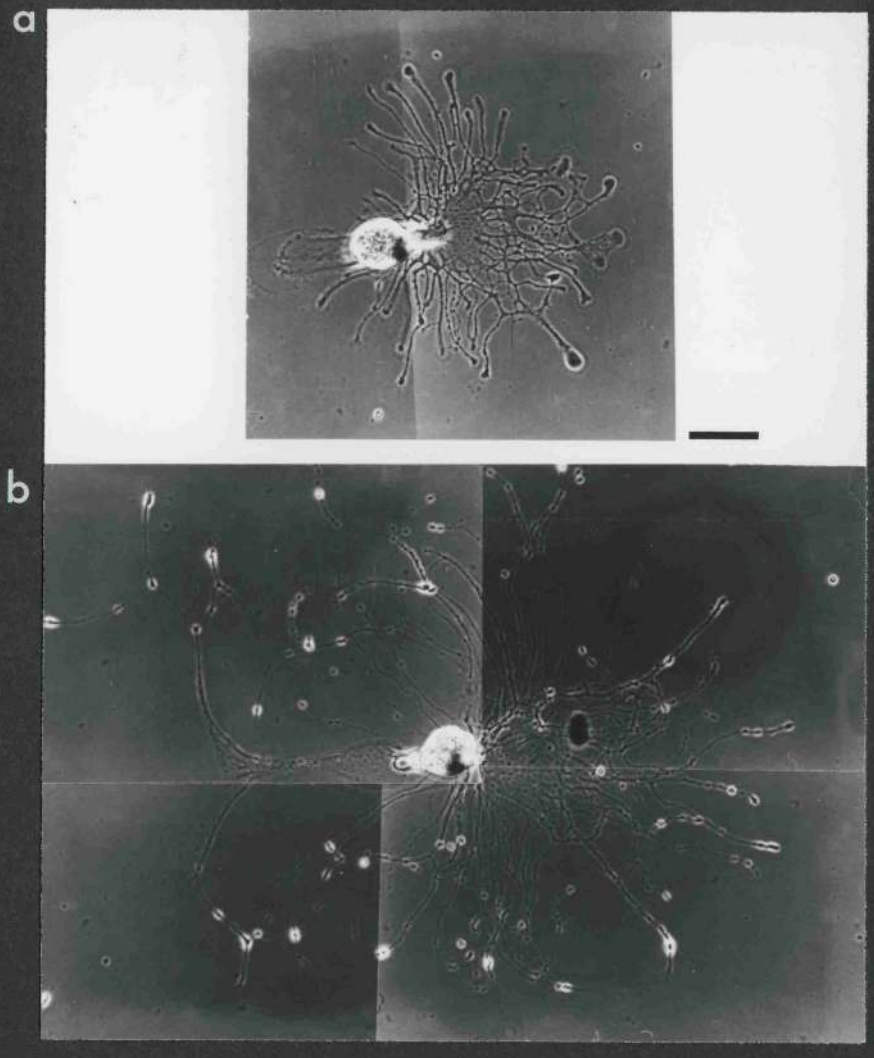

a
b

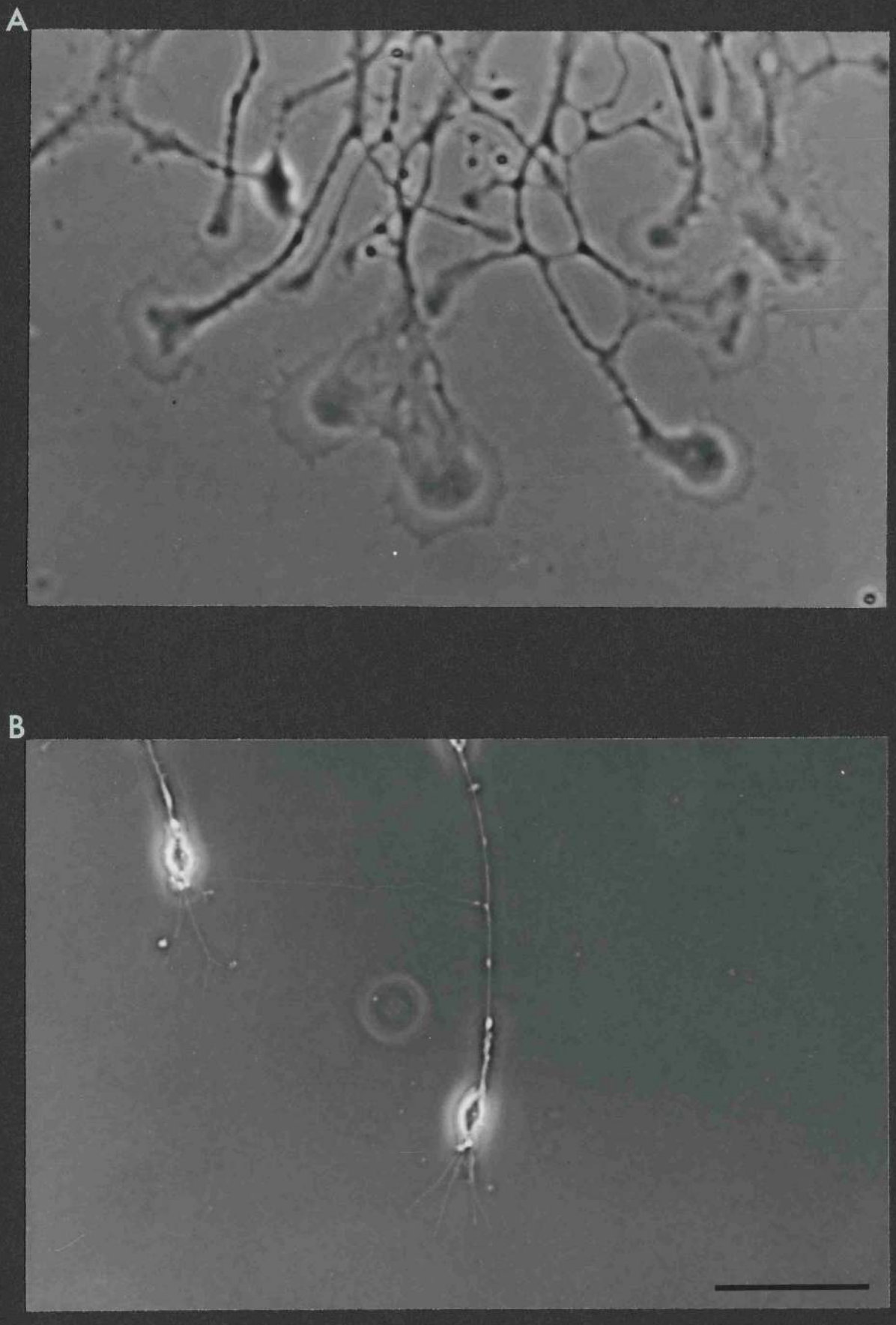
Figure III.7.

This figure shows at a higher magnification the growth cones of the GDN described in figure III.6.

a. Actively extending growth cones of the GDN after 18 hours in culture. Note the large size and the morphology typical of actively extending growth cones.

b. Inactive growth cones on the same neurone after 4 days in culture. Note the typical rounded phase bright appearance and long filopodia extending from the tips.

Scale bar 50μm


A
B

III.4.2. Effect of substrate on neurite outgrowth

Although the GDN did not display any distinctive pattern of neurite outgrowth, it was found during the course of the experiments that the composition of the substrate had a large effect on the pattern of neurite outgrowth. Cells maintained on Con-A exhibited a very different pattern of processes to those maintained on poly-L-lysine with molecular weights between 5,000 and 25,000 (cf. Grumbacher-Reinert 1987). The molecular weight of the poly-L-lysine was also seen to have an effect on the pattern of neurite development.

The effect of different substrates on neurite outgrowth is shown in figure III.8., where GDNs were plated out under identical conditions onto the bases of Falcon 3001 dishes with CM as the culture medium but the substrate was varied. Poly-L-lysine produced a branching network of fine neurite processes. As the molecular weight of the poly-L-lysine was reduced, the neurites became thinner and more branched. When maintained on Con-A, the pattern of outgrowth tended towards broad flat areas of lamellipodia with little evidence of neurites. It appears that the more sticky the substrate, the broader the neurites. In the extreme case of Con-A, neurites give way to lamellipodia, i.e. very broad flat processes.

III.5. NEURONE-NEURONE CONNECTIONS

In the intact preparation, the GDN from *P. corneus* has been shown to have multiple post-synaptic actions on different follower neurones, particularly in the visceral and left parietal ganglia (Berry & Cottrell 1975). These actions include slow inhibitory and excitatory effects, a fast excitatory effect and electrotonic coupling. In *H. trivolvis* during the course of this work, only the

Figure III.8.

The effect of different substrates on the pattern of GDN neurite outgrowth. All neurones were plated out onto Falcon 3001 dishes, the culture medium was CM. Only the substrate coating differed.

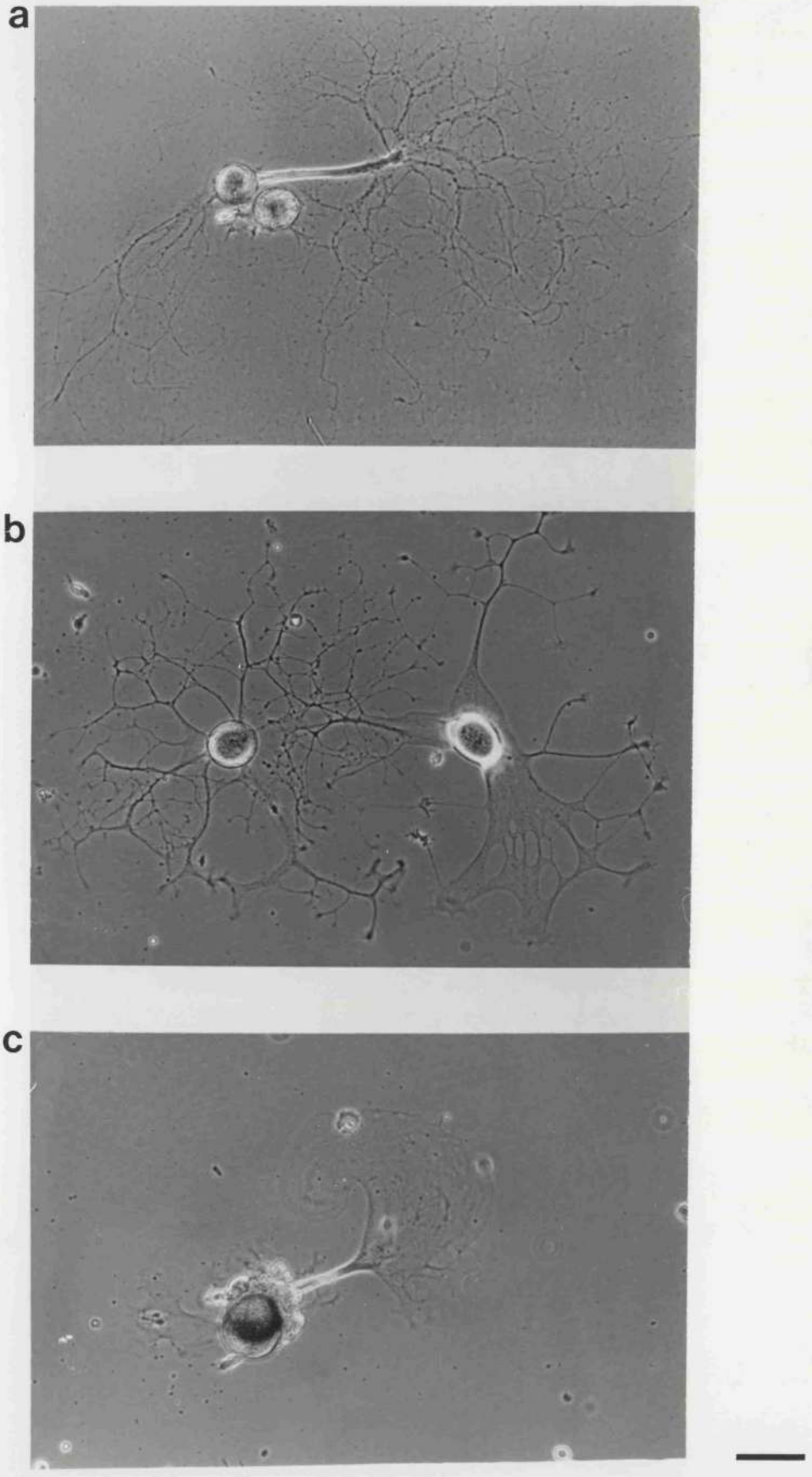
a. Poly-L-lysine MW. 5000

b. Poly-L-lysine MW. 26,000

c. Con-A

Note that with increasing molecular weight poly-L-lysine the neurites become thicker and less branched. With Con-A the neurites are replaced by a large lamellipodium. A large area of lamellipodium can be seen extending from the process to the right of the soma.

Scale bar 100μm.


a
b
c

slow inhibitory effect of the GDN has been recorded *in situ*, but this effect has been observed on many neurones in both the visceral and parietal ganglia (Fig, III.9.). *In situ* the GDN has been shown to make a mutually inhibitory connection with the LSN and is inhibited by the VD4, an interneurone of the visceral ganglion (Syed et.al. 1993).

Attempts were made to recreate these synapses in culture. The GDN was plated out with putative follower neurones, and intracellular current clamp recordings were made from both neurones simultaneously to see if a connection had formed. Recordings were made at periods between 18 hours and 6 days after isolation.

III.5.1. Connections with unidentified visceral and parietal neurones

When the GDN was plated out with unidentified neurones from the visceral or parietal ganglia, it was selective with which neurones it formed connections. Of 20 cell pairs tested only 2 chemical connections were observed. One with a visceral neurone and one was with a parietal neurone. Both of these connections were a slow inhibition by the GDN (Fig. III.9.).

The connection from the GDN to the visceral neurone was similar in duration in culture and *in situ*, but the connection in culture was slower in onset and smaller in amplitude. The connection from GDN to parietal neurone in culture, appeared as fast in onset and of similar amplitude to connections *in situ*, but had a longer duration. Two weak electrical connections were also observed between the GDN and unidentified parietal neurones.

Figure III.10. shows a pair of neurones in culture in which neurites from an unidentified visceral neurone appeared to actively avoid approaching the

Figure III.9.

Chemical connections between the GDN and visceral and parietal neurones.

a. The pair of traces on the left show an inhibitory chemical connection from the GDN to an unidentified visceral neurone recorded *in situ*. The traces on the right also show a GDN to unidentified visceral connection. The two connections bear a close resemblance in both time course and sign, but the connection recorded in culture has a later onset and much smaller amplitude.

b. Inhibitory chemical connections from the GDN to unidentified parietal neurones, both *in situ* and in culture. Comparison of the two connections is difficult but the inhibition in culture does appear to have a longer duration than that observed *in situ*.

soma of a GDN. It is possible that this type of avoidance mechanism may play a role in selectivity of junction formation, although in many cases where formation of junctions did not occur there was extensive overlap of neurites between neurones.

It has been suggested, regarding chemical junction formation, that neurones may be divided into two categories. Those which will form chemical junctions with many different neurones regardless of whether a chemical junction exists in the intact preparation and those which will only form connections with specific targets (Camardo et. al 1983; Schacher et. al. 1985; Arechiga et. al. 1986; Haydon & Zoran 1989; Kleinfeld et. al. 1990). The GDN falls into the second of these categories. This agrees with the observations of Syed, Lukowiak & Bulloch (1992).

III.5.2. Chemical connections with identified neurones

GDNs were plated out with either the VD4 neurone or the LSN. It is known that the GDN forms chemical connections with these neurones *in situ*. 55% of all such pairs formed chemical connections (which may have subsequently become electrical), 24% formed purely electrical connections and 21% did not form a detectable connection (total n=53 pairs).

The chemical connections that formed with the GDN as both the pre- and post-junctional neurone were of the same sign as those observed *in situ*. Inhibitory connections formed from the GDN to the LSN and from the LSN to the GDN. Inhibitory connections were observed from the VD4 to the GDN (Fig. III.11.). Although the junctions formed were of the same sign as those observed *in situ*, there were some differences: (1) Onset of the responses in culture was slower (Syed et.al. 1993). (2) Chemical connections were not stable

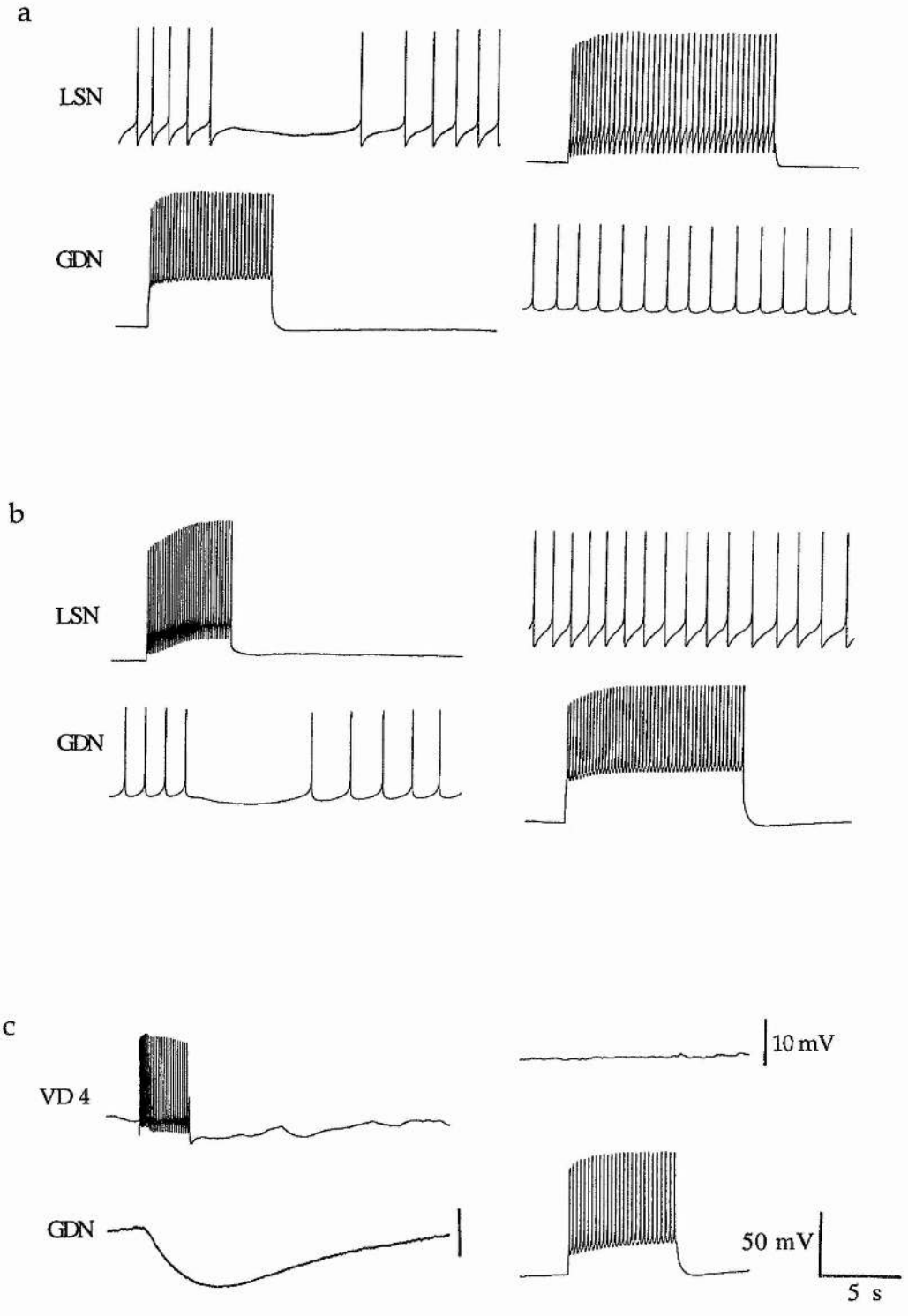
Figure III.11

The three chemical connections involving the GDN which were reliably formed in culture.

a. Stimulation of the GDN, by injection of a depolarising current pulse, results in an inhibition of the LSN. There is no reciprocal connection.

b. Stimulation of the LSN, by injection of a depolarising current pulse, results in an inhibition of the GDN. There is no reciprocal connection.

c. Stimulation of the VD4, by injection of a depolarising current pulse, results in an inhibition of the GDN. There was no reciprocal connection.


a
LSN
GDN
b
LSN
GDN
c
VD 4
GDN
10 mV
50 mV
5 s

and usually broke down leaving electrical connections. (3) Junctions between the GDN and LSN could form in either direction, but rarely were such connections simultaneously bidirectional. These differences are described in greater detail in the following sections.

III.5.3. Novel electrical connections

Novel electrical connections, not observed *in situ,* formed between the GDN and the identified neurones in some cases. These connections were characterised by a post-junctional event simultaneous in onset with the pre-junctional spike. The post-junctional event was a fast depolarisation with a duration of approximately 200 ms. The connections were reciprocal and pulses of hyperpolarising current were passed in both directions (Fig. III.12.).

It was thought possible that the fast post-junctional depolarisation was chemical in origin and related to the fast depolarisations produced by the GDN in some follower neurones in *P. corneus.* However, the electrical post-junctional potentials were not blocked when the neurones were bathed in a zero calcium saline, which blocked the slower inhibitory chemical junction that followed (Fig. III.13.). Furthermore the post-junctional response was not blocked by d-tubocurarine (Fig. III.13.), a drug which has been shown to block the fast depolarising response of follower neurones to GDN stimulation in *P. corneus* (Cottrell et. al. 1974).

The formation of chemical connections between the identified neurones did not prevent the subsequent formation of an electrical connection. From 14 chemical connections recorded, 10 became solely electrical after 48 hours in culture (Fig. III.12.). Others either remained chemical or were completely lost. In some cases transient chemical/electrical connections were observed in the

Figure III.12.

Change from a chemical to an electrical connection with time, in a pair of neurones in culture.

a. Recordings from a GDN and LSN after 18 hours in culture. Stimulation of the LSN results in a hyperpolarisation of the GDN. There is no reciprocal connection. Hyperpolarising pulses injected into one neurone do not pass to the other. Unmarked scale bars 5mV.

b. Recordings from the same neurone pair after 48 hours in culture. Stimulation of the LSN now results in a wave of depolarisation in the GDN, with transient depolarisations in the GDN simultaneous in onset with the action potentials in the LSN. The connection is reciprocal and hyperpolarising current injected into one neurone passes into the other. Unmarked scale bars 5mV.

c. Further recordings at 48 hours showing the post-junctional depolarisations and their pre-junctional action potentials on a faster time-base. Unmarked scale bar 2mV.

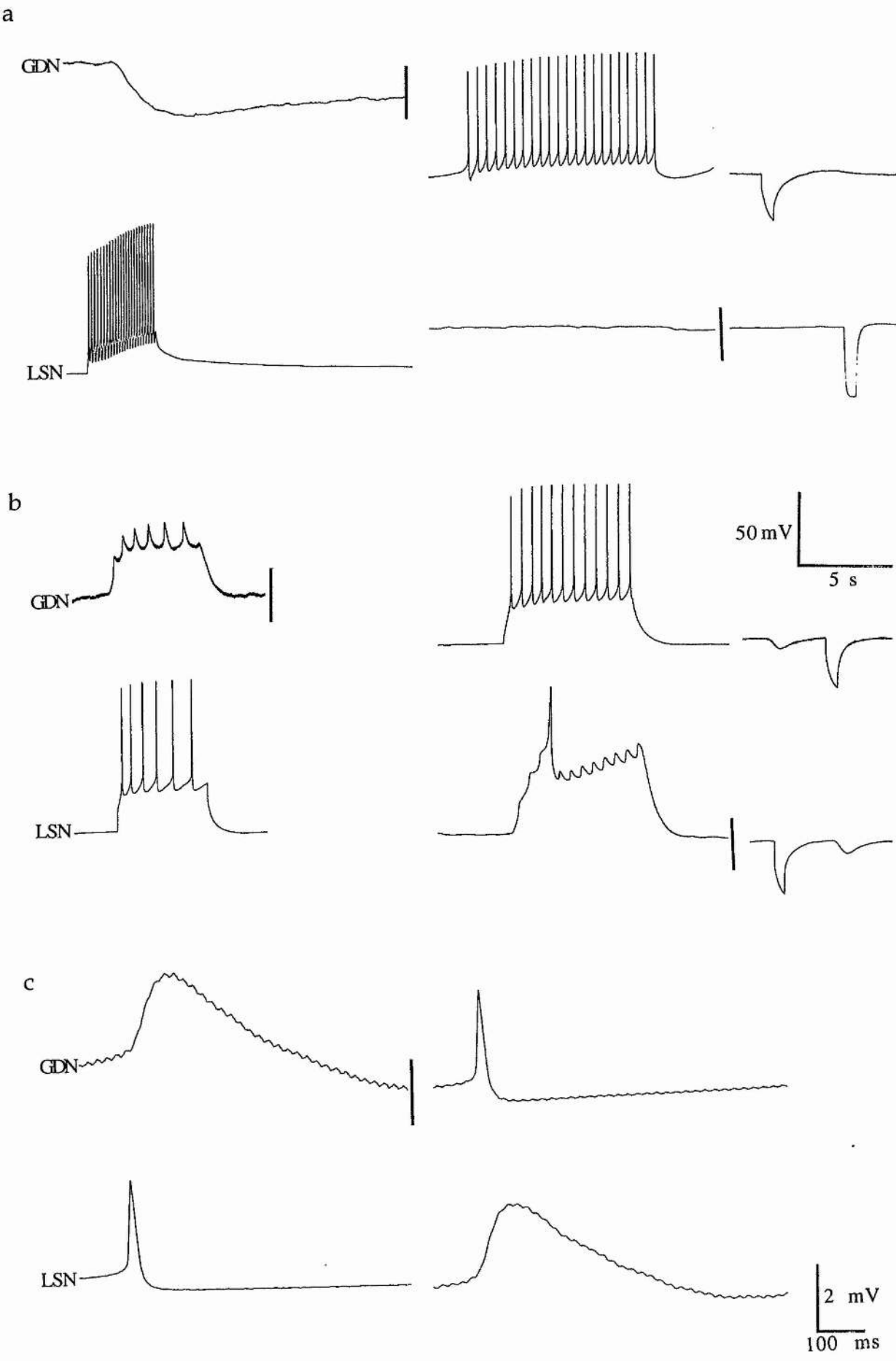

a
GDN
LSN
b
GDN
LSN
50 mV
5 s
c
GDN
LSN
2 mV
100 ms

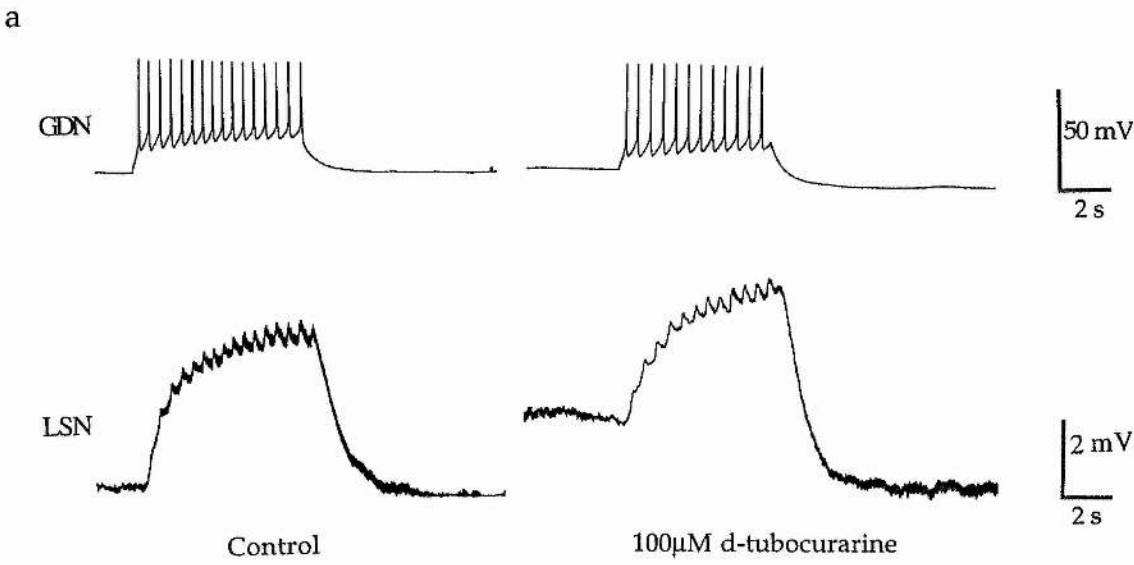
Figure III.13.

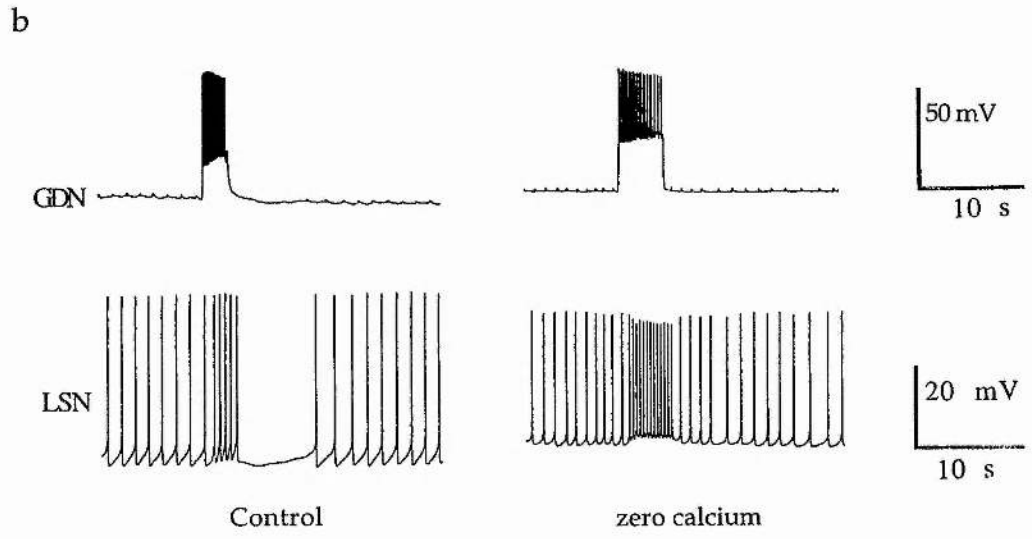
The fast depolarisations elicited by the GDN in follower neurones are not blocked by d-tubocurarine or zero calcium saline.

a. Stimulation of the GDN elicits a wave of depolarisation in its follower neurone (LSN), a fast transient depolarisation is produced by each action potential in the GDN. Bath application of 100μM d-tubocurarine has no effect on this connection.

b. Stimulation of the GDN elicits an increase in the rate of firing in its follower neurone (LSN), followed by a prolonged inhibition. Following perfusion with zero calcium saline, stimulation of the GDN still results in an increase in the rate of firing of the LSN, however the following inhibition is blocked.

The electrical connection in 'b' was reciprocal and each action potential of the LSN produces a very small transient depolarisation in the GDN. These depolarisations were not blocked by zero calcium.


a
GDN
LSN
50 mV
2 s
2 mV
2 s
Control
100µM d-tubocurarine


b
GDN
LSN
50 mV
10 s
20 mV
10 s
Control
zero calcium

changeover from one form of connection to another. Neurone pairs whose junction changed from chemical to electrical did not regain a chemical junction after up to six days in culture.

III.5.4. Polarity of chemical junction formation between GDN and LSN

The chemical junctions which formed between the GDN and the LSN were mostly unidirectional (89% n=42). The direction in which the connection formed was random when the neurones were plated out at the same time. However, *in situ* the synaptic connection between the GDN and LSN has been shown to be reciprocal (Syed et al 1993). The reason for this difference is unknown but is probably related to culture conditions.

III.5.4.1. *The influence of the form of physical contact*

The way in which neurones make contact in culture has been shown to affect the way in which synapses form. Nicholls et. al. 1990 showed that the type of synapse which developed between a pair of leech Retzius cells in culture varied depending on whether contact was made between initial segments, soma/soma or through a combination of both. A reciprocal chemical synapse was found to develop if both initial axon segments made contact, but the synapse was unidirectional if the connection was from the initial axon segment of one neurone onto the soma of another. The synapse was predominantly chemical if the cells made contact via their somas.

The direction in which chemical junctions formed between the GDN and the LSN was not affected by the way in which they made physical contact. Chemical junctions were recorded between the GDN and LSN in pairs where the contact was soma/soma, axon/soma, axon/axon, via neurites or a

combination of the above (Fig. III.14.). The direction in which the chemical junction formed could not be determined by examining the physical contact between the pairs. Of the three reciprocal junctions observed, two were soma/soma and one was axon/soma.

The formation of unidirectional junctions between the GDN and LSN can be summarised as follows:

Soma/soma only	4
Soma/soma with neurites	4
Neurites only	9
Axon/soma only	4
Axon/soma with neurites	4
Axon/axon only	4

These results show that unidirectional synapses could form in any physical configuration. However the absolute numbers are unimportant because they are determined by the number of cell pairs that were plated out in each of the configurations. i.e. unidirectional junctions between neurites only were most common because more cell pairs were recorded in which neurite only contact was made.

Neurone pairs in which only electrical connections formed also did not appear to fall into any particular category of physical contact. Purely electrical connections were seen between soma/soma contact and between axon/soma contact.

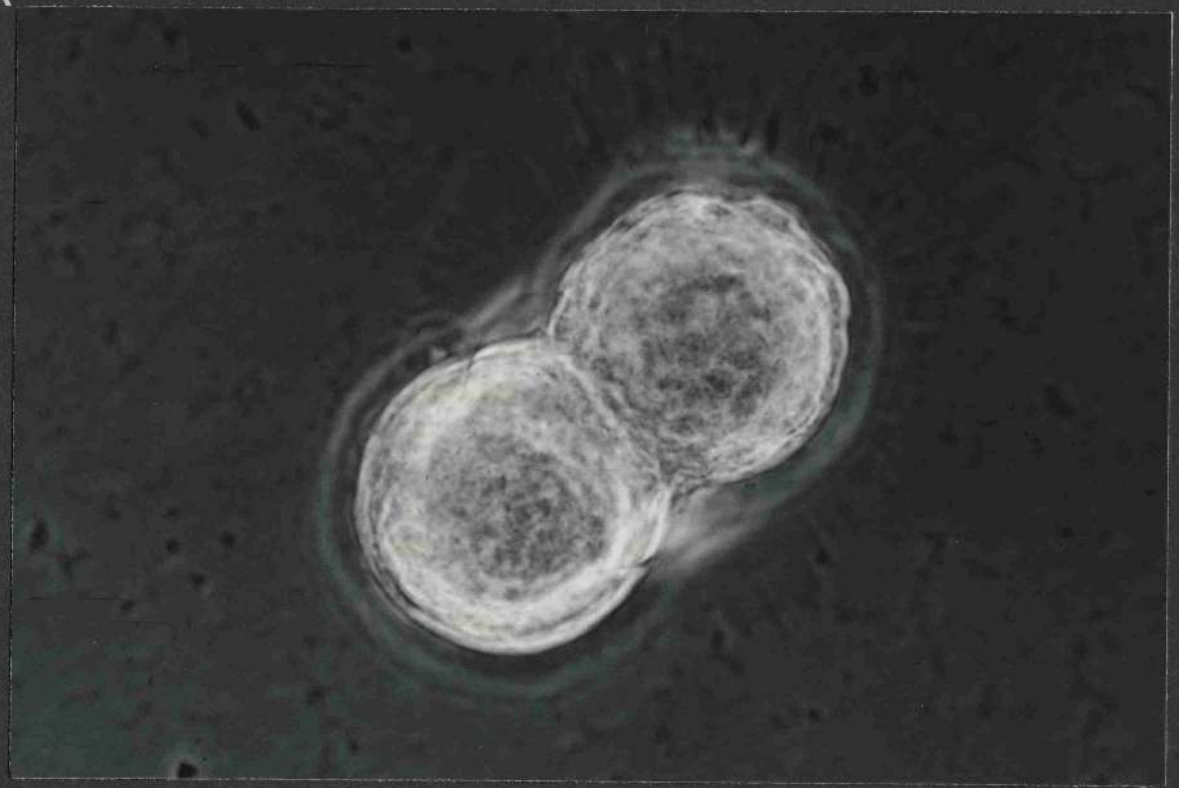
Figure III.14.

Unidirectional chemical junctions formed between the GDN and LSN regardless of their form of physical contact.

A. A GDN and LSN making a soma/soma contact after 16 hours in culture. A unidirectional chemical connection existed from the GDN to the LSN.
Scale bar 50μm

B. A GDN and LSN making contact purely via neurites after 24 hours in culture. A unidirectional chemical junction existed from the LSN (uppermost) to the GDN.
Scale bar 100μm

A



B

III.5.4.2. *Influence of plating the neurones on the same plate but at different times*

There is evidence that the degree of activity of neurite extension affects synapse formation. For example Hadley et.al. 1986 showed that mutual neurite elongation was required for the formation of electrical synapses in *H. trivolvis* neurones. Therefore an attempt was made to try to influence the direction in which the chemical junction formed between the GDN and the LSN by plating the neurones out at different times. Either GDNs or LSNs were plated out and allowed to extend neurites for a period of 24 hours. The putative follower neurone (either the GDN or LSN) was then plated out onto the neuritic arbour of the neurone first isolated. The direction in which any subsequent chemical junction formed was recorded (Table III.2.).

When the neurones were plated out simultaneously, half of the pairs formed a synapse from the GDN to the LSN and half formed a synapse from the LSN to the GDN, indicating that the direction of formation was random. The same result was obtained when the GDN was plated out first. However, when the LSN was plated out first, it became the presynaptic cell in every pair. In only three pairs was a very weak chemical connection from the GDN to the LSN observed.

Table III.2.

Direction of chemical junction formation when the GDN and LSN were plated out on the same plate but at different times

	Polarity of chemical junctions formed		
	GDN to LSN	LSN to GDN	Reciprocal
Cells plated simultaneously (n=10)	3	3	0
GDN plated 24 hours before LSN (n=11)	4	4	0
LSN plated 24 hours before GDN (n=21)	11	0	3

III.5.5. Ultrastructure of the GDN in culture: evidence of classical synapses?

The chemical junctions observed electrophysiologically during the course of this work, differ from those observed *in situ*. Although they bear some similarities, they were usually very weak, slower in onset and only transient in their appearance. It is feasible that this form of transmission could take place at non-specialised areas of contact between the two neurones.

A number of neurone pairs which had formed chemical junctions were processed for electron microscopy in an attempt to find regions of specialised synaptic contact. No contacts resembling chemical synapses were observed, although numerous varicosities with vesicles were found at intervals along the neurites of the GDN (FigIII.15.). These vesicles ranged in diameter from 50-170 nm. They closely resembled the dopamine containing vesicles observed in EM studies of *P. corneus* ganglia (Pentreath & Berry 1975).

Figure III.15

Transmission electron micrograph of a vesicle filled varicosity on a neurite from a cultured GDN. The vesicles vary in diameter from 50 to 170 nm.

Scale bar 500 nm.

CHAPTER IV

RESULTS II: CHARACTERISATION OF THE 'FAST RESPONSE'

IV.1 THE FAST RESPONSE; AN EFFECT OF DOPAMINE ON THE GDN

The Giant Dopamine Neurone, when in the intact preparation has a variety of different post synaptic effects on its many follower neurones. One of these effects is a fast depolarisation, mimicked by iontophoresis of dopamine onto the follower neurones (Berry & Cottrell 1975). In *H. trivolvis* 100μM dopamine applied to the GDN *in situ* produced no discernible effect. However application of dopamine to the GDN in culture elicited a biphasic response. The first phase of the response was a fast depolarisation, when recorded at a membrane potential of about -60mV. This rapid response evoked by dopamine was chosen for more detailed study; it resembled the fast dopamine response observed in follower cells (Berry & Cottrell 1975).

IV.1.1. Time course of the Fast Response

Dopamine applied by pressure to the GDN in culture evoked a rapid onset Fast Response. The delay in onset was typically less than 50ms from the start of the pressure pulse when the tip of the application pipette was positioned 15-20μm from the cell membrane (Fig. IV.1.). The time taken from the start of the response until it reached half its maximum amplitude varied from 40 to 140ms. This large variation in onset was studied in more detail and described in a later section. The time taken for the response to decay could not be measured because of the onset of the slow inhibitory phase of the biphasic response.

Figure IV.1.

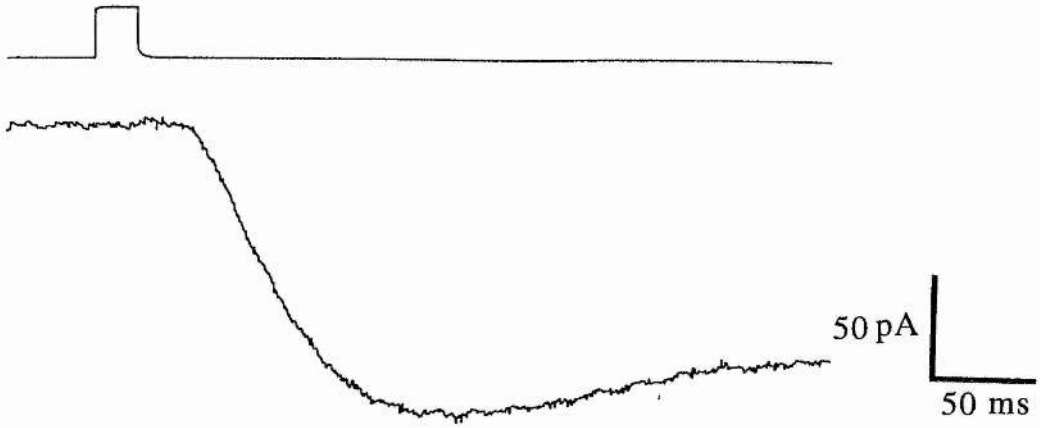
Properties of the dopamine Fast Response

a. Whole cell patch clamp recording showing the time course of the Fast Response evoked by application of dopamine to a GDN in culture. The square pulse in the upper trace indicates a 20ms pressure application of 100μM dopamine. The lower trace shows the response. Onset of the response occurred 45ms after the start of the pulse. Half maximum response was reached 40 ms after the start of the response. Application protocol was 100μM dopamine, 20ms, 20psi.

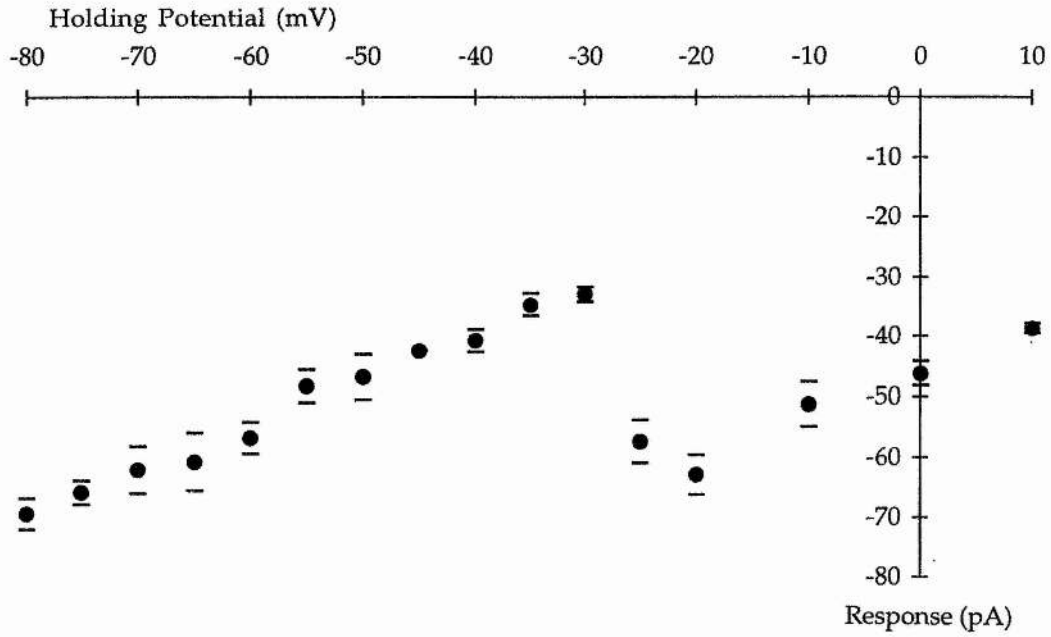
b. Current voltage relationship of the Fast Response, using whole cell patch clamp. Between -80 and -30mV the relationship is linear with an extrapolated reversal of the response close to 0mV. The size of the response increases sharply between -30 and -25mV, then decreases again with more positive holding potentials. Three responses were measured at each potential, the plot shows mean and standard deviation.

c. Mean current voltage relationship for the Fast Response recorded from 9 GDNs in culture using two electrode voltage clamp. The plot shows mean and standard deviation. The error is large because of variation in the size of the response between individual neurones and the decreased signal to noise ratio using this recording technique. However as in part 'b' the response shows an increase in size at about -30mV.

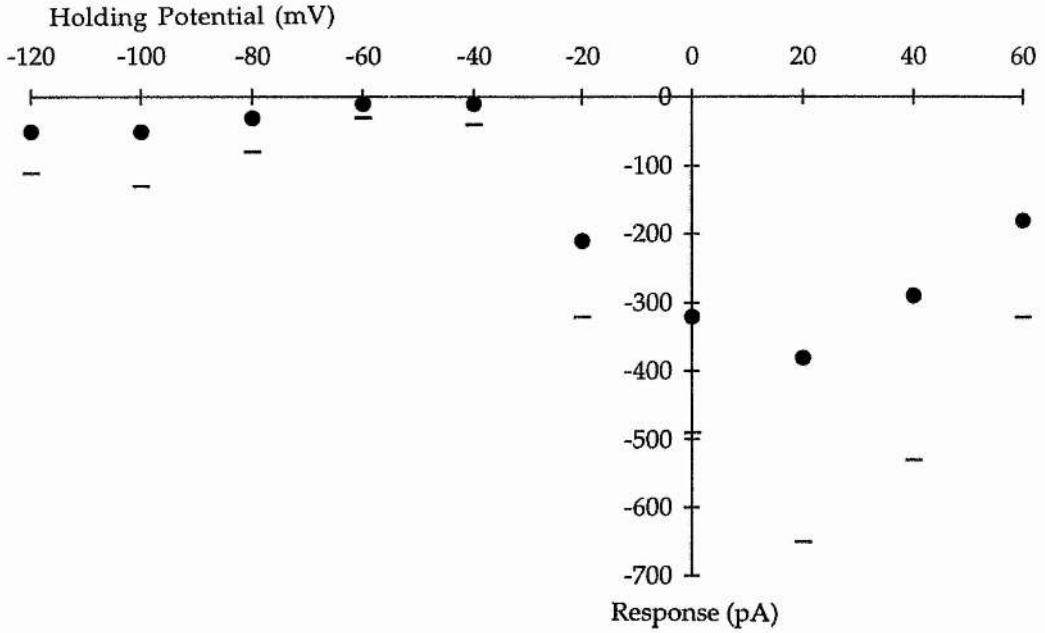
a


50 pA
50 ms

b


Holding Potential (mV)
-80
-70
-60
-50
-40
-30
-20
-10
0
10
0
-10
-20
-30
-40
-50
-60
-70
-80
Response (pA)

c


Holding Potential (mV)
-120
-100
-80
-60
-40
-20
0
20
40
60
0
-100
-200
-300
-400
-500
-600
-700
Response (pA)

IV.1.2. Size and voltage dependence of the Fast Response

The response of the GDN to dopamine was very small. Pressure application of 100μM dopamine at a membrane potential of -60 mV elicited a Fast Response with a size of 3-4 mV when recorded in current clamp, and 50-60 pA when recorded using voltage clamp. This indicates that the GDN in culture has an input resistance of about 50-80 MΩ at a membrane potential of -60 mV. This is similar to an input resistance determined from the I/V curve in figure III.4.

Determination of the voltage dependence of the Fast Response produced some interesting results. Using the continuous single electrode voltage clamp technique, the Fast Response appears in most GDNs to have a linear current voltage relationship with an extrapolated reversal of around 0mV. Using this technique GDNs can only usually be clamped at membrane potentials more negative than -30 mV. However with some favourable preparations it was possible to make recordings at potentials more positive than -30 mV. It was noted that at these more positive potentials the Fast Response abruptly increased in size before diminishing again as more positive potentials were reached (n=3) (Fig. IV.1.).

Further experiments were performed using the double electrode voltage clamp technique. This has the advantage that the clamp is more efficient and the holding potential is more accurately controlled. The disadvantage is that the signal to noise ratio is significantly reduced making recordings of such small responses particularly difficult. Using this technique GDNs could be routinely voltage clamped at potentials more positive than -30mV. These experiments also showed an increase in the size of the Fast Response at positive potentials (Fig. IV.1.) adding further evidence that the I/V curve of

the Fast Response becomes non-linear at potentials more positive than -30 mV (n=9).

The increase in the size of the Fast Response at more positive potentials could come about for two reasons. (1) The response seen at more negative potentials may show some form of voltage dependent block or gating similar for example to the NMDA receptor (Nowak, Bregestovsky & Ascher 1984). (2) There could be two separate responses one of which predominates at more negative potentials, and another which is voltage dependent, only being activated at more positive potentials. The two response theory is supported by an experiment shown in figure IV.2. in which the time course of the response at -70mV and -20mV is compared. The latency of the response is the same at both holding potentials, but at -25mV the rate of rise of the response is faster and the overall duration of the response increased. At -25mV it appears that there are two inward components to the response.

IV.1.3. Is the Fast Response an artefact of pressure ejection?

At first it was thought possible that the Fast Response was not an effect due to dopamine, but was an artefact caused by the method of application. For example the pressure pulse may have caused mechanical disturbance of the cell membrane resulting in the activation of mechanosensitive ion channels.

100 μM dopamine in normal saline was pressure applied to GDNs and a typical Fast Response was recorded. The application pipette was then replaced by one containing only normal saline. The same application protocol was followed, but no response was observed (Fig. IV.3.). In a second series of experiments dopamine was applied to GDNs using iontophoresis rather than pressure (Fig. IV.3.). The Fast Response was also observed using this

Figure IV.2.

Comparison Fast Response, recorded at holding potentials of -70 and -25mV using whole cell patch clamp.

a. The square pulse indicates the time course of the dopamine pressure application pulse. 50μM, 100ms, 20psi.

b. Timecourse of the Fast Response recorded at a holding potential of -70mV. Onset of the response occurs at approximately 70ms and peaks 240 ms after the start of the pulse. Time from onset to half maximum response is approximately 75ms.

c. Timecourse of the Fast Response recorded at a holding potential of -25mV. Onset of the response occurs at approximately 70ms, similar to the response recorded at -70 mV. However the rate of rise is faster, with time to 1/2 max. of 60 ms, and the response peaks later at 410ms.

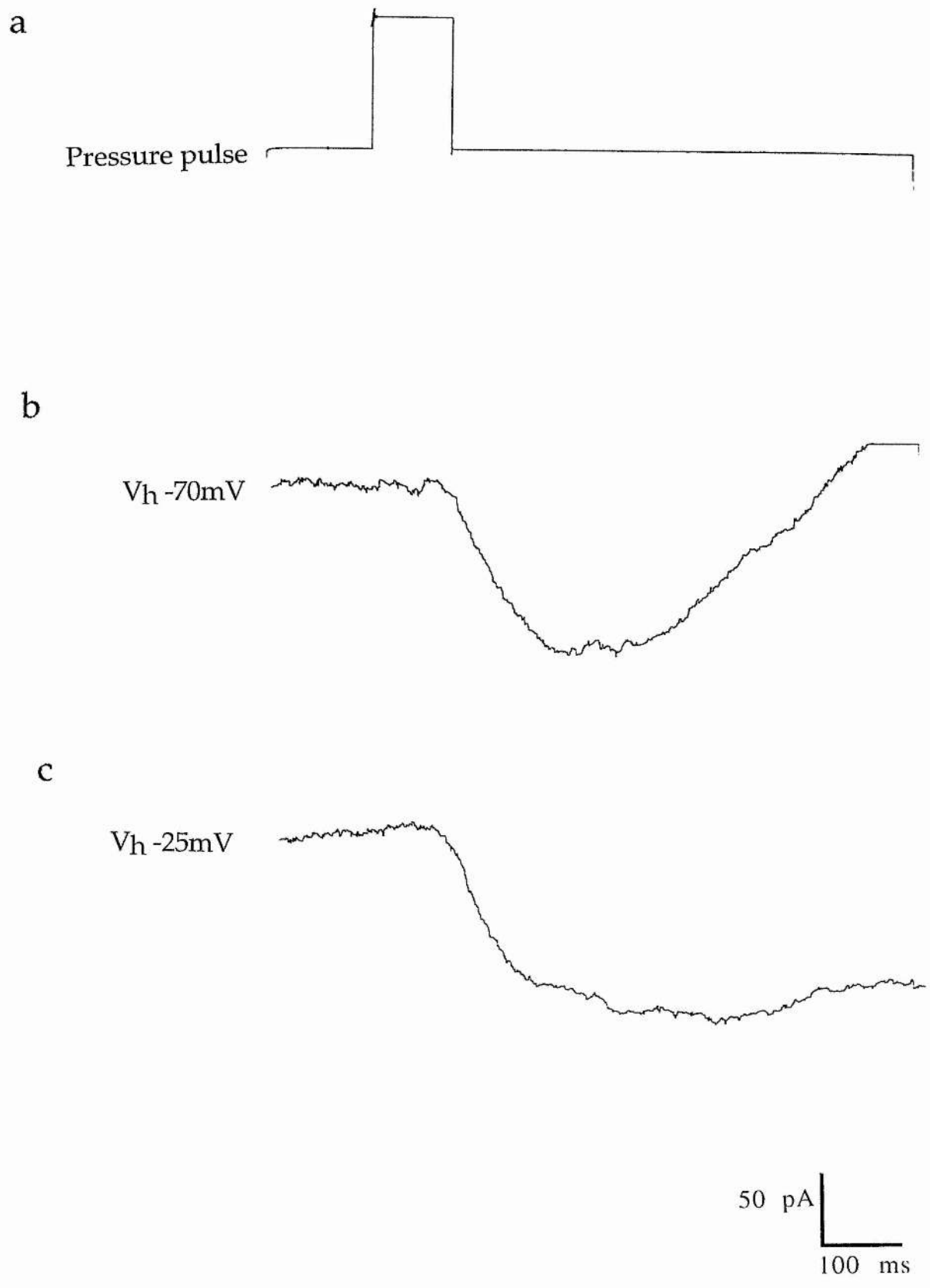

a
Pressure pulse
b
Vh -70mV
c
Vh -25mV
50 pA
100 ms

Figure IV.3.

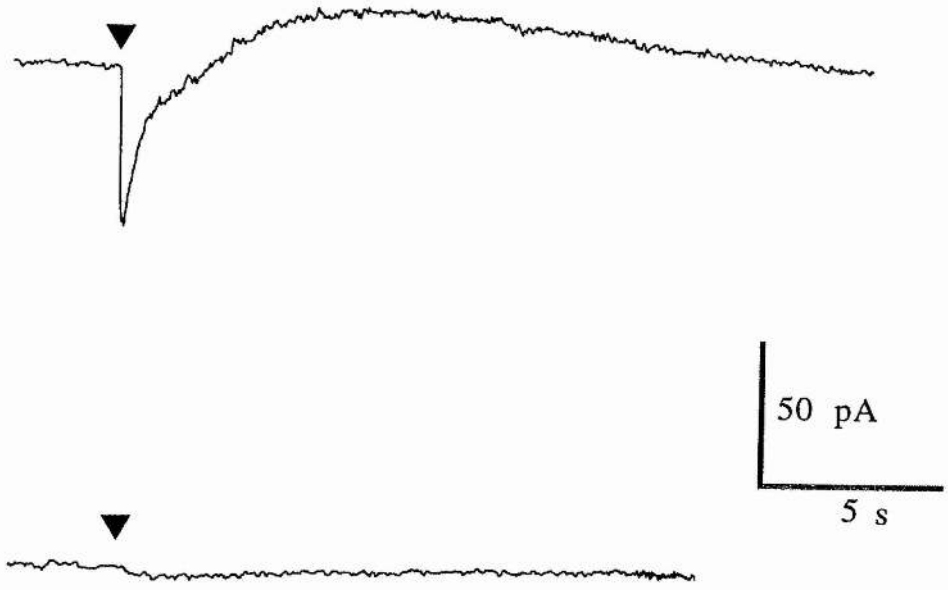
Confirmation that the Fast Response is not an artefact of pressure application.

a. Whole cell voltage clamp recording. The upper trace shows the typical effect of 100μM dopamine dissolved in normal saline applied by pressure to a GDN in culture using a 20ms, 20psi pulse. The trace below shows the application of normal saline, following the same application protocol. It is clear that the pressure pulse has little effect on the GDN current trace. Holding potential of -65mV.

b. Current clamp recording. Application of dopamine to a GDN in culture by iontophoresis. The recording was made using single electrode current clamp. Application evokes a brief depolarisation, followed by a slow hyperpolarisation. The application pipette contained 10mM dopamine in distilled water. The application was 400nA for 500ms.

Black triangles indicate point of application.

a



b

technique. These results confirm that the Fast Response is an effect of dopamine and not one of the method of application.

IV.1.4. Desensitisation of the Fast Response

A feature of the Fast Response is that it is markedly desensitised after a single application of dopamine. This caused some practical difficulties during recording, because even a small leak of dopamine from the tip of the application pipette caused profound desensitisation of the response. Leakage of dopamine from pressure application pipettes was minimised in two ways. Firstly, application pipettes were only filled with a small volume of dopamine containing saline, minimising the height of the column of liquid in the pipette. Secondly a suitable tip diameter through which dopamine could be reliably ejected, but would not leak, was determined by trial and error. Pressure application pipettes were then manufactured to this specification.

The first application of dopamine elicited a Fast Response. The size of the response elicited by subsequent applications was determined by the dose of dopamine applied, and the interval between applications. In a typical experiment, in which dopamine was applied at 50μM with a 100ms pressure pulse, complete desensitisation of the Fast Response was observed with an interval of 10 seconds between applications (Fig. IV.4.). With an interval of 20 seconds, desensitisation was reduced to 40%. No desensitisation was observed when the interval between applications was increased to 45 seconds.

IV.1.5 Variation in the rate of rise of the Fast Response

Casual observation of the Fast Response showed significant variation in the rate of rise of the response. This led to the view that there may be more than

Figure IV.4.

Desensitisation of the Fast Response.

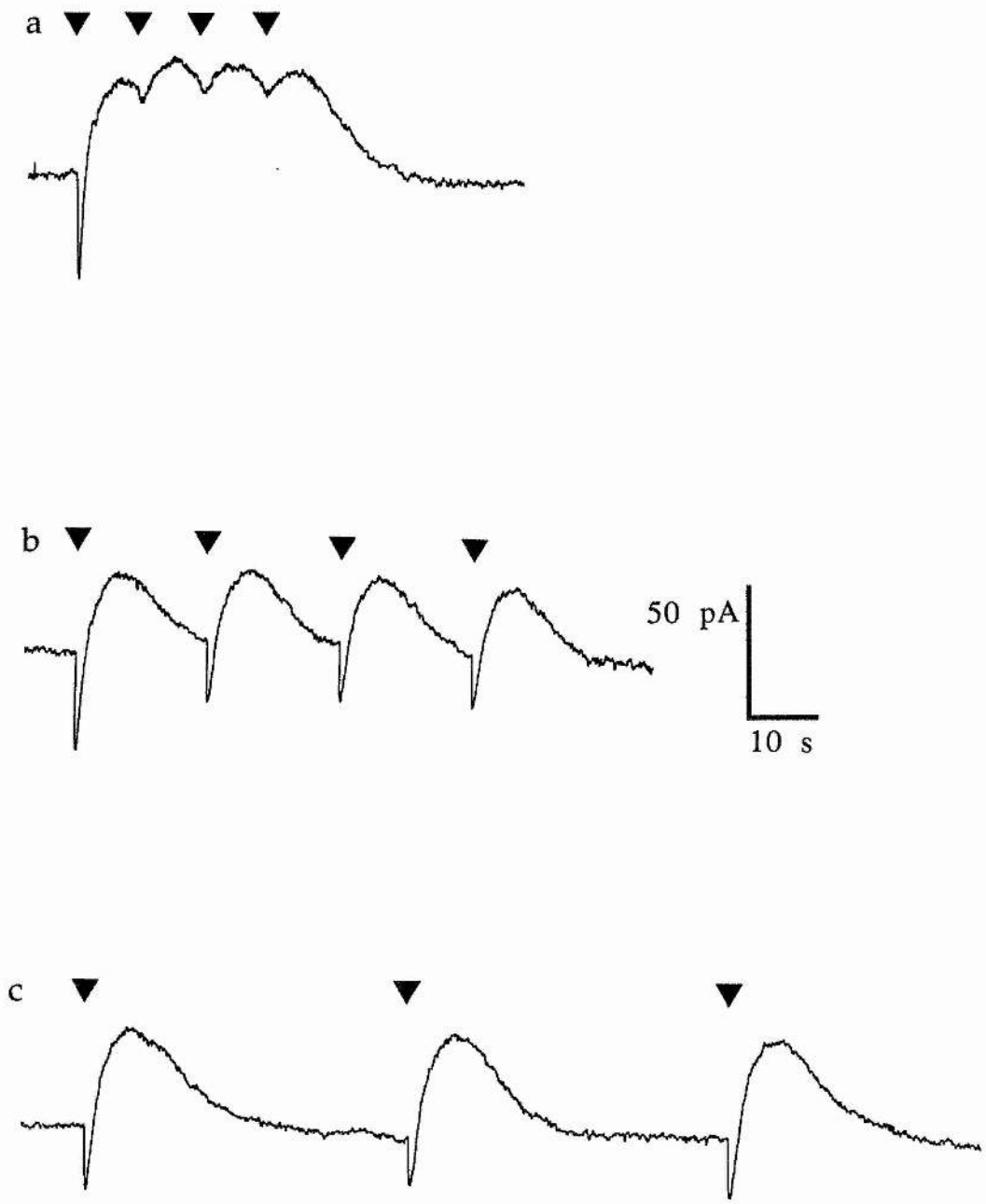
a. Applications of dopamine at 10 second intervals result in complete desensitisation of the Fast Response after the first application.

b. Applications of dopamine at 20 second intervals allows some recovery of the Fast Response after each application.

c. Applications of dopamine at 45 second intervals shows almost complete recovery of the Fast Response between applications.

In each case 50μM dopamine was pressure applied with a 50ms, 20psi pulse. All experiments were performed on the same GDN in culture which was constantly perfused with normal saline.

Recordings were whole cell patch clamp, holding potential -65mV.


a
b
50 pA
10 s
c

one current involved in the response, one of which had a faster rate of rise than the other. A frequency distribution curve showing the rate of rise of the Fast Response in different GDNs is plotted in (Fig. IV.5.). The frequency distribution curve confirms the wide variation in the rate of rise of the Fast Response in different neurones. The data do not provide clear evidence for two responses with different rates of rise, but this does not rule out the possibility that there are two currents, the reasons for which will be discussed.

Further evidence for the presence of two components to the Fast Response came from GDNs in which the Fast Response had two phases. This was most clearly seen in the neurone described in figure IV.6. Application of dopamine elicited a fast inward current which peaked and then started to decay, a further increase and peak in the inward current was then observed before the slow outward current predominated.

The rate of rise of the Fast Response varied depending on the part of the neurone to which dopamine was applied (Fig IV. 7.a.). It is possible that this effect was produced in one of two ways: (1) There may be different receptors present on different parts of the neurone. (2) Diffusion of dopamine over large areas of the membrane may cause an apparent slowing of the response. Close examination of the responses suggests that diffusion is the more likely cause. This will be discussed.

Figure IV.5.

Frequency distribution curves of the rate of rise of the FMRF amide and dopamine fast inward currents in the GDN in culture.

The curve for dopamine shows a large variation in the time for the response in different neurones to reach 1/2 maximum amplitude. Most responses fall into the 40-49ms bin, however the relatively small sample number and the broad spread of response times do not appear to show the single peak that would be expected from a standard distribution.

The curve for FMRFamide shows less variation in the time to 1/2 maximum response. Most responses fall into the 60-69ms bin, and this appears to be a distinct peak in the distribution.

Frequency distribution of the rate of rise of the fast inward current to dopamine and FMRFamide in the GDN

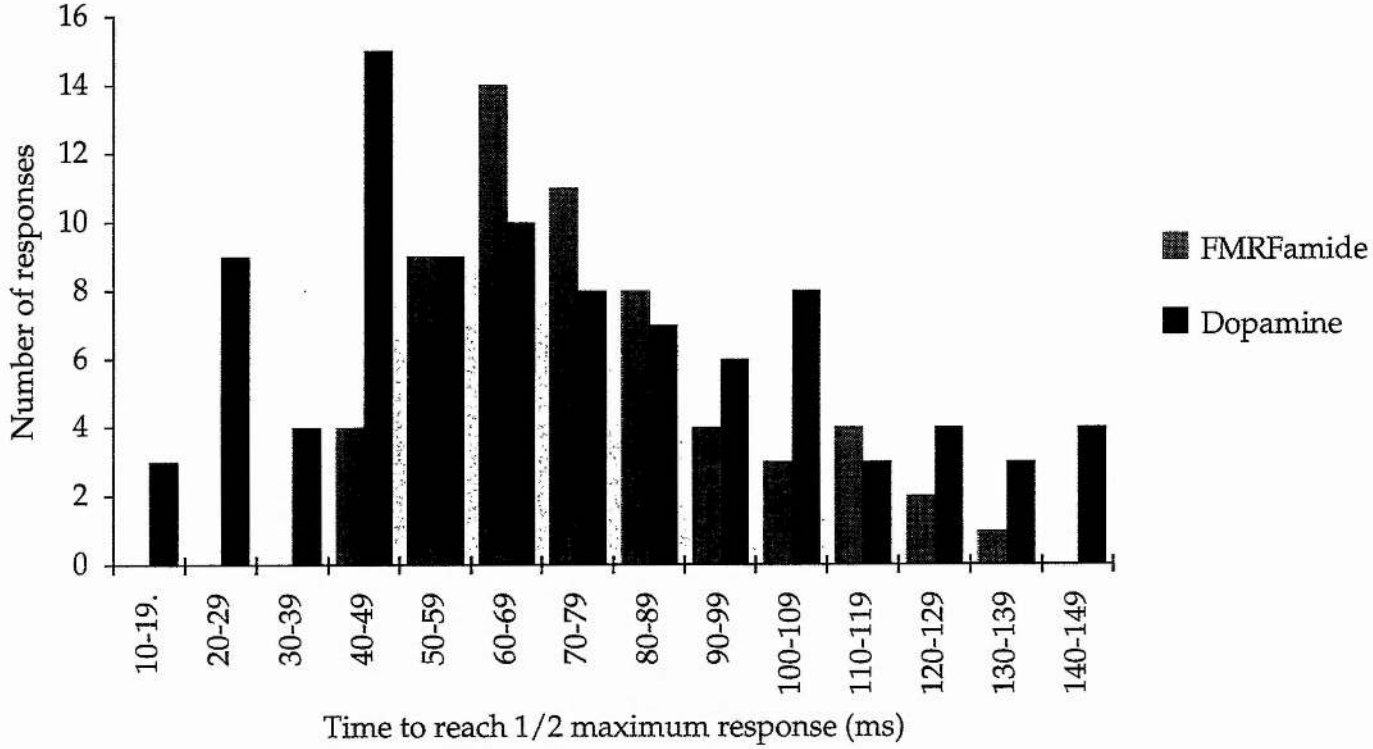


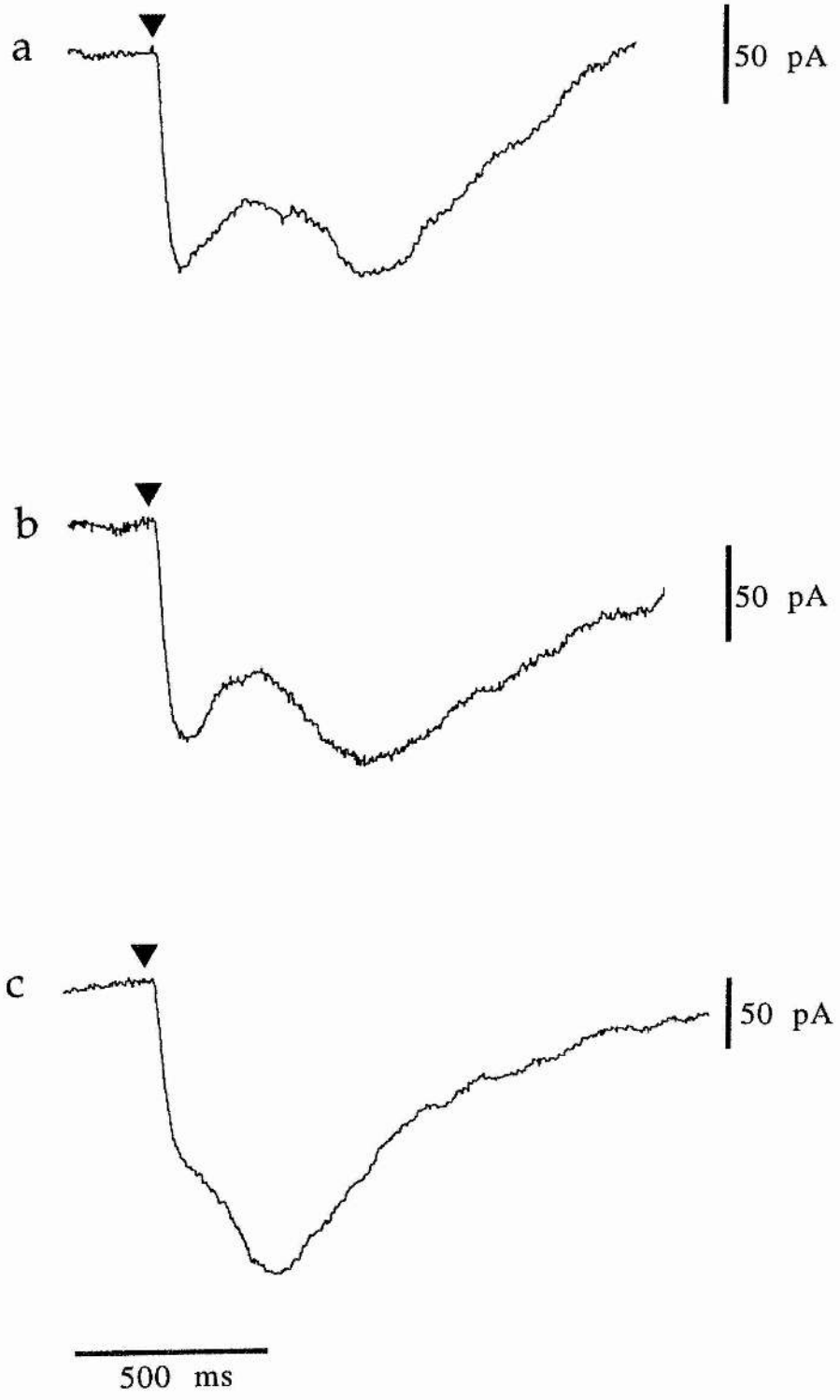
Figure IV.6.

Two components of the Fast Response.

a. At a holding potential of -70 mV application of dopamine elicited a fast inward current which started to decay. Before the onset of the slow outward current, a second, slower inward current was observed. The response was very similar when observed at -80 mV (b). Application 100μM dopamine, 20ms, 20psi.

c. Another application of dopamine at a holding potential of -80mV. However the duration of the application pulse is increased from 20 to 60ms. The increase in pulse duration had little effect on the size of the first inward component but the second inward component was increased in amplitude, it also peaked earlier.

Recordings were made using whole cell patch clamp. Triangles indicate applications.


a
50 pA
b
50 pA
c
50 pA
500 ms

IV.1.6 Differential distribution of receptors over the surface of the GDN

With practice, it became possible to routinely dissect the GDN from the pedal ganglion and plate it out in culture with up to 400μM of axon intact. Focal pressure application of dopamine to different points on the surface of the axon and soma of the GDN, provided a crude indication of the distribution of receptors within the membrane.

The general trend observed was that the total response to dopamine, Fast Response and slow outward response, were much smaller when dopamine was applied to the soma compared to the axon or axon hillock (n=6) (Fig. IV.7.). The ratio of the size of the Fast Response:slow outward response varied at different points of application. For the neurone shown in figure IV.7. the trend was for the Fast Response to make up a larger proportion of the total response at the axon tip compared with the soma. On the soma the Fast Response made up 26% of the total, whereas at the axon tip the Fast Response made up 60% of the total.

These results indicate that receptors for dopamine on the GDN are localised predominantly on the axon and axon-hillock (c.f. Ascher 1972). This may explain why there is no response to dopamine observed *in situ*, where only the soma is exposed. The axon and axon-hillock are buried in the neuropile and the receptors thus masked from the dopamine.

Figure IV.7.

Variation in the amplitude of the dopamine response with different points of application.

The photograph shows the GDN from which the recordings were made. The soma is at the top of the photograph, and extending from it is a section of axon which was dissected out intact. Above, is an outline drawing of the neurone, with arrows indicating the different points of application of dopamine. Alongside the arrows are the responses to dopamine observed at the respective points.

On the soma, the Fast Response and to a certain extent the slow inward current were relatively small in amplitude. Both responses showed an increase in amplitude with applications to the axon and the axon hillock.

Scale bar 100pA 10s
Soma is 65μm in diameter.

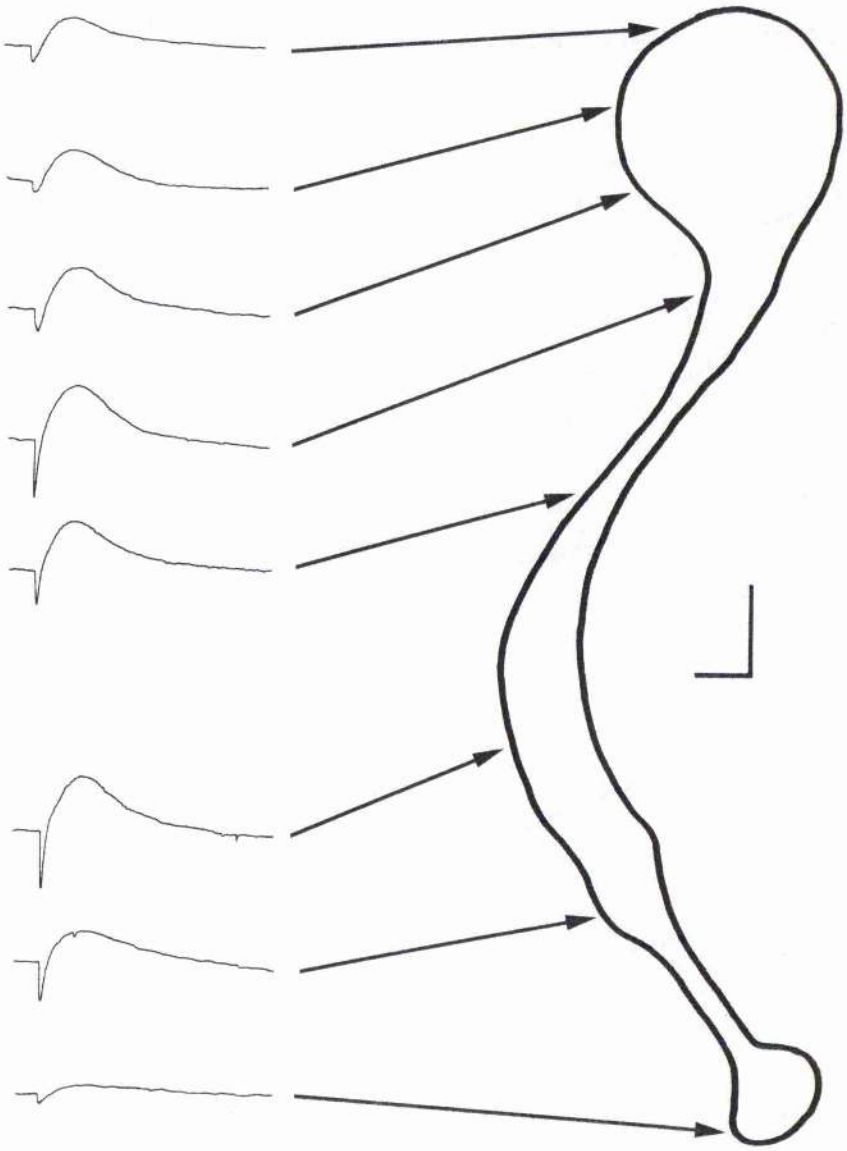
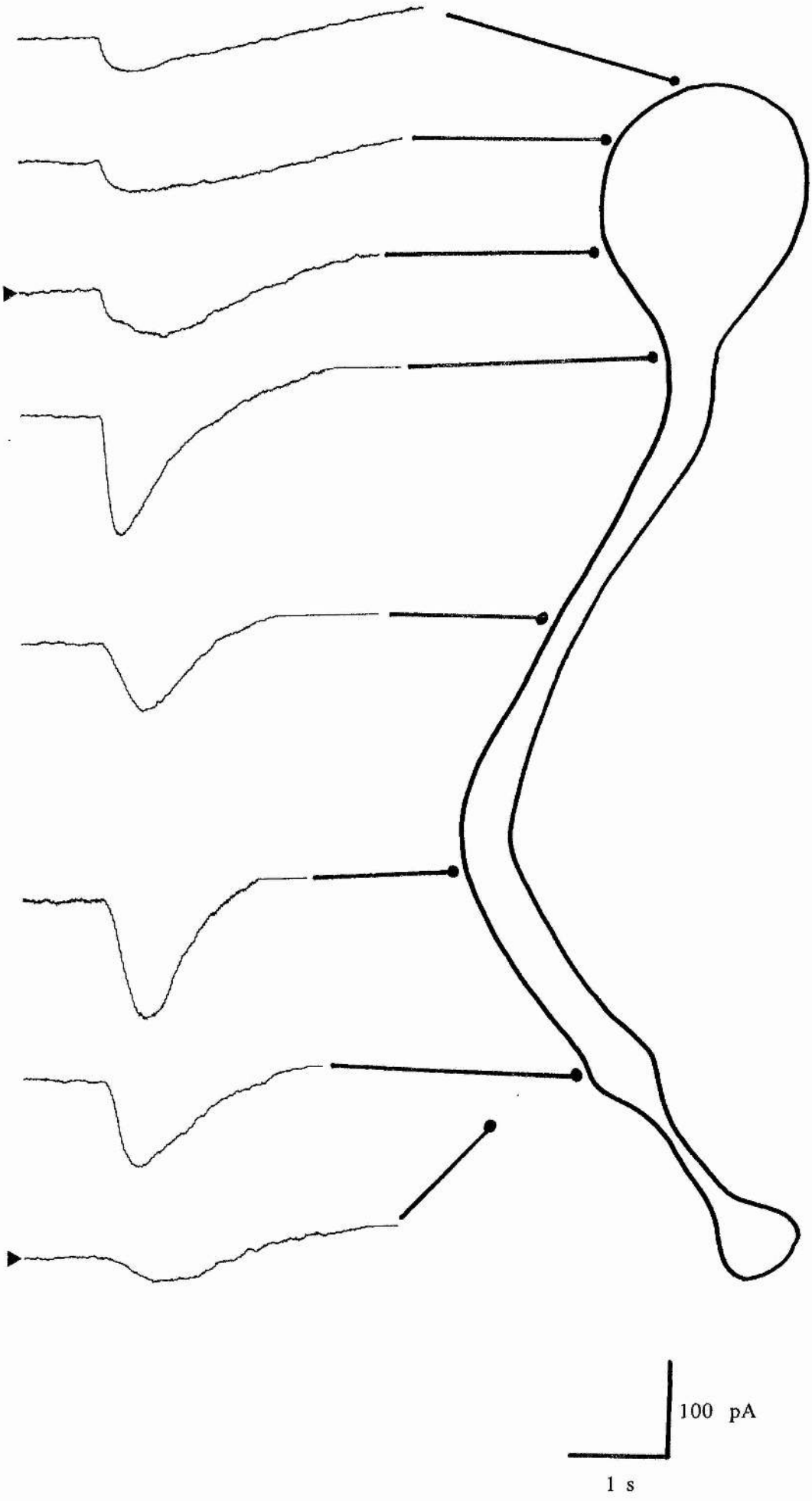


Figure IV. 7.a.

Variation in the rate of rise of the Fast Response with different points of application.

The results are from the same neurone as Figure IV.7.The Fast Response evoked by dopamine application to different parts of the neurone are shown using a fast time-base. The upper response marked by a triangle is bi-phasic. and the lower response marked by a triangle has a slow rate of rise. Both of these effects are thought to be due to diffusion of the applied dopamine.


100 pA
1 s

IV.2. EVIDENCE THAT THE FAST RESPONSE IS DIRECTLY LIGAND GATED

The onset and rate of rise of the Fast Response is comparable to that of the nicotinic type action of acetylcholine on molluscan neurones (Kehoe 1972) and to the direct gating of an ion channel by the peptide FMRFamide in the snail *Helix aspersa*, described by Cottrell et al. (1990). This suggests that the Fast Response may also be the result of direct ligand activation of an ion channel.

IV.2.1. Effect of inactivating G-proteins

So far all dopamine receptors which have been described exert their actions through a family of GTP binding proteins (Gingrich & Caron 1993; Grandy & Civelli 1992). The possibility that the Fast response was not G-protein coupled was explored. The G-protein analogue GDP-ß-S was introduced at a concentration of 1mM into the recording solution of patch pipettes. As a result, during recordings made in the whole cell patch configuration GDP-ß-S diffused into the intracellular space of the GDN.

Recordings made for up to 35 minutes in this configuration did affect the amplitude of the Fast Response (n=5), but the slow phase of the response was completely abolished during this time (Fig. IV.8.). Abolition of the slow component of the dopamine response acted as a convenient control, showing that sufficient GDP-ß-S had entered the neurone to produce an effect.

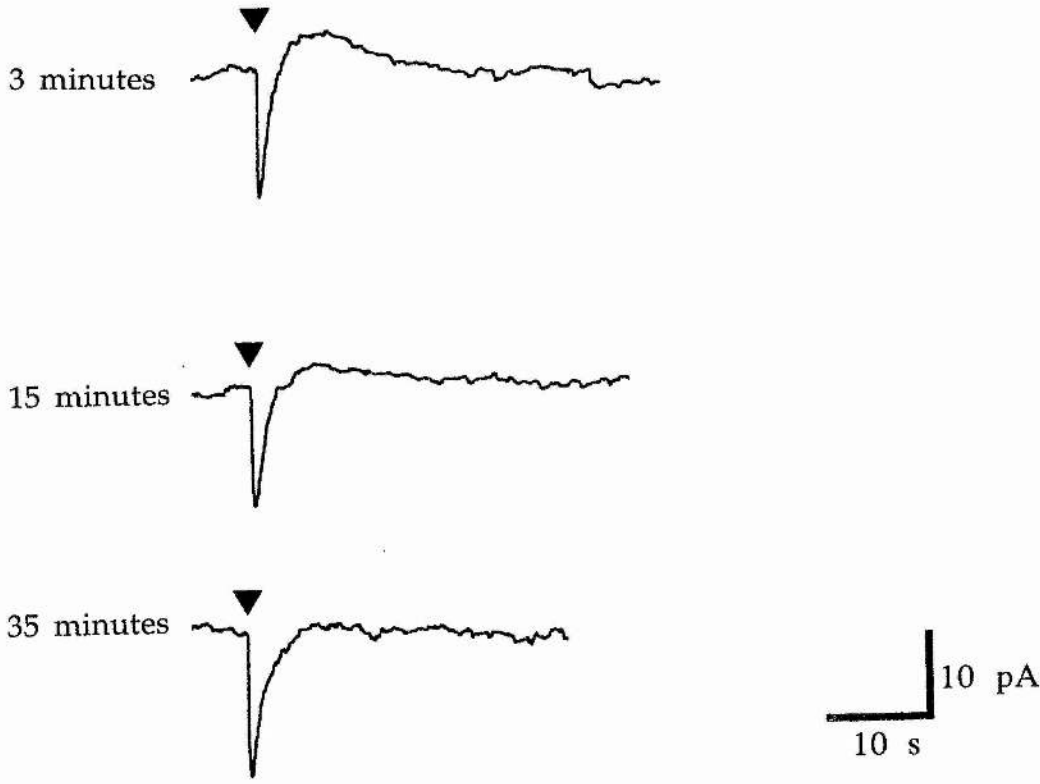
Figure IV.8.

GDP-ß-S did not block the Fast Response

Whole cell patch recording from a GDN in culture in which the recording pipette contained 1mM GDP-ß-S. Three minutes after the start of recording, dopamine evoked a Fast Response followed by a slow outward current. As recording continued the slow outward response diminished, until it was completely abolished at 35 minutes. The Fast Response was not affected.

100µM dopamine pressure application 20ms, 20psi.

Scale bar 10pA 10s


3 minutes
15 minutes
35 minutes
10 pA
10 s

IV.2.2. Unitary current recordings from outside-out patches

Outside-out patches were prepared from cultured GDNs. Three different pipette (intracellular) solutions were used:

High sodium (mM); NaCl 54, KCl 3, $MgCl_2$ 1, EGTA 5, HEPES 10, pH7.3 with NaOH.

High potassium (mM); NaCl 2, KCl 54, $MgCl_2$ 1, EGTA 5, HEPES 10, pH 7.3 with KOH.

High caesium (mM); NaCl 2, CsCl 54, $MgCl_2$ 1, EGTA 5, HEPES 10, pH 7.3 with NaOH.

The bath solution contained (mM) NaCl 52.9, KCl 0.1, $CaCl_2$ 4.1, $MgCl_2$ 1.5, HEPES 10, pH 7.3 with NaOH. This was similar to normal saline, but had a lowered potassium concentration to reduce interference from spontaneously active potassium channels. The high caesium pipette solution was also used in an attempt to reduce interference from potassium. None of these solutions contained GTP.

Patches were taken from both the soma and the axon of the GDN in culture. Responses to dopamine were not observed in patches taken from the soma ($n>60$). However in approximately 2% of all stable patches taken from the axon region ($n>250$), small rapidly activating, multi-channel inward current responses were evoked by dopamine (Fig. IV.9.). As a control normal saline applied to the responsive patch shown in figure IV.9., had no effect. The low probability of finding a responsive patch and the small size of the channels have made analysis of the responses extremely difficult. It was noted however, that in all responsive patches, dopamine elicited a multi-channel response. These results further indicate that the receptors for dopamine are located on the axon, but not on the soma of the GDN. The low chance of

Figure IV.9.

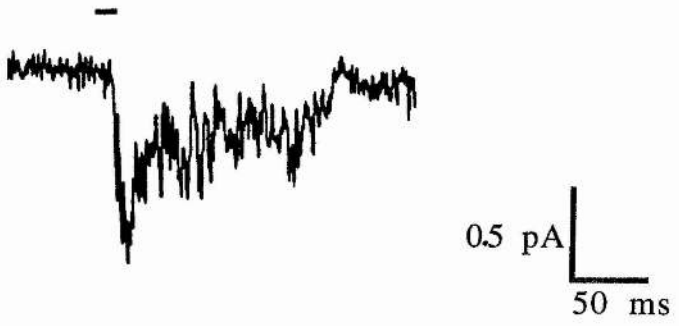
Records taken from outside-out patch recordings.

a. Small unitary inward currents evoked by dopamine. Recorded in an outside-out patch taken from the axon of an isolated GDN. Holding potential -60 mV

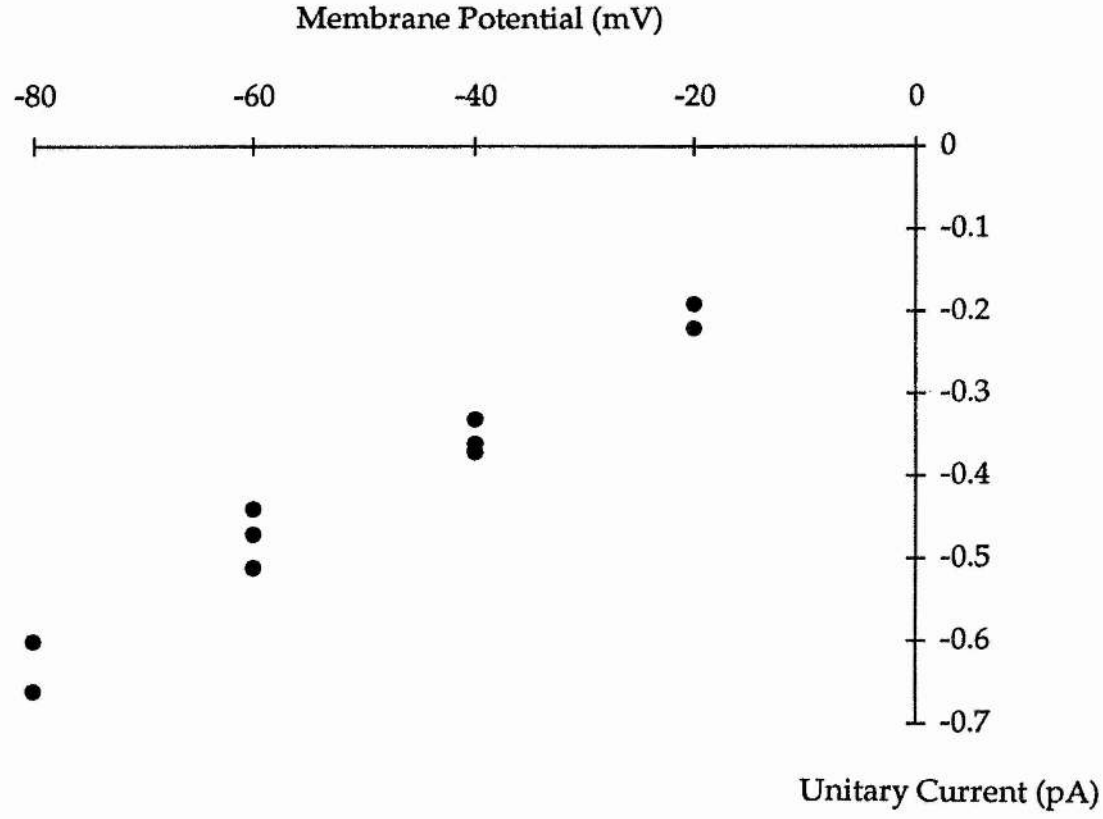
The bar represents dopamine application, 50 μM. 20 psi. 10 ms.

b. Current voltage relationship for unitary currents evoked by dopamine. Recorded in outside-out patches taken from *H. trivolvis* parietal neurones in the intact preparation.

a


0.5 pA
50 ms

b


Membrane Potential (mV)
-80
-60
-40
-20
0
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
Unitary Current (pA)

finding a responsive patch and their multi-channel nature suggest a clustering of the channels.

In this laboratory, Dr. K. Green has performed experiments on neurones from the left parietal ganglion of *H. trivolvis in situ*. Parietal neurones were used in an attempt to find neurones in which dopamine evoked a Fast Response, but the receptors were more numerous. In some of these neurones dopamine elicited a whole cell, fast inward current which was similar to that seen in the GDN. Dopamine activated small multi-channel inward currents in outside-out patches taken from the soma of these neurones. The success rate and stability of patches made in this way are improved and a crude unitary current/voltage relationship plot has been constructed (Fig. IV.9.). This suggests a channel conductance of about 2pS and an extrapolated reversal close to 0mV.

It is assumed that the unitary current responses observed in the parietal neurones are the same as those seen on the GDN. If this is the case, then the shape of the single channel current/voltage curve from the parietal neurones can be compared to the whole cell current/voltage curve from the GDN. The single channel relationship bears a close resemblance to that of the whole cell for holding potentials more negative than -30mV, both have an extrapolated reversal close to 0 mV. However in the unitary current curve there is no sign of the sudden increase in current which is observed in the whole cell recording at more positive potentials.

IV.3. PHARMACOLOGY OF THE FAST RESPONSE

IV.3.1. Inhibitors of the Fast Response

A selection of antagonists and channel blocking drugs have been applied to the GDN in culture to start to determine the pharmacology of the response to dopamine.

IV.3.1.1. ±Sulpiride, a selective D2 receptor antagonist was bath applied to the GDN in culture at concentrations of up to 20μM. At concentrations of 10 and 20μM sulpiride had no effect on the size of the Fast Response. However, it produced a 65% block of the slow outward current (Fig. IV.10.) (n=6). The effect of sulpiride was readily reversible with washing.

IV.3.1.2. Chlorpromazine, a phenothiazine and D2 antagonist which also has many other actions (e.g. Ogata, Yoshii & Narahashi 1990), was bath applied to GDNs at concentrations from 5 to 50μM (n=4). At all concentrations chlorpromazine reduced the size of both the Fast Response and the slow outward current. At 5μM there was a 50 % block of the Fast Response and a 40 % block of the slow inward current (Fig. IV.10.). Preferential block of a slow component of the outward current was observed. A further effect was to induce a sustained underlying inward current throughout the period of application. The effects were reversible with washing.

IV.3.1.3. Apomorphine, a partial agonist at peripheral D1 receptors and agonist at central D2 receptors, was bath applied to the GDN at concentrations from 10-50 μM (n=5). At 10μM there was a complete and irreversible block of the slow outward current. The Fast Response was blocked by 40 %. At this concentration blockade was reversible with washing (Fig. IV.10.).

The block by apomorphine may have revealed the presence of more than one component of the Fast Response. In the presence of apomorphine the Fast Response had a smaller amplitude and decayed relatively quickly within 2 s. The decay was easily observed because the slow outward current was blocked. After washing off the apomorphine, the Fast Response returned to its original amplitude but the slow outward current did not recover. The decay of the Fast Response was slower after washing than in the presence of apomorphine. There appeared to be a slower component of the Fast Response which was blocked by apomorphine and was usually masked by the onset of the slow outward current.

IV.3.1.4. Strychnine, a competitive antagonist of glycine receptors was bath applied to the GDN at a concentration of 100μM. Its effect was to produce an almost complete block of the Fast Response whilst having little effect on the slow outward component (n=3). The effect was reversible (Fig. IV.11).

IV.3.1.5. Amiloride is a diuretic which exerts its effect by blocking epithelial sodium channels and which has been shown to block certain monovalent cation and calcium channels in neurones (Garty & Benos 1988; Sanchez-Armass, Merz, & Drapeau 1991; Tang, Presser & Morad 1988). Amiloride was bath applied at concentrations from 50 to 100μM (n=4). At 50μM it produced a

Figure IV.10.

Whole cell patch clamp recordings from GDNs in culture showing the effect of antagonists of the dopamine response. Antagonists were bath applied in normal saline.

a. 10μM ±sulpiride had little effect on the Fast Response, but produced a 65% reduction in the amplitude of the slow outward current. The effect was reversed with washing. Dopamine 50μM, 20ms, 20psi.

b. 5μM Chlorpromazine produced a partial block of both the inward and outward components of the response and also produced an underlying inward current. The shape of the outward current component was altered, possibly due to it consisting of two components, the slower of which was preferentially blocked. The effect was reversed on washing. Dopamine 50μM, 20ms, 20psi.

c. 10μM apomorphine completely and irreversibly abolished the outward component of the response, and also evoked an underlying inward current. There was a 40% reduction in the amplitude of the Fast Response. On washing the amplitude of the Fast Response recovered and the rate of decay of the response decreased. Dopamine 20μM, 50ms, 20psi.

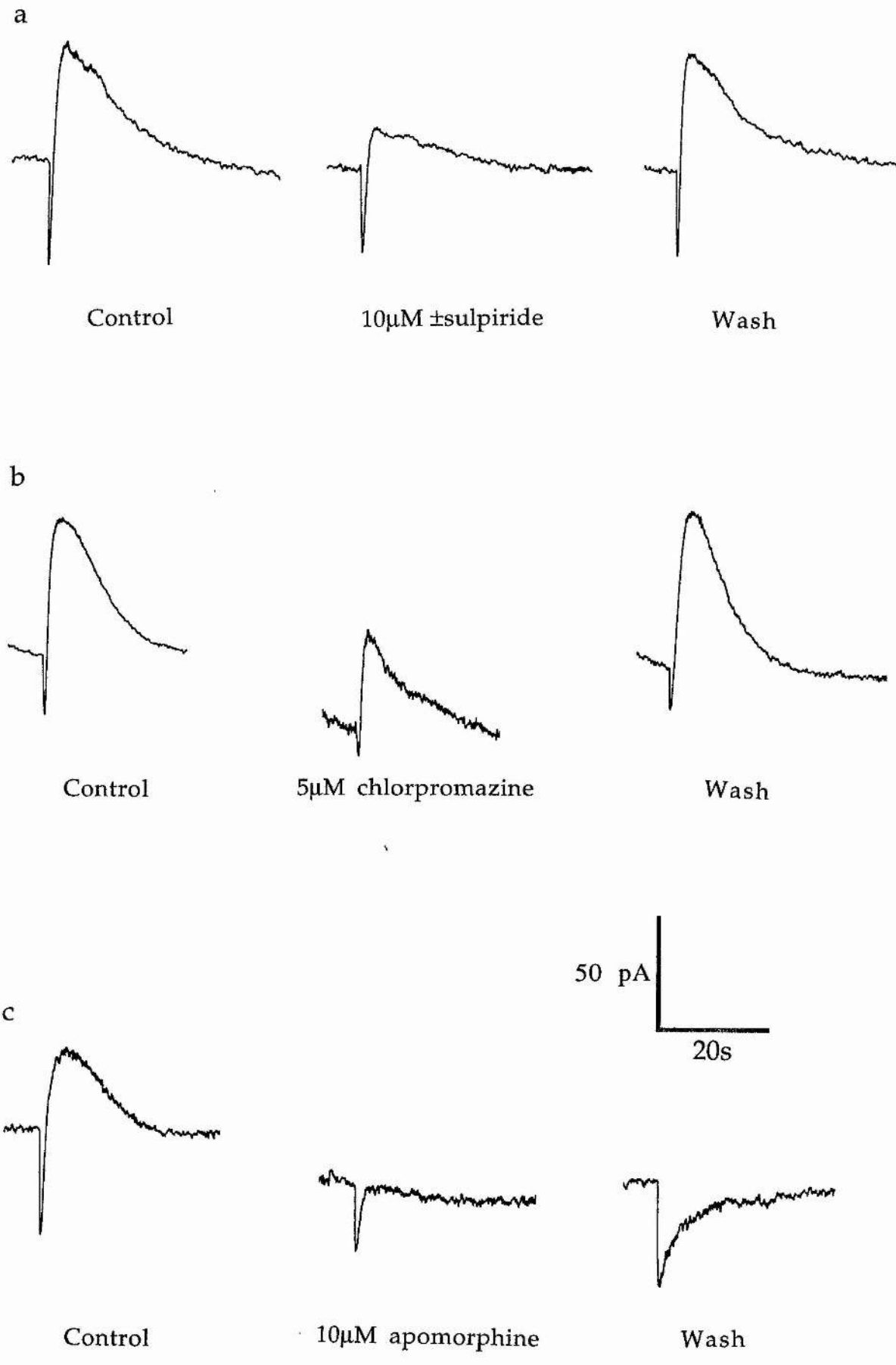

a
Control
10μM ±sulpiride
Wash
b
Control
5μM chlorpromazine
Wash
50 pA
20s
c
Control
10μM apomorphine
Wash

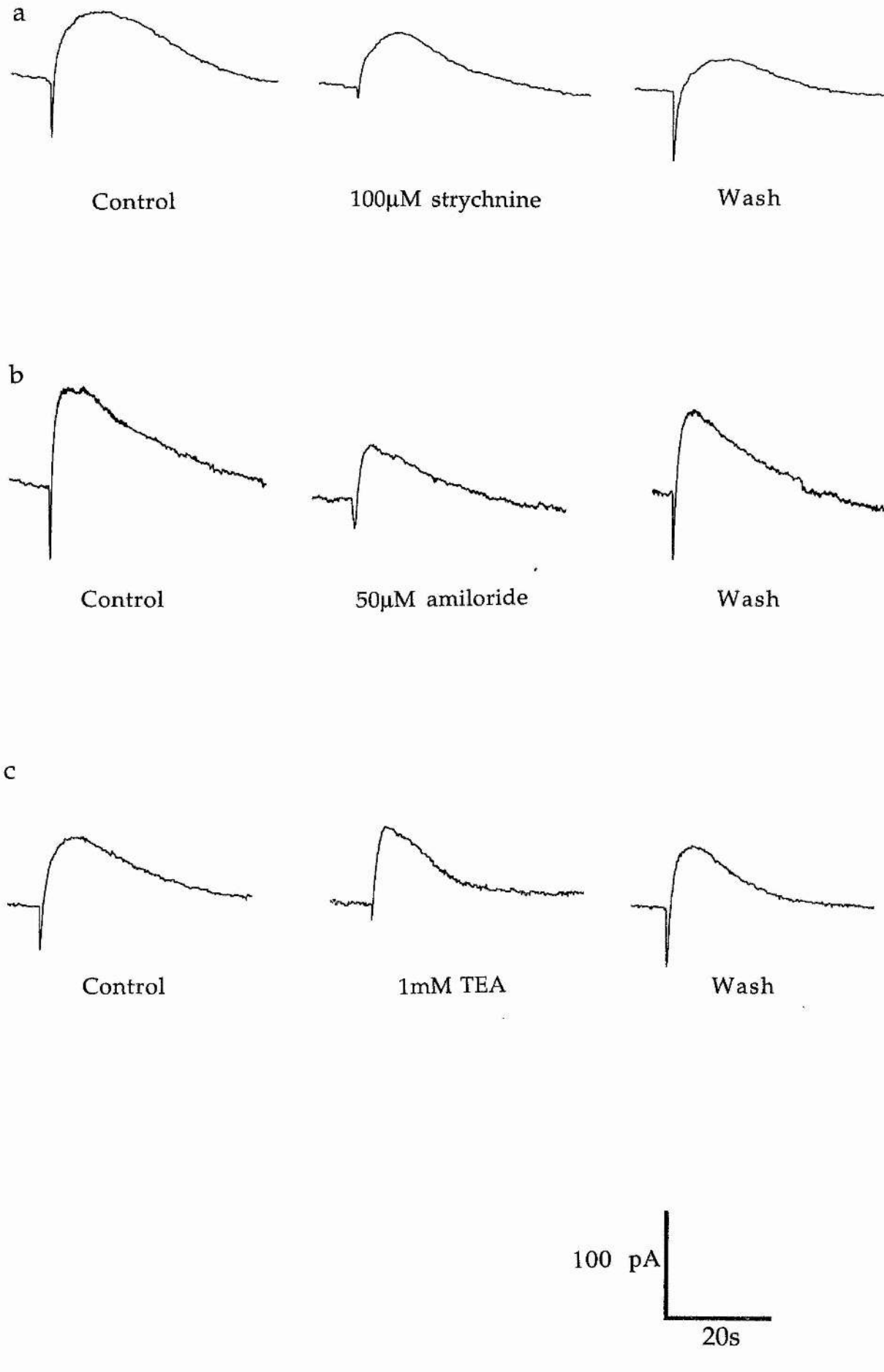
Figure IV.11.

Whole cell patch clamp recordings from GDNs in culture showing the effects of antagonists of the dopamine response. Antagonists were bath applied in normal saline.

a. 100μM strychnine almost completely abolished the Fast Response whilst leaving the slow outward current little affected. The effect was reversible on washing. Dopamine 100μM. 20ms, 20psi.

b. 50μM amiloride caused a reduction in amplitude of both the inward and outward components of the response. The effect was reversible with washing. Dopamine 100μM, 20ms, 20psi.

c. 1mM TEA produced an almost complete but reversible block of the Fast Response, whilst having little effect on the amplitude of the slow outward current. The shape of the slow outward component was changed, this appears to be due to preferential block of a more slowly decaying outward current. Both effects were reversed with washing. Dopamine 50μM, 40ms, 20psi.


a
Control
100μM strychnine
Wash
b
Control
50μM amiloride
Wash
c
Control
1mM TEA
Wash
100 pA
20s

60 % reduction of the Fast Response and a 45% reduction of the slow outward current (Fig. IV.11.). Both effects were reversible with washing.

IV.3.1.6.Tetraethylammonium (TEA), a large cation which is usually associated with the blockade of voltage gated potassium channels was bath applied at concentrations between 1 and 10mM (n=6). 1mM TEA produced an almost complete block of the Fast Response but had little effect on the amplitude of the slow outward current (Fig. IV.11.). This block may resemble TEA blockade of nicotinic acetylcholine receptors which have been observed (Adler et al. 1979, Wright, Kline & Nowak 1991). At higher concentrations, block of the slow outward current was also observed. Again there appeared to be preferential block of a later, slower, part of the response revealing a more rapidly inactivating part. All the effects of TEA were reversed with washing. TEA was allowed to diffuse into two GDNs via the recording patch pipette (n=2). The pipette solution consisted of (mM); NaCl 1.7, KCl 51.3, $MgCl_2$ 4.5, EGTA 1, HEPES 5, TEA 5. This had no effect on the Fast Response. The slow outward current was also not affected which may indicate that insufficient TEA entered the cells.

IV.3.1.7. d-tubocurarine (curare), a competitive antagonist of the nicotinic acetylcholine receptor was bath applied to the GDN in culture at concentrations of 100μM. Its effect was to completely abolish the Fast Response whilst leaving the slow outward current unaffected (Fig. IV.12.) (n=8). The effect of curare was reversed with washing. The curare experiments shown in figure IV.12. also make a comparison between dopamine applied by iontophoresis and pressure. Both methods elicited a fast excitatory response followed by a slow inhibitory response, and in both cases the Fast Response was completely blocked by curare with the slow response unaffected.

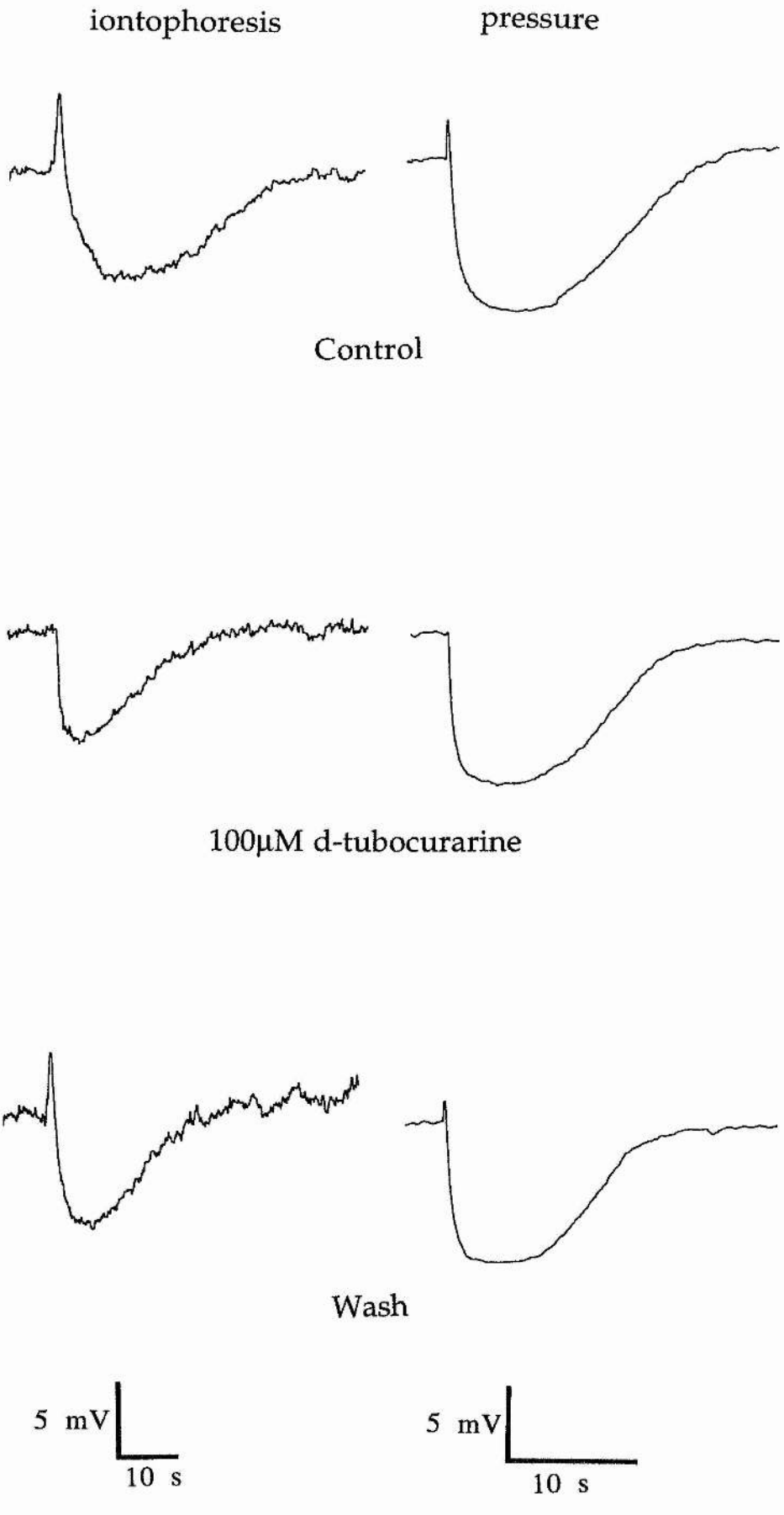
Figure IV.12.

Single electrode current clamp recordings showing the effect of d-tubocurarine on the dopamine response of the GDN.

Two experiments are shown, in the left hand column dopamine was applied by iontophoresis (10mM, 500ms, 400nA) and in the right hand column by pressure (100μM, 20ms, 20psi). In both cases 100μM tubocurarine abolished the Fast Response but had no effect on the slow hyperpolarising response.

These results also demonstrate that the Fast Response can be elicited using both iontophoresis and pressure application.

Scale bars 5mV, 10s.


iontophoresis
pressure
Control
100μM d-tubocurarine
Wash
5 mV
10 s
5 mV
10 s

IV.4. EFFECT OF AGONISTS ON THE GDN

IV.4.1. 5-Hydroxytryptamine (5-HT)

It is possible that the Fast Response to dopamine is not the action of dopamine on a dopamine receptor, but is a non-specific effect on another type of receptor. A 5-HT receptor was thought to be a likely candidate for three reasons. 1, 5-HT is present in the central ganglia of *H. trivolvis*. 2, The 5-HT molecule shares structural similarities with dopamine. 3, 5-HT has been shown to directly gate an ion channel in the 5-HT_3 receptor.

50 μM 5-HT was applied to the GDN both *in situ* (n=3) and in culture (n=3). There was no evidence of a fast excitatory response. In all cases it elicited a depolarising response which was slow in onset and long in duration (Fig IV.13.). The time from onset to half maximal response was about one second and the duration was 20-25 seconds.

IV.4.2. Acetylcholine

The Fast Response was blocked by the cholinergic antagonist curare and by TEA which blocks nicotinic acetylcholine receptors. This suggested that the dopamine Fast Response may be due to nonspecific activation of an acetylcholine receptor. Application of acetylcholine to the GDN in culture elicited a fast inward current, similar in onset and rise time to the dopamine Fast Response. There was no evidence of a slow outward component (Fig. IV.14.).

Figure IV.13

Current clamp recording showing the effect of 5-HT on the GDN in culture.

Application of 5-HT (50μM, 15ms, 10psi.) evoked a depolarising response which was slow in onset and long in duration.

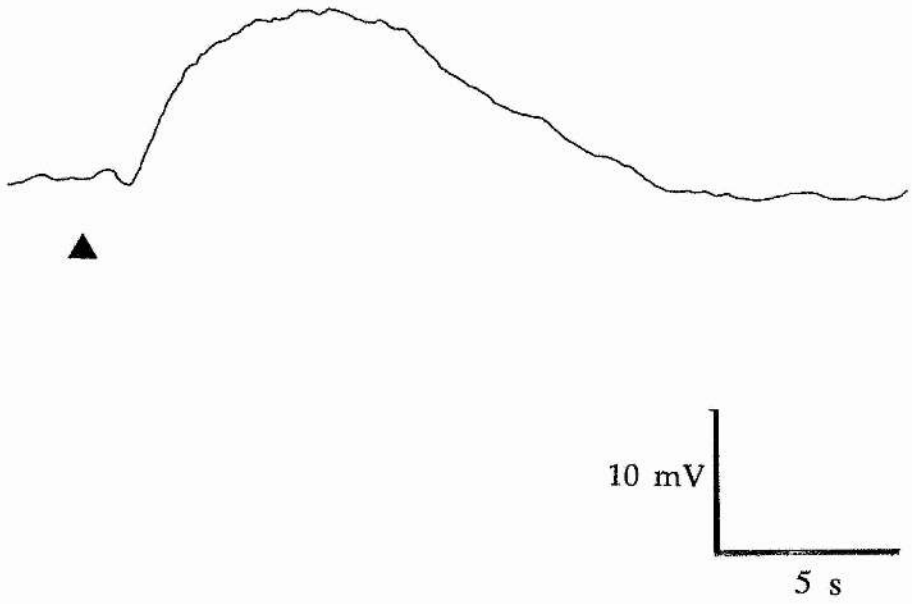

10 mV
5 s

Figure IV.14.

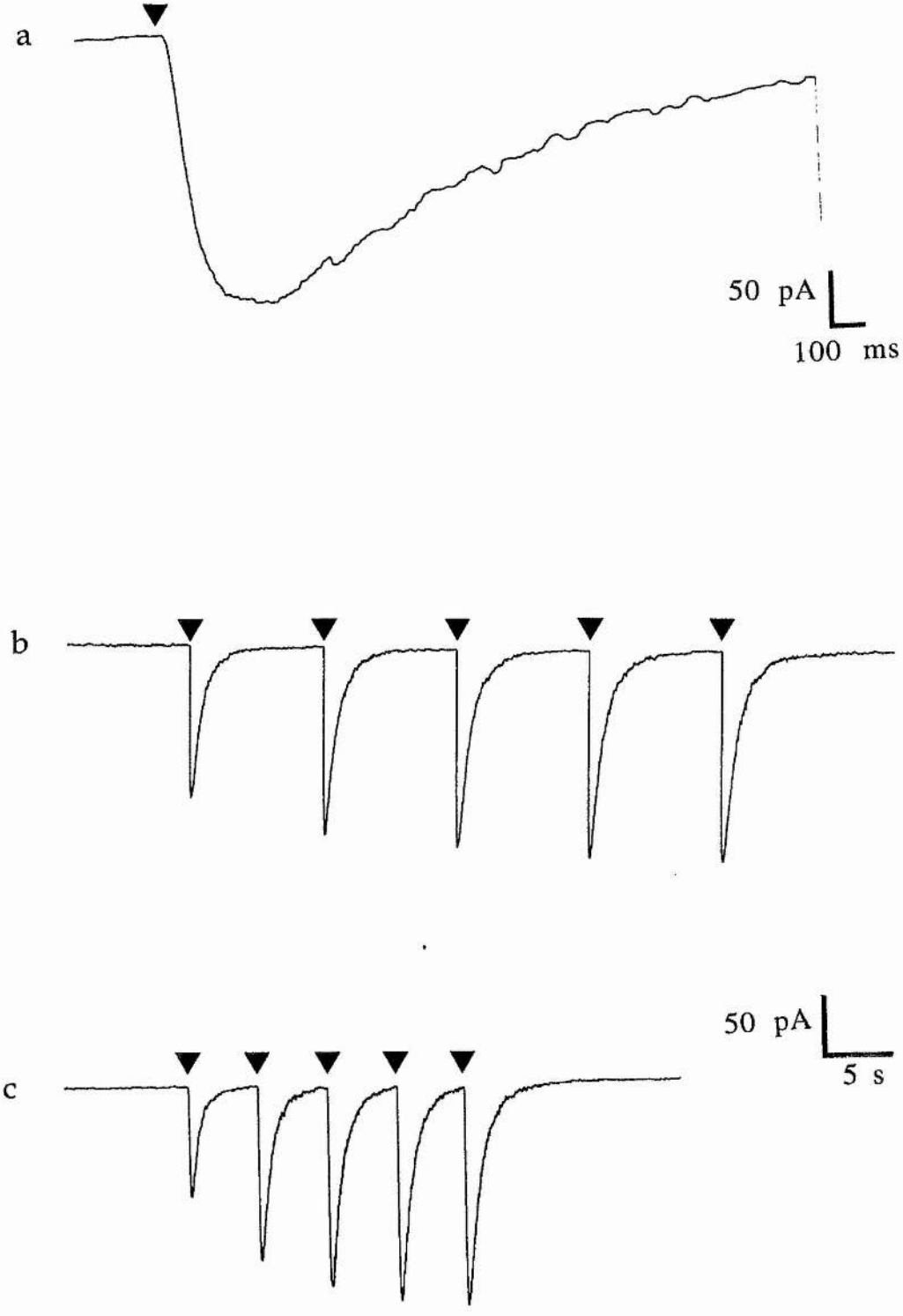
Whole cell voltage clamp recordings showing the effect of acetylcholine on the GDN in culture. Holding potential -60mV.

a. Pressure application of acetylcholine evoked a fast inward current similar in time course to the dopamine Fast Response, however there was no evidence of a slow outward current.

b. Repetitive application of acetylcholine at 10s intervals showing the potentiation of the response with successive applications.

c. Repetitive application of acetylcholine at 5s intervals showing more pronounced potentiation of the response.

Acetylcholine pressure applied 10μM, 10ms, 20psi.


a
50 pA
100 ms
b
50 pA
5 s
c

The acetylcholine fast differed from the dopamine response in two ways. (1) The response was much larger, even with low concentrations of acetylcholine. A typical response was 0.2-0.3 nA at -60mV in response to a 10ms pulse of 10μM acetylcholine, a ten fold increase over the equivalent dopamine response. (2) The response to acetylcholine did not show desensitisation. Repetitive application of acetylcholine resulted in potentiation of consecutive responses (Fig. IV.14.). This second difference strongly suggests that dopamine is acting on a different receptor to acetylcholine to produce its effect.

IV.4.3. FMRFamide

The molluscan neuropeptide FMRFamide has been shown to directly gate two ion channels in the snail *Helix aspersa* (Green, Falconer & Cottrell 1994). Application of 100μM FMRFamide to the GDN in culture produced a response very similar to that seen with dopamine, a fast inward current followed by a slow outward current. The response was also seen when FMRFamide was leaked onto the GDN from a broken pressure application pipette (Fig. IV.15.). The FMRFamide fast response showed marked desensitisation (Fig. IV.15.) and a rate of rise comparable to that of the dopamine Fast Response (Fig 3.2.5). The response was abolished by bath application of 100μM amiloride (n=3).

There were differences between the FMRFamide and dopamine Fast Responses. The FMRFamide fast response was not affected by bath application of 100μM curare (n=3) (Fig. IV.15.) which completely abolished the dopamine Fast Response. A more significant difference was the distribution of receptors for dopamine and FMRFamide.

Figure IV.15.

FMRFamide on the GDN in culture.

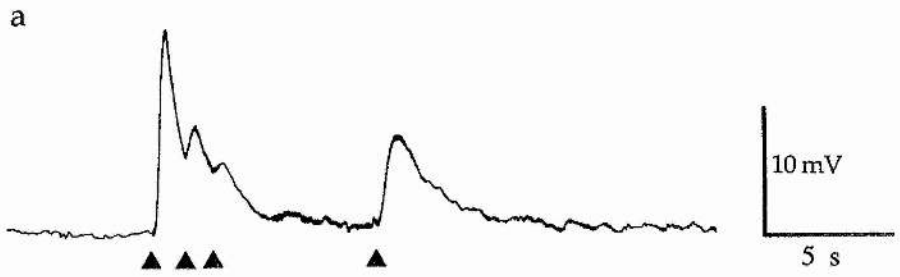
a. Current clamp recording showing the effect of FMRFamide on a GDN with the membrane potential hyperpolarised to -75mV. FMRFamide evoked a fast depolarising response, with successive applications resulting in desensitisation. (scale bars 10mV, 5s.)

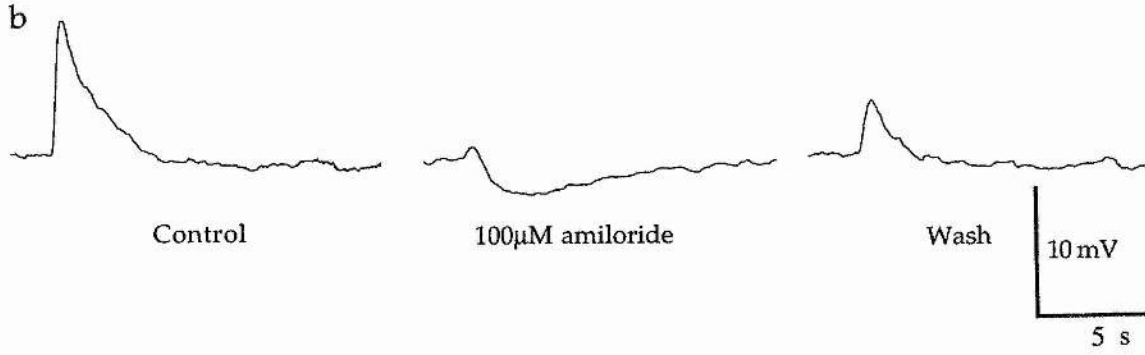
b. 100μM amiloride reversed the sign of the FMRFamide response. Partial recovery is observed with washing (scale bars 10mV, 5s).

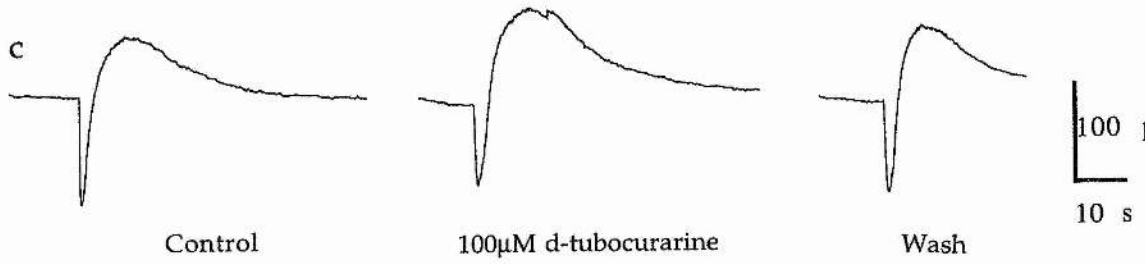
c. Whole cell patch clamp recording of the FMRFamide response on the GDN (holding potential -60mV). Bath application of 100μM d-tubocurarine had no effect on the response (scale bars 100pA, 10s).

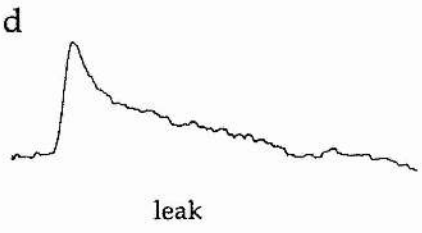
d. Fast inward current evoked in a GDN by leaking 100μM FMRFamide from a broken pressure application pipette onto the neurone. (Scale as for a)

FMRFamide pressure applied 100μM, 20ms, 20psi.


a
10 mV
5 s


b
Control
100µM amiloride
Wash
10 mV
5 s


c
Control
100µM d-tubocurarine
Wash
100
10 s


d
leak

FMRFamide was focally applied by pressure to a variety of different points on the surface of GDNs in culture (n=4). The fast inward current in response to FMRFamide was largest on the soma and smallest on the axon. This contrasts with the effect of dopamine which was largest on the axon, and smallest on the soma. This difference is apparent in figure IV.16. where both dopamine and FMRFamide were applied to the same neurone in culture. The difference in the distribution of the fast responses suggests that FMRFamide and dopamine act on different receptors to produce a fast response.

Although it appears that dopamine and FMRFamide act on different receptors to produce a fast response in the GDN, there is evidence of some interaction between the two responses. Both the dopamine and FMRFamide response are desensitised with repetitive applications . However it is possible for dopamine to cause desensitisation of the FMRFamide fast response and vice-versa. Either dopamine or FMRFamide was applied to GDNs in culture. Either FMRFamide or dopamine was then bath applied and its effect on the response to pressure application of the other drug recorded. Bath application of FMRFamide abolished the dopamine Fast Response, and bath application of dopamine abolished the FMRFamide fast response (Fig IV.17.).

Figure IV.16.

Variation in the amplitude of the response to FMRFamide of the GDN with point of application. Comparison with the dopamine response.

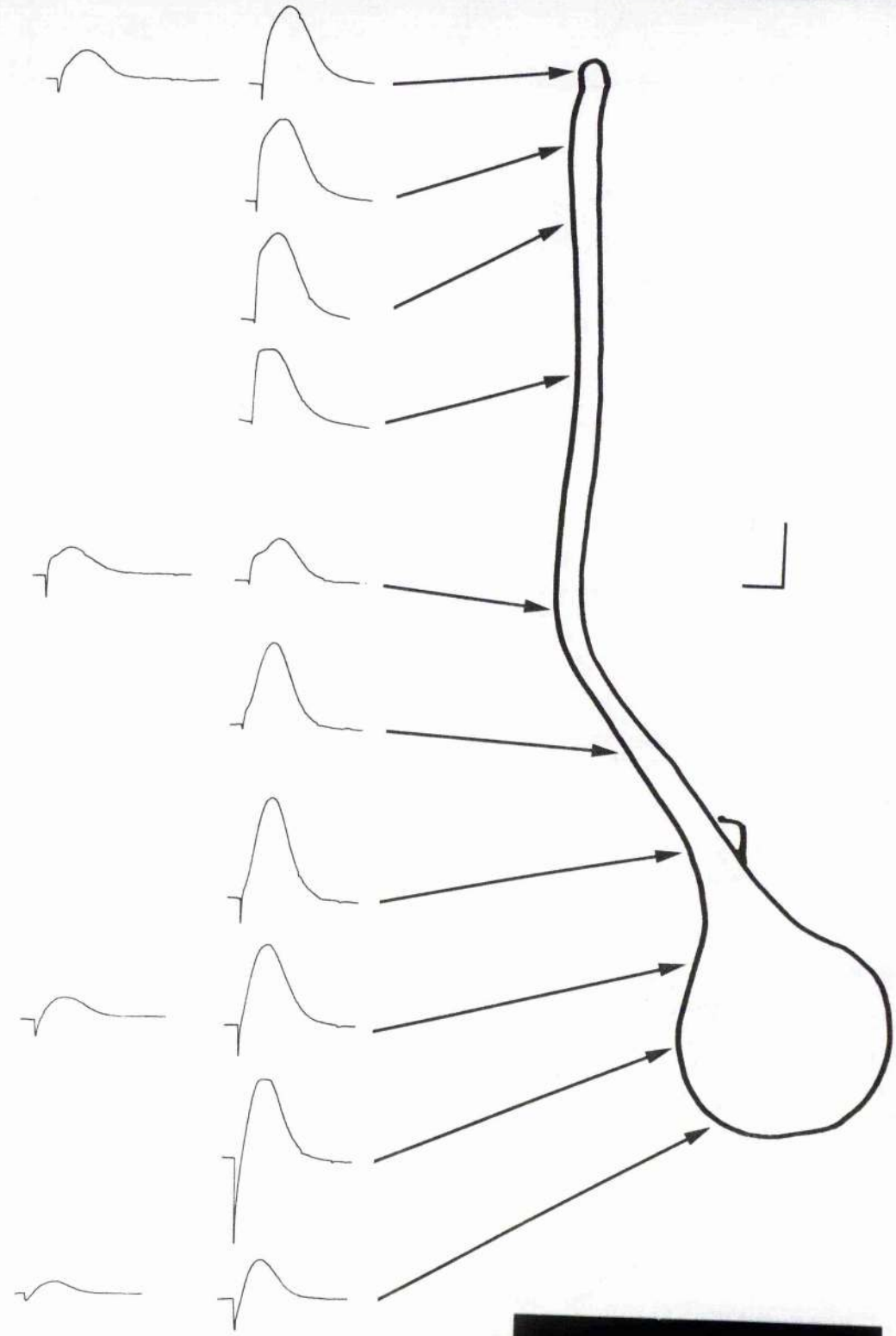
The photograph shows the GDN on which the experiment was performed. In the lower right is the soma, and extending from it is a section of axon which was dissected out intact. Above, is an outline drawing of the neurone with arrows indicating the point of application of FMRFamide and dopamine. To the left of the arrows are responses recorded from these applications.

The right hand column of responses show the effect of FMRFamide. The amplitude of the inward current was largest with applications onto the soma, and almost non-existent with applications to the axon, although the response appeared to increase in amplitude slightly towards the end of the axon. The slow outward current shows less variation in amplitude, but there is variation in its shape.

The effect of dopamine is shown to the left. In contrast to the FMRFamide effect, the dopamine Fast Response is largest on the axon and smallest on the soma.

Scale bars 200pA 10s

The diameter of the GDN soma is 50μm



Dopamine FMRFamide

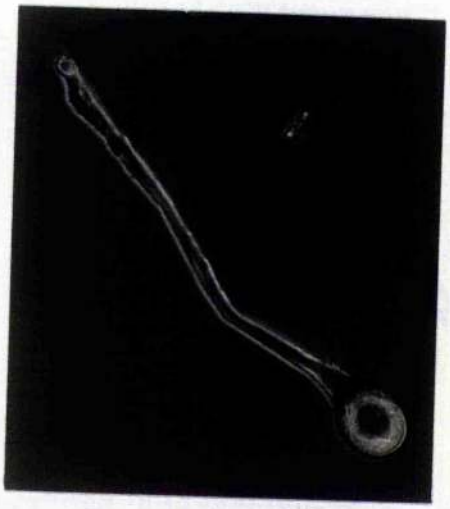
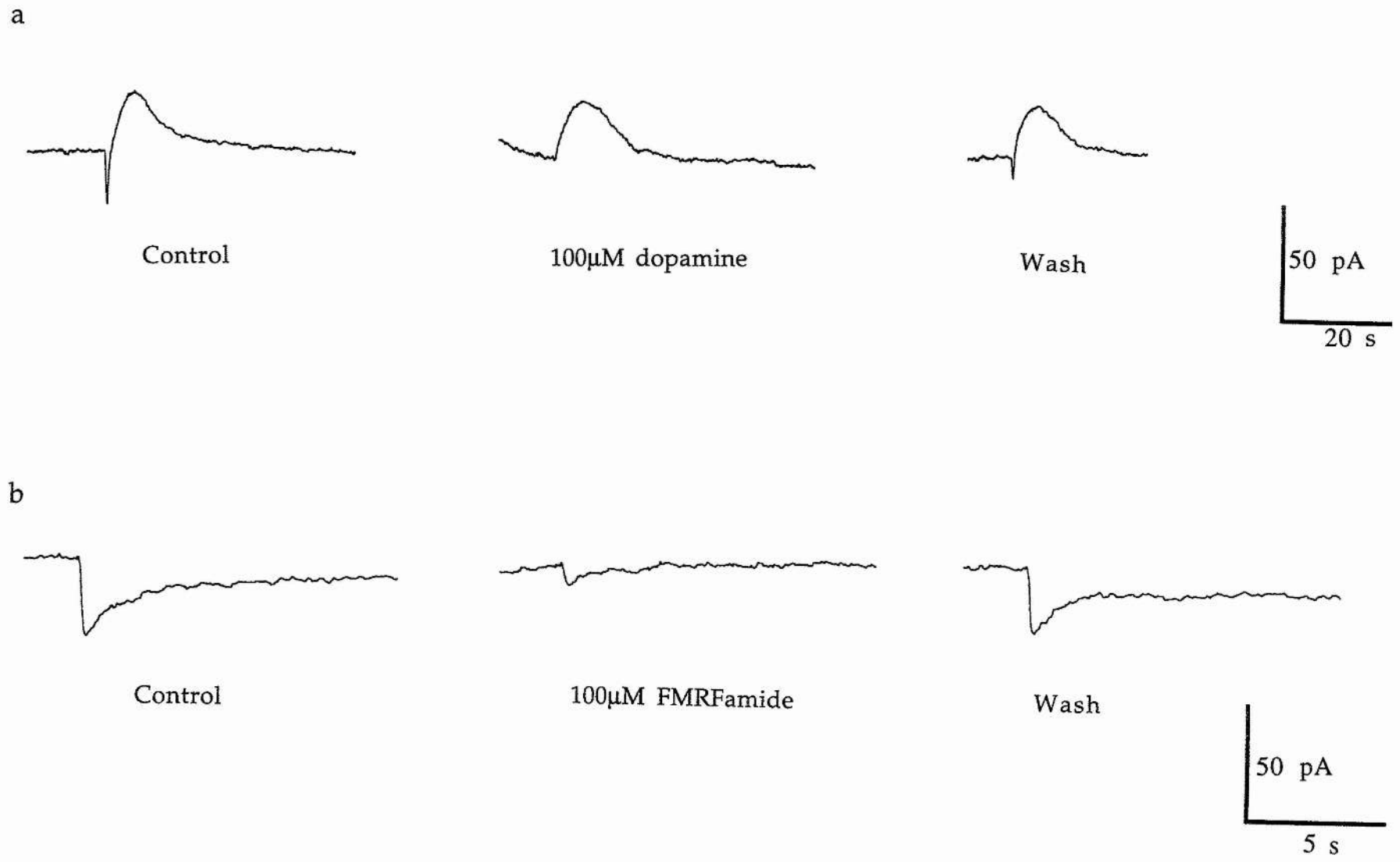


Figure IV.17.

Whole cell voltage clamp recordings showing cross desensitisation of dopamine and FMRFamide.

a. Application of 100μM FMRFamide to a GDN in culture elicited a biphasic response. Bath application of 100μM dopamine abolished the inward component, leaving the outward component unaffected. The effect showed partial reversal with washing. Holding potential -60 mV.

b. Application of 100μM dopamine to a GDN in culture elicited a fast inward current (holding potential -75mV). Bath application of 100μM FMRFamide desensitised the response. The effect was reversed with washing.


a
Control
100μM dopamine
Wash
50 pA
20 s
b
Control
100μM FMRFamide
Wash
50 pA
5 s

CHAPTER V

DISCUSSION I: CELL CULTURE AND JUNCTION FORMATION

V.1. Identification of the GDN in *H. trivolvis*

The work in this thesis has been based around properties of a single identified neurone located within the left pedal ganglion of the snail *Helisoma trivolvis*. At the start of this work, a giant dopamine containing neurone in the closely related snail *Planorbis corneus* was already well characterised in terms of morphology, transmitter phenotype and *in-situ* synaptic connections (Berry & Cottrell 1975, Cottrell 1977). The homology between the giant neurone of *H. trivolvis* and the GDN of *P. corneus* has been confirmed both morphologically using Lucifer Yellow and histochemically using glyoxylic acid. Electrophysiological evidence showing follower neurones of the *H. trivolvis* GDN in the visceral and left parietal ganglia, also provide evidence of homology with *P. corneus*. More recently Syed et al. have revealed by HPLC analysis the presence of dopamine at a concentration of approximately 0.42 mM in the GDN of H. trivolvis (Syed et al. 1993).

V.2. Development of neurone culture conditions

Neurone culture was based on the techniques described by Wong et al. 1981. In initial experiments neurones were plated out onto poly-l-lysine coated glass coverslips which would allow high resolution differential interference contrast microscopy if this was required in future experiments. However although neurones survived with good resting membrane potentials under these conditions, they did not extend neurites.

The key to extensive neurite outgrowth of *H. trivolvis* neurones is the use of a conditioned medium derived from the isolated central ganglionic rings of *H. trivolvis*. Recently, an active component in *H. trivolvis* conditioned medium has been identified (Miller & Hadley 1991; Miller, Hadley & Hammond 1994).

It is a laminin like molecule similar to that which is already known to promote neurite extension from leech neurones in culture (Chiquet et al. 1988). It is also thought that conditioned medium also contains a nerve growth factor-like molecule (Ridgway et al. 1991). During this study pre-treatment of coverslips with conditioned medium had little effect on the quantity of neurite outgrowth. This conflicts with the increase in neurite extension observed by Wong et al. 1981 with similar treatment.

The use of concanavalin A as a substrate increased the percentage of neurones extending neurites. Concanavalin A has been shown to stimulate neurite outgrowth in neurones of several different species including *A. Californica,* leech and chick dorsal root ganglion neurones (Lin & Levitan 1987, Chiquet & Acklin 1987, DeGeorge et al. 1985). Concanavalin A also appears to promote neurite outgrowth in *H. trivolvis* neurones. The mechanism by which this occurs is not clear. However concanavalin A has been shown to increase the number of calcium channels in the growth cones of cultured leech neurones (Ross, Arechiga & Nicholls 1988). There is evidence that for *H. trivolvis* growth cones to be active, their intracellular calcium concentration must be controlled to within a very narrow band (Kater & Mills 1991). Concanavalin A may, through increasing the number of calcium channels raise growth cone calcium concentrations to within the permissive range. Concanavalin A has the ability to alter synaptic specificity between neurones (Lin & Levitan 1987) causing neurones to form electrical connections where they would usually form chemical connections. As a result concanavalin A was ruled out as a suitable substrate.

The switch from a glass to a plastic base appeared to be critical in order to obtain sufficient neurite outgrowth using poly-l-lysine as a substrate. The reason for this is unclear. As glass is used successfully in other laboratories it

seems likely that some apparently trivial difference in methods has a profound effect on the number of neurones extending neurites. A second possibility is that the switch from plates pretreated with CM to plates in which the culture medium was CM produced the effect. However this seems unlikely as pretreatment should confer significant growth promoting properties to the poly-l-lysine substrate (Wong et al. 1981).

V.3. Morphology of the GDN in culture

When plated out in culture under suitable conditions the GDN developed broad flat growth cones and extended neurites. After a period of approximately three days, active neurite extension ceased and the growth cones became rounded, inactive, phase bright terminals. This sequence of events is well documented and is typical of that observed for many other *H. trivolvis* neurones under similar culture conditions (Cohan et al. 1985; Hadley et al. 1985). Reasons for the spontaneous cessation of neurite extension in these neurones is unclear as the extracellular environment does not change during this period. The change is linked to an alteration in ion channel activity in the growth cone which appears to be brought about via an intracellular signal (Cohan et al. 1985).

There is evidence that when isolated in culture, certain invertebrate neurones extend a distinctive pattern of neurites which varies from one neurone type to another, and may even be unique for individual identified neurones (Chiquet & Nicholls 1987; Graf & Cooke 1994; Howes, Cheek & Smith 1991). Earlier unpublished work in this laboratory (A. Thewlis. Hons Thesis) indicated that the GDN of *P. corneus* developed a distinctive pattern of neurite outgrowth. In this study neuritic pattern of the GDN could not be distinguished from that of the LSN indicating that the presence of dopamine in the neurone did not

affect its morphology. However the growth cones of the GDN and the LSN were particularly large, distinguishing them from many other neurones in culture.

The type of substrate had a profound effect on the neuritic growth pattern of the neurones in culture, an effect which was far greater than differences due to the type of neurone. Even subtle changes in the substrate such as increasing the molecular weight of the poly-l-lysine had a detectable effect on the neuritic extension of these cells. Variation in the pattern of growth of neurones on different substrates has been widely reported (e.g. Grumbacher-Reinert 1989; Chiquet & Nicholls 1987). The variation in neuritic pattern observed here in the absence of any change in the soluble cues for neurite outgrowth, underlines the significance of the physical environment of the neurones in addition to any genetically pre-programmed instruction in determining its morphology. It is easy to imagine how changes in a the local environment of a neurite developing *in-situ* could help to define its final morphology.

V.4. Electrical properties of the GDN in culture and *in-situ*

It is often assumed that in culture the electrical properties of a neurone are maintained, but studies have shown that changes do occur (e.g. Fuchs, Nicholls & Ready 1981; Cohan, et al. 1985). The results of the present study showed that the resting membrane potential and the current voltage curve of the GDN were unchanged. However the peak spike amplitude was increased and the half width was reduced when in culture. These differences may represent real changes in the membrane properties of the neurones, possibly due to a reduction in branching, or they could be due to changes in the environment. A difference in local ionic concentration around the cell in

culture and *in-situ* may produce these effects. For example a lowered extracellular calcium concentration may result in a narrowing of the action potential and a reduction in its amplitude. It has been reported that the action potential width of certain identified buccal neurones from *H. trivolvis* increases when in culture (Cohan et al. 1985). This change is not attributable to neurite extension and is opposite to the effect I have observed. It is possible that this reflects a neurone specific difference in the membranes of the different neurones tested. A more likely cause of the observed differences is the lack of synaptic input onto the neurone when in culture. In the intact preparation an unobserved tonic synaptic input may cause subtle membrane changes.

The most striking difference between the GDN in culture and *in-situ* was in its firing pattern. In culture the GDN had a rapid regular firing pattern, but *in-situ* the neurone was silent, interspersed with bursts of activity, although the resting membrane potential was not different. The fact that at the same membrane potential the GDN in culture fires action potentials but *in-situ* is silent suggests a change in the firing threshold of the neurone, or the development of some pacemaker activity in a region of the neurone too distant from the site of recording for any underlying potential changes to be recorded.

The bursting activity *in-situ* was the result of an external synaptic input. Many of the follower neurones of the GDN showed bursting activity simultaneous with that observed in the GDN. However the fact that this did not originate in the GDN is shown in III.5. where stimulation of the GDN by depolarising current injection results in hyperpolarisation of such a follower neurone. If the GDN was initiating the bursting then stimulation of the GDN would have resulted in depolarisation of the follower neurone. It was thought that the

bursting activity was due to excitation by input 3 interneurone (IP3I), a neurone involved in the generation of the respiratory rhythm (Syed, Bulloch & Lukowiak 1990). However removal of the Right Parietal Ganglion in which IP3I is situated did not affect the bursting activity of the GDN, suggesting that the rhythm originated elsewhere.

V.5. Neurone-Neurone connections

V.5.1. Selectivity of connections

This study has shown that when isolated in culture with a selection of unidentified neurones from the left parietal and visceral ganglia the GDN of *H. trivolvis* forms a chemical connection with only 10% of these neurones. When the GDN is plated out with neurones with which it is known to form connections *in-situ,* the success rate for chemical junction formation is increased to 55%. This shows that the GDN under these conditions maintains a degree of selectivity in the connections it forms.

Other studies have shown that certain 'selective' neurones including the GDN never form chemical connections with non follower neurones in culture (Chiquet & Nicholls 1987; Schacher, Rayport & Ambron 1985; Syed et al. 1992; Zoran, Doyle & Haydon 1990), whereas here the GDN formed chemical connections with 10% of the unidentified neurones. *In-situ,* the GDN forms synapses with a large number of visceral and parietal neurones and it is probable that the chemical connections observed in culture with unidentified neurones from these ganglia also existed *in-situ.* A more suitable way to have shown the selectivity of the GDN would have been to culture it with identified neurones with which its branches made close physical contact but no chemical connection *in-situ.*

The reasons for the apparently low success rate in junction formation with identified neurones is not clear as pairs were only recorded from if there was considerable physical contact between the neurones. It is possible that in pairs where contact was made via neurites, a connection made at a point electrically distant from the soma would not be observed. Also many connections observed in culture were extremely weak and it is possible that some connections were too weak to be observed using the techniques employed. The first possibility could be eliminated in experiments in which the neurones are plated out in conditions specifically designed to prevent neurite outgrowth and contact is made only through the soma (Haydon 1988). Recording from the follower neurone using voltage clamp technique may help to improve the sensitivity of recordings to observe very weak connections.

V.5.2. The nature of the chemical connections observed in culture

It has been shown that the GDN retains some selectivity with which neurones it forms chemical connections when in culture. However the chemical connections which have been observed in culture differ considerably from those which have been observed *in-situ*. There are three main differences. First the latency of the onset of the post-junctional response is slower in culture. Second, the connection between the GDN and the LSN is rarely reciprocal in culture. Third the chemical connections are not stable and are usually lost to be replaced by electrical connections. These differences are discussed in following sections.

The slow timecourse of onset of the post-junctional response in culture may be due to a different spatial arrangement of the synaptic terminals in cultured

neurones. For example terminals may be arranged at the end of long multi-branched neurites. These terminals may not be stimulated by the initial action potentials of a burst due to failure of transmission of the impulse at some point along the neurite. This would result in an apparently slow junctional latency. Alternatively, the connections formed may not be true synapses, but rather sites of close proximity, where any released transmitter may diffuse to the receptors of the adjacent cell to produce the response.

Electron microscopic studies of cultured neurone pairs in which chemical junctions had been demonstrated electrophysiologically did not reveal the presence of membrane specialisations such as pre and post synaptic thickenings associated with synaptic contacts (Descarries et al. 1987; Heuser 1989; Karle et. al 1994). Varicosities containing vesicles with diameters ranging in size from 50-170 nm, similar to those observed in the presumed preterminal regions of the GDN of *P. corneus* (Pentreath & Berry 1975) were seen at points along neurites of the cultured GDN. However these varicosities were not observed in close opposition to the membrane of other cultured neurones. The electron microscopic study was not exhaustive and it is possible that sites of synaptic contact were overlooked. This electron microscopic study was unable to confirm or refute the presence of synaptic connections between the GDN and other neurones in culture. A more efficient study may be performed on connections between spherical somata without neurites where the point of physical contact between the neurones is better known.

V.5.3. The chemical connection between the GDN and LSN

Chemical junctions formed in both directions between the GDN and the LSN in culture, although such connections were rarely bidirectional as is the case

in-situ. The reason for this difference is unknown, but may relate to culture technique. Syed et al. (1993) have reported reliable formation of a bidirectional junction between the GDN and LSN in culture under similar conditions. The formation of this connection in culture was studied in greater detail to see if the direction in which the unidirectional junction formed could be influenced.

It has been shown that the type of physical contact made between neurones can influence the type of synapse which forms between a neurone pair. For example specific regions of leech neurones such as the soma, initial segment and axon tip, preferentially develop chemical or electrical synapses (Nicholls et al. 1990). *A. californica* neurones L10 and RUQ will not normally synapse, but if they are forced into sufficient contact by intertwining their axons a chemical synapse will form (Hawver & Schacher 1993). The type of physical contact between the GDN and LSN did not influence the direction of junction formation. A unidirectional junction could develop with any form of contact for example soma/soma, axon/soma, neurite/neurite etc. The same appeared to be true of the bidirectional junctions.

The state of neurite outgrowth has been shown to affect junction formation. It has been shown that electrical synapses will only form between a pair of identified neurones if they undergo a period of "temporally coincident, and spatially overlapping, active neurite extension" (Haydon, McCobb & Kater 1987; Hadley, Bodnar & Kater 1985). It was thought that by plating out one neurone of the GDN/LSN pair 24 hours before the other, that there would be a period of approximately 24 hours where the neurone plated first was inactive whilst the second neurone was still actively extending neurites. It was hoped that this period of differential growth would influence junction formation. Plating the GDN out first had no effect on the polarity of junction

formation, but when the LSN was plated first, it changed from being prejunctional in 50% of cell pairs, to being prejunctional in all cell pairs.

It seems unlikely that the difference between the GDN and LSN when plated out in this way can be attributed to activity of neurite extension. Both neurones undergo neurite extension and growth cone collapse over a similar time period. Therefore if a difference in the degree of neurite extension was responsible for the change in junctional polarity, the GDN would be expected to become prejunctional when it was plated first. The formation of weak reciprocal connections by the GDN indicates that the GDN does not lose the ability to form a connection onto the LSN when it is plated second. It appears that the change occurs in the LSN (the prejunctional site) to cause the alteration in junction formation.

V.5.4. Novel electrical connections

In most cases in which chemical junctions formed in culture, the junctions developed within 18 hours of the neurones being plated out, but were not sustained. They were replaced by an electrical connection within 48 hours and the chemical connection did not return. This is contrary to many published results, in which chemical junctions form after periods of days and are maintained, possibly with electrical connection before synapse formation (Camardo, Proshansky & Schacher, 1983; Ambron, Den & Schacher, 1985; Haydon 1988; Vylicky & Nicholls, 1988). The late appearance of the novel electrical connections in culture is unexpected because the formation of electrical connections by *H. trivolvis* neurones in culture requires mutual neurite elongation (Hadley et al. 1985), and when electrical connections appear between the GDN and other neurones in culture, neurite extension has almost, if not completely ceased.

When electrical connections formed, a prejunctional action potential elicited a postjunctional depolarisation. The long duration of the postjunctional response relative to the prejunctional spike suggested that there may have been a chemical component of the response. The depolarising postjunctional potentials were similar in duration to the fast chemically mediated depolarising potentials observed in some GDN follower neurones *in-situ* (Berry & Cottrell 1975; Winlow, Haydon & Benjamin 1981). d-Tubocurarine and a calcium free bathing medium had no effect on the fast depolarisations observed in culture, suggesting that they are purely electrical. The relatively long timecourse of decay of the response, may be due to slow capacitative decay rather than to any chemical element. It is unlikely that the electrical connections mask the effect of any previously formed chemical junction because the chemical response is much longer in duration and would be observable after the end of any electrical effect. Therefore it appears that after 48 hours the chemical connections are usually lost to be replaced by electrical connections.

The reason for the loss of the chemical connection is not known and to my knowledge has not been reported with chemical connections formed by the GDN in culture. The sustained high rate of activity by the neurones in culture may result in transmitter depletion, or the downregulation of postsynaptic receptors. Neurone activity has long been know to influence the formation of synaptic connections between neurones. The development of ocular dominance columns is probably the most widely studied (e.g. Wiesel & Hubel 1963; Archer, Dubin & Stark 1982;), but any direct action of neural activity on chemical synapse development in invertebrates is not described. The effect of neuronal activity on the formation of redeveloping electrical connections has been studied in *H. trivolvis* (Berdan & Bulloch 1990), but was found to have no

effect on the selection or stability of transient novel electrical synapses but was found to increase the coupling coefficient of an appropriate electrical connection between neurones B5.

CHAPTER VI

DISCUSSION II: THE FAST RESPONSE

VI.1. FINDING THE FAST RESPONSE

It was known that the GDN of *P. corneus* evoked dopamine mediated fast depolarising post-synaptic potentials in follower neurones (Berry & Cottrell 1975). The GDN of *H. trivolvis* has also been shown to have similar post-synaptic effects (Syed et al. 1993). The fast time course of the post-synaptic events intimated a possible ligand gated response. This prompted a search for an identifiable neurone in which applied dopamine evoked a response which was comparable to the fast depolarising response evoked by the GDN in certain follower neurones. Such a response was observed first in unidentified follower neurones in the parietal ganglion of *H. trivolvis*, and also surprisingly in the cultured GDN. More recently a larger dopamine evoked fast depolarising response has been observed in the C2 neurone of *H. aspersa* (K. Green, unpublished).

Initially the Fast Response in the GDN was extremely small and was only observed in a small percentage of GDNs. Improved application pipettes reducing the leakage of dopamine, and the observation that the response was bigger when dopamine was applied to the axon, allowed more successful recording of the Fast Response. However the possibility that the Fast Response was an artefact of pressure ejection remained. The observations that the Fast Response was evoked by iontophoresed dopamine and that pressure application of normal saline had no effect, confirmed that the response was evoked by dopamine.

VI.2. THE FAST RESPONSE; A LIGAND GATED ION CHANNEL?

To date, two major classes of dopamine receptors have been described. D1-like receptors are subdivided into D1 and D5, and D2-like subdivided into D2,

D3 and D4 (Gingrich & Caron 1993; Grandy & Civelli 1992). All of these receptors exert their actions through a family of GTP binding proteins, a result of which is that their effect is relatively slow. Over recent years the family of receptors which directly gate ion channels has expanded to include those activated by acetylcholine, GABA, glycine, glutamate, 5-hydroxytryptamine, histamine, ATP and FMRFamide (Sargent 1993; Schofield et al. 1987; Grenningloh et al. 1987; Zorumski & Thio 1992; Derkach et al. 1989, Hardie 1989; Edwards et al. 1992; Cottrell et al. 1990; Green et al. 1994). Evidence presented here suggests that the Fast Response is evoked by an ion channel directly gated by dopamine.

VI.2.1. Time course of the Fast Response

The latency and the rate of rise of the Fast Response were comparable to ligand gated channels which have been described in molluscan neurones. These include channels gated by acetylcholine and FMRFamide (Kehoe 1972; Cottrell et al. 1990). There was considerable variation in the rate of rise of the Fast Response recorded in different neurones, and from different parts of a single neurone. This variation may have arisen for several reasons:

1. It is possible that dopamine elicited more than one type of inward current, one of which was slower. This was supported by GDNs in which the Fast Response showed two distinct phases. If individual neurones had predominantly one receptor type or the other, then the frequency distribution curve for rise times would be bimodal (Fig. IV.5.). The curve is not clearly bimodal indicating that if two currents exist, they are distributed in different ratios on different GDNs. Dopamine applied to different points on an individual GDN elicited responses which varied in magnitude and rate of

rise. This variation in rate of rise may be due to different types of receptor predominating on different regions of the neurone.

2. The time course of a Fast Response elicited by a single current may vary due to the rate of diffusion of applied dopamine. For example, dopamine applied to an area of membrane with few receptors would initiate a small response, but diffusion of the dopamine to another area of membrane rich in receptors would elicit a further, delayed increase in the size of the current. This would cause an apparent slowing of the response. Evidence for this appears in Fig. IV.7a. Dopamine applied to a point on the soma close to the axon hillock elicited a response which appeared to have two phases. The first phase was similar in time course to the responses observed from other areas of the soma. The second, slower phase according to this theory was elicited by diffusion of dopamine to the axon hillock, an area rich in receptors. Dopamine application at a greater distance from the cell surface also produced a slowing of the response. This effect can account for biphasic responses and variation in the rate of rise.

3. An apparent variation in the rate of rise of the Fast Response may have been caused by the onset of the slow outward current. The rate of rise of the Fast Response was measured by the time taken for it to reach half of its maximum amplitude. In some neurones the onset of the slow outward current was relatively fast. This resulted in an effective masking of the Fast Response before it reached its maximum. It is possible that this early masking of the Fast Response resulted in artificially fast or slow estimates for the rate of rise. This effect alone can not explain the distinctly biphasic inward current seen in some neurones.

It is clear that examination of the rate of rise of the Fast Response does not provide conclusive evidence for the existence of more than one inward current. Unitary current recordings may provide evidence for two distinct channel types. However particular difficulties in recording the unitary currents elicited by dopamine in the GDN are discussed in a later section. Initially an attempt at pharmacological dissection of two different currents may be the way forward.

VI.2.2. Desensitisation

The Fast Response showed profound desensitisation after a single application of dopamine. The desensitisation was fast in onset and could remain in evidence for over 45s. Desensitisation is a property common to most ligand gated ion channels (Huganir & Greengard; 1990). It is usually fast in onset and for AMPA/kainate, desensitisation is rapid enough to account for the decay of synaptic currents (Trussell & Fischbach 1989). The rate of decay of the Fast Response due to desensitisation was not observed. Prolonged application of dopamine to the GDN evoked a Fast Response which was terminated by the onset of the slow outward current.

There is evidence that desensitisation of certain ligand gated ion channels is modulated by phosphorylation of the receptor molecule (Yakel, Shao & Jackson 1991; Swope, Moss, Blackstone & Huganir 1992; Lozovaya, Vulfius & Ilyn 1993), although the precise mechanism is not clear. Raised intracellular calcium concentration has also been shown to increase the rate of desensitisation of the 5-HT_3 and nicotinic acetylcholine receptors (Chemeris et.al. 1982; Yakel et al. 1993). It is suggested that the influx of calcium through the open channel contributes to the rapid desensitisation of the 5-HT_3 receptor. These effects on the desensitisation of ligand gated channels suggest

further experiments to examine the desensitisation of the Fast Response. This would aid comparison with other ligand gated ion channels.

VI.2.3. Voltage dependence of the Fast Response

VI.2.3.1. Recording Techniques

Most voltage clamp recordings of the Fast Response were made using the whole cell patch configuration and continuous single electrode voltage clamp. The main advantage of this technique for recording the Fast Response was the high signal to noise ratio that could be achieved. However this technique does have certain limitations which must be taken into account. The command voltage is set at the top of the recording pipette. The same electrode is used to simultaneously pass current and record voltage. The result is that the voltage recorded at the top of the electrode is the sum of the membrane potential plus the voltage drop across the electrode tip resistance. The end result is that the membrane potential at which the cell is held, is not precisely known.

The error was minimised in three ways when recording the Fast Response: 1, The access resistance to the cell was very low (typically 1MΩ). 2, Series resistance compensation usually > 50% was employed. 3, The currents observed were relatively small. However control experiments using a second voltage recording electrode showed that the error increased to unacceptable levels when large currents were passed. For example the current required to hold the GDN at very depolarised potentials.

The use of a two electrode voltage clamp to record the current voltage relationship of the Fast Response overcame the problems encountered with the continuous single electrode clamp. It enabled the GDN to be clamped at

very positive potentials without error. This is because current injection and voltage recording are performed by two separate electrodes. Therefore there is a negligible voltage drop across the tip of the recording electrode. The disadvantage of this method was that the signal to noise ratio was relatively poor.

VI.2.3.2. I/V Curves

Both methods of recording, produced a dopamine current/response relationship with a sudden increase in the size of the Fast Response at around -30mV, which peaked, then started to reduce again at more positive potentials. The shape of the curve is suggestive of the voltage dependent block by magnesium ions of the NMDA receptor and certain cloned 5-HT3 receptors (Nowak et al. 1984; Maricq et al. 1991). A similar voltage dependence has also been observed for certain slow dopamine responses in insect neurones and molluscan neurones (Davis & Pitman 1991; Matsumo et al. 1988). There are however important differences:
1, Both the NMDA and 5-HT_3 receptors activate a non-specific cation channel, the reversal potential for which is close to zero mV. 2, The reversal potential for the insect dopamine response is also close to zero mV. 3, At potentials more negative than -30 mV the current voltage relationship of the Fast Response is relatively linear, with an extrapolated reversal potential close to 0 mV. This indicates a non-selective cation channel or a chloride channel. However the response does not reverse at zero mV. An extrapolation from the more depolarised region of the curve indicates a very depolarised reversal potential, possibly due to a sodium or calcium selective channel.

The appearance of two, apparently different reversal potentials within the current voltage relationship of the Fast Response, again raises the possibility

that dopamine activates more than one type of ion channel. The first does not display voltage dependence and predominates at potentials more negative than -30mV. It has an extrapolated reversal potential close to 0 mV. The second shows voltage dependence, being activated at potentials more positive than -30 mV. It has an extrapolated reversal potential more positive than +60 mV. The activation of a different current at more positive membrane potentials is supported by a change in the shape of the Fast Response recorded at positive potentials. The presence of two different currents may be identified pharmacologically. For example, at negative potentials the Fast Response is blocked by a variety of agents including curare, strychnine and TEA. However the effect of these drugs has not been determined on the Fast Response at depolarised potentials. It is feasible that if there are two different currents, that they are blocked by different drugs.

The voltage dependent, dopamine evoked, inward currents observed in cockroach and *A. californica* neurones (Davis & Pitman 1991; Matsumo et al. 1988) were both slow inward currents. This suggests that these responses were second messenger mediated. The *A. californica* response was mimicked by intracellular injection of cyclic AMP.

VI.3. DIRECT EVIDENCE FOR A LIGAND GATED RESPONSE

The results discussed so far reveal the presence of a fast, rapidly desensitising current evoked by dopamine on the GDN. These are characteristics indicative of a ligand gated ion channel, but this evidence is indirect. More direct evidence is now discussed, in which the possible involvement of a second messenger linked response is eliminated.

VI.3.1. Inactivation of G-proteins

G-protein linked receptors represent a vast number of different cell surface receptors, the effects of which vary widely depending on the enzyme or ion channel activated. To date, all dopamine receptors and most non-ligand gated membrane ion channels have been shown to operate via a G-protein (Alberts et.al 1994; Grandy & Civelli 1992). The non-hydrolysable GTP analogue GDP-ß-S was used as a tool to examine the possible involvement of G-proteins. GDP-ß-S blocks the action of G-proteins by competing with GTP for binding to the α subunit (Eckstein et.al. 1979). Intracellular perfusion with GDP-ß-S had no effect on the Fast Response. The slow outward current was abolished showing the involvement of a G-protein in that part of the response, and acting as a control to show the entry of sufficient GDP-ß-S into the cell. This confirms that a G-protein is not involved in the activation of the Fast Response.

The lack of involvement of a G-protein in the Fast Response is not direct evidence of a ligand gated ion channel. It is possible that the Fast Response is mediated by, for example an enzyme linked receptor. This however is unlikely considering the ubiquity of G-protein involvement in the activation of neurotransmitter mediated metabotropic ion channels.

VI.3.2. Unitary current recordings from outside-out patches

Unitary inward currents have been evoked by dopamine in excised outside-out patches taken from the GDN. The pipette (intracellular) solutions did not contain GTP. This is the strongest single piece of evidence that dopamine directly gates an ion channel.

Channel activity in response to dopamine was only observed in patches taken from the axon of isolated GDNs. This agrees with the predicted distribution of dopamine receptors from the whole cell recordings. The combination of a small whole cell current and the clustering of the ion channels, have combined to make recording of the unitary currents extremely difficult. As a result little specific information about the properties of the unitary currents on the GDN has been obtained.

More recently in this laboratory, information on dopamine evoked unitary currents has been obtained by Dr. Kevin Green *in-situ* from outside out patches taken from both *H. trivolvis* parietal neurones and the C2 neurone from *H. aspersa*. The results obtained from the C2 neurone are particularly promising for future study of the Fast Response because the whole cell response is much larger than that observed in the GDN and dopamine activated ion channels appear to be widely distributed on the soma. Unitary currents evoked by dopamine in these neurones appear to have a conductance of about 2pS. This is relatively small. However the single channel conductance of ligand gated ion channels varies widely even among a single receptor subtype e.g. 0.31-16.6pS for 5-HT_3 receptors (Peters, Malone & Lambert 1992).

The current/voltage curve for unitary currents recorded in parietal neurones and the C2 are similar to the whole cell GDN current at potentials more negative than -30mV, but it does not show the voltage dependence of the whole cell current at more positive potentials. This difference between the two curves can be reconciled in at least two ways; 1, A voltage dependent channel block of the kind observed with NMDA channels does not affect the unitary conductance. It reduces the channel open time by causing the opened channel to flicker rapidly between the open and closed states (Nowak et al.

1984). 2, At more depolarised potentials a different, voltage dependent channel may be activated which is not present in the area of the excised patch. The data collected at present are insufficient to determine which, if any of these possibilities occurs.

VI.4. PHARMACOLOGY

VI.4.1. Inhibitors of the Fast Response

The Fast Response was antagonised by a wide range of different compounds. The effect of each compound is discussed in turn.

Strychnine, a competitive antagonist of the glycine receptor produced a reversible block of the Fast Response. There was little effect on the slow outward current. Strychnine has long been known to block the excitatory effects of dopamine in molluscan neurones (Ascher 1972). The glycine receptor is a member of the ligand gated ion channel super family which also includes GABAa, nicotinic ACh and 5-HT3 receptors. The strychnine binding site is thought to be within an extracellular loop of the α1 subunit. This loop is conserved in other members of the ion channel family, but slight changes in the amino acid residues in the loop are thought to confer different specificity for certain antagonists (Vandenberg et al. 1992). Antagonism of the fast response by strychnine may indicate a similarity between the dopamine and the glycine receptor.

d-Tubocurarine (curare) completely abolished the Fast Response, but did not affect the slow outward current. Curare acts as a competitive antagonist at the nicotinic ACh receptor, but at higher concentrations it may act in a non-competitive manner on the ion channel. Curare is also a potent antagonist of

the 5-HT3 receptor (Maricq et al. 1991). Importantly, curare is known to block fast excitatory synaptic potentials mediated by the GDN (Cottrell, Berry & Macon 1974). The block of the Fast Response by curare helps to link it with the fast depolarising post synaptic potentials evoked by the GDN in-situ and secondly may indicate similarities between the dopamine and nicotinic acetylcholine receptors.

Tetraethylammonium (TEA) blocked both the Fast Response and the slow outward current, but the antagonism of the Fast Response was more potent. TEA is usually associated with the block of potassium channels and it is probably this effect which antagonised the slow outward current. TEA has also been shown to block nicotinic acetylcholine, glutamate, NMDA and 5-HT3 receptor mediated currents (Adler et al. 1979; Lingle 1989; Wright et al. 1991; Kooyman et al. 1993). TEA is thought to inhibit these currents by a voltage dependent block of the ion channel, the block being greatest when the membrane is hyperpolarised. There is also evidence of competition by TEA for the agonist binding site (Adler et al. 1979; Kooyman et al. 1993). The effect of TEA on the Fast Response is likely to be due to channel block, the main effect of TEA is nonspecific block of non-selective cation channels.

Amiloride blocks both the Fast Response and the slow outward current. It has been shown to block certain monovalent cation and calcium channels in neurones (Garty & Benos 1988; Sanchez-Armass et al. 1991; Tang et al. 1988). Amiloride has also been used in snail neurones to distinguish between two components of a ligand gated FMRFamide response (Green et al. 1994). The block by amiloride may in the future reveal the presence of two components of the Fast Response. The nonspecific block of both the Fast Response and the slow outward current make meaningful interpretation of the results difficult.

Apomorphine acts on mammalian neurones as a partial agonist at peripheral D1 receptors, and as an agonist on central D2 receptors. Contradictory effects of apomorphine have been reported on snail neurones. Macdonald & Berry 1978, found that apomorphine did not block the slow inhibitory effect of dopamine on *P. corneus* neurones, but Bokisch & Walker (1988) reported an irreversible inhibition of the inhibitory current in *Helix* neurones. However Macdonald & Berry did report that apomorphine blocked the fast excitatory phase of dopamine evoked biphasic potentials. I have observed irreversible inhibition of the slow outward response and reversible inhibition of the Fast Response. The reason for the apparently different effects of apomorphine on different preparations is not clear.

The irreversible effect of apomorphine on the slow outward current may prove to be a useful tool in the study of the Fast Response. It is possible that apomorphine may be used to eliminate the complicating effects of the slow outward current on the Fast Response.

Chlorpromazine produced a potent block of both the Fast Response and the slow outward current. The clinical effects of chlorpromazine as an antipsychotic, are thought to be due primarily to its antagonism of dopamine D2 receptors. It seems likely that it was this effect of chlorpromazine that blocked the slow outward current. Chlorpromazine has a wide range of other actions which include block of ATP sensitive potassium channels (Muller, Deweille & Lazdunski 1991), block of certain voltage gated sodium and calcium channels (Ogata et al. 1990) and block of the nicotinic receptor channel (Beniot & Changeux 1993). It is clear that chlorpromazine blocks a wide range of cation channels, and it seems likely that the effect of chlorpromazine on the Fast Response is due to channel block.

Sulpiride, a selective antagonist of mammalian D2 like dopamine receptors has also been shown to antagonise inhibitory responses evoked by dopamine in snail neurones (Stoof et al. 1986; Bokisch & Walker 1988). Dopamine receptors in molluscs can not easily be classified as D1-like or D2-like on the basis of their pharmacology. However inhibition of the slow outward current by sulpiride suggests that it is mediated by a receptor with D2-like properties. The effect of sulpiride showed selectivity and did not antagonise the Fast Response.

It is clear that the pharmacological characterisation of the Fast Response is far from complete. It would be useful to test if other agonists of dopamine receptors can mimic the response. This would help to test that the response is due to activation of a dopamine receptor and not the nonspecific activation of another receptor type. It is also hoped that the effect of certain antagonists can be repeated on the unitary currents observed in outside-out patches.

VI.4.2. Effect of agonists on the GDN

The effect of certain neurotransmitters were screened on the GDN. They were chosen due to the likelihood that they may have activated the same receptor as dopamine to evoke the Fast Response.

5-Hydroxytryptamine (5-HT) is present in the CNS of *H. trivolvis*, it bears structural similarity to dopamine and it has been shown to activate a ligand gated ion channel. This made it a likely candidate as an agonist of the Fast Response. However, it evoked a slow depolarising response when applied to the GDN, showing that the Fast Response is not due to activation of a 5-HT receptor.

Acetylcholine is another transmitter present in the CNS of snails where it can evoke fast depolarising responses blocked by curare. Acetylcholine evoked a fast inward current in the GDN, but the response was different to that evoked by dopamine. The main difference was that the acetylcholine response was potentiated by successive applications, rather than desensitised. This difference suggests that acetylcholine acts on a different receptor to produce its effect.

The molluscan neuropeptide FMRFamide has been shown to directly gate two ion channels in snail neurones (Cottrell et al. 1990; Green, Falconer & Cottrell 1994). Its effect on the GDN appeared very similar to that of dopamine, a fast inward current followed by a slow outward current. The fast inward current also showed marked desensitisation and was blocked by amiloride. Finally bath application of FMRFamide desensitised the dopamine Fast Response, and bath application of dopamine desensitised the FMRFamide fast response. These observations combined appeared to suggest that dopamine and FMRFamide activated the same receptors on the GDN. However, differences were observed between the two responses which made this unlikely. Curare had no effect on the FMRFamide response, and the receptors for the two agonists had completely different distributions on the surface of the GDN.

The cross desensitisation between the two responses is interesting. It suggests that the fast responses evoked by the two different agonists have a common mechanism of desensitisation. Most ligand gated channels described have been shown to be regulated by protein phosphorylation (Swope et al. 1992). Binding of both dopamine and FMRFamide to cell surface receptors, not necessarily those producing the Fast Response, may result in activation of a common protein kinase which is able to phosphorylate sites on both the

FMRFamide and dopamine receptors producing desensitisation of them both. This interaction is further complicated by the second effects (slow outward currents) of both dopamine and FMRFamide.

VI.5. PHYSIOLOGICAL SIGNIFICANCE OF THE FAST RESPONSE

The Fast Response of the GDN was always accompanied by a slow outward current. Fast excitatory post synaptic responses observed in follower neurones of the GDN are often also part of a similar biphasic response. This is particularly true of those responses with a similar time course and pharmacology to the Fast Response of the GDN. These two responses appear to be linked, but it seems unlikely that both responses are mediated via the same receptor. It is possible to preferentially block one or other of the responses using antagonists which exert their effect by influencing agonist binding (e.g. sulpiride).

The effect of the combined response is to produce a very brief excitation of the follower neurone, followed immediately by prolonged inhibition. The inhibition is two fold, due to hyperpolarisation by the slow response and inactivation of the Fast Response by desensitisation. It can be envisaged that a prolonged dopaminergic input onto a follower neurone with the biphasic dopamine response, could be converted into a single action potential followed by an extended period of inhibition in the follower neurone.

The Fast Response of the GDN and of its follower neurones is extremely small in amplitude when there is a localised input. As a result the effect of the Fast Response on the neurones' excitability is minimal. However it may be possible to evoke a large response with a simultaneous dopaminergic input at multiple sites on the neurone. The effect of this would be that different,

temporally spaced inputs would merely inhibit the neurone, but the simultaneous arrival of many inputs would briefly excite the neurone before producing inhibition.

The voltage dependence of the Fast Response has so far only been observed in the GDN and it is not clear whether the response is due to the activation of one or more different receptors. The depolarised potential at which the Fast Response starts to increase in amplitude means that this part of the conductance would only be active during stimulation of the GDN resulting in action potentials. Activation of the NMDA receptor whose associated current shows voltage dependence, is linked with the initiation of long term potentiation (Alberts et al. 1994). The generation of a region of negative slope conductance has been shown to confer bursting properties on some neurones (Wilson & Watchell 1978), but the rapid desensitisation of the Fast Response makes this effect improbable.

There is a long history of observations made on molluscan neurones being carried forward to mammalian systems. In the longer term it is hoped that the presence of a ligand gated dopamine receptor will be revealed within the mammalian CNS.

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