University of Southampton

SYNAPTIC INHIBITION IN THE RAT HIPPOCAMPUS: INVESTIGATION OF THE ACTIONS OF 2-HYDROXY-SACLOFEN.

by

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A thesis submitted for the degree of
Doctor of Philosophy

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University of Southampton

September 1993

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE
DEPT. PHYSIOLOGY & PHARMACOLOGY

Doctor of Philosophy

SYNAPTIC INHIBITION IN THE RAT HIPPOCAMPUS: INVESTIGATION OF 2-HYDROXY-SACLOFEN

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In the hippocampus, γ aminobutyric acid (GABA) acts as an inhibitory neurotransmitter via ligand gated $GABA_A$ and G protein -coupled $GABA_B$ receptors. GABA can also inhibit synaptic transmission via activation of presynaptic $GABA_B$ receptors on the terminals of local interneurones and excitatory afferents. These multiple sites of control allow a number of possibilities for modulation of physiological and pathological processes.

In order to address the role of inhibition in the modulation of excitatory transmission, standard extracellular techniques were used to record population spikes from CA1 pyramidal cells in response to stimulation of afferent fibre tracts. Two components of inhibition, $GABA_A$ (10-30msec) and $GABA_B$ (300msec) were measured which could be blocked by bicuculline and phaclofen respectively. The selective $GABA_B$ antagonist 2-hydroxy-saclofen (2-OH-S) was used to investigate inhibition mediated by $GABA_B$ receptors. In addition to attenuating the late inhibition 2-OH-S unexpectedly depressed the conditioning response and the early inhibition. These effects could be blocked by phaclofen but not bicuculline suggesting that increased activation of $GABA_A$ receptors due to disinhibition of inhibitory interneurones was not involved.

To investigate these anomalous effects and the physiological role of pre and postsynaptic inhibition mediated by $GABA_B$ receptors, isolated IPSC's were recorded from single CA1 pyramidal cells. The $IPSC_A$ and $IPSC_B$ were both depressed by 2-OH-S. The latency to peak of the $IPSC_A$ correlated with the latency to peak of the early inhibition. However the latency to peak of the $IPSC_B$ (175msec) was less than the latency to peak of the late inhibition (300msec), which was similar to the latency to peak of the paired pulse depression of the EPSC. With the postsynaptic K^+ channel blocked, both 2-OH-S and baclofen depressed the EPSC via activation of presynaptic $GABA_B$ receptors. Unlike baclofen, there appeared to be no evidence for an action of 2-OH-S at the postsynaptic site in any of these experiments. These results could be explained by a partial agonist action of 2-OH-S at presynaptic $GABA_B$ receptors.

Within the intact slice, activation of afferent fibres leads to an EPSC in the pyramidal cells and subsequent cell firing. Activation of postsynaptic $GABA_A$ receptors produces a fast inhibition and is followed by a slow postsynaptic $GABA_B$ inhibition, both of which prevent repetitve firing. Activation of presynaptic $GABA_B$ receptors on excitatory terminals by a 'spillover' of GABA in the synapse, appears to account for the depression, at a longer latency, of the EPSC and the late phase of paired pulse inhibition in the intact hipocampal slice.

To My Parents

Acknowledgements

"To (Two) Be (B's) or Not To (Two) Be (B's) ?" While the answer to the second remains controversial, the other one is answered in the completion of this thesis, with the help of my parents, who supported me through the good, the bad and the ugly....
I love you both.

I also want to say thank-you to my supervisor, John Chad, for giving me a lot of support throughout the three years, to Lars Sundstrom and Howard Wheal, for listening and advising and to everyone else in the lab past and present (who I'm sure know their own names by now) for their individual and endearing qualities!

It's been a great three years!

Preface

Extracellular recordings of a population of cells, coupled with whole cell patch recordings of single cells, were used to study the mechanisms of GABAergic inhibition mediated by pre- and postsynaptic $GABA_B$ receptors, in the CA1 area of the hippocampal slice. Investigations of the pre- and postsynaptic $GABA_B$ receptors, in particular, the actions of 2-hydroxy-saclofen, were focused on. **Chapter 1** gives an overview of the anatomy and physiology of the hippocampus. **Chapter 2** provides an explanation of the methods and materials used. **Chapter 3** describes the isolation of the inhibitory phases using paired pulse stimuli of the population of cells. **Chapter 4** deals with the actions of 2-OH-S on the extracellular responses. **Chapter 5** outlines the recording of EPSC's and IPSC's from single cells using the whole cell voltage clamp technique. **Chapter 6** looks at the actions of 2-OH-S and other ligands on the isolated IPSC's and EPSC's, paired pulse depression of the EPSC and the mechanisms of pre- and postsynaptic inhibition in a single cell. Finally, **Chapter 7** brings together the results of chapters 3-6 and discusses their relevance in respect of what is currently known about GABAergic inhibitory mechanisms mediated by $GABA_B$ receptors in the hippocampus.

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Abbreviations

ACSF	artificial cerebro-spinal fluid
AMPA	α–amino-3-hydroxyl-5-methyl-isoxole proprionic acid
3-APPA	3-amino-propylphosphinic acid
D-APV	D-2 amino-5 phosphonovavaleric acid
ATP	adenosine tri-phosphate
Bac	(-) baclofen
Bic	bicuculline
C	conditioning response
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
Cd^{2+}	cadmium
CNQX	6 cyano, 7 nitroquinoxaline 2,3 dione
CNS	central nervous system
CPP	3 ((R)-carboxypiperizine-4-yl)-propyl-1-phosphinic acid
CSLM	confocal scanning laser microscope
DAVA	8-aminovaleric acid
EGTA	ethyl eneglycol bis(β-aminoethylether)NNN',N' tetraacetic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
GABA	γ-amino butyric acid
GAD	glutamic acid decarboxylase
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
IR	input resistance
IV	Current/Voltage
L/M	lacunosum/moleculare
LTP	long term potentiation
mOsM	milliosmoles
min	minute
mM	millimole
μM	micromole
mRNA	messenger ribonucleic acid
msec	millisecond
mV	millivolt
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
O/A	oriens/alveus
PCP	phencyclidine
PDS	paroxysmal depolarising shift
Phac	phaclofen
PKC	protein kinase C
PKA	protein kinase A
PTX	pertussis toxin
QX-314	(N-(2,6 dimethyl-phenylcarbamoylmethyl)-triethylammonium bromide
2-OH-S	2-hydroxy-saclofen
s.e.m.	standard error of the mean
SOM	somatostatin
SR	stimulus/response
T	test response
THA	tetrahydroaminoacridine
THIP	4,5,6,7-tetrahydro-iso-oxazolo (5,4-c)pyridine 3-ol)
TLE	temporal lobe epilepsy
TRANS	transverse axis

trans ACPD	trans D,L-amino 1,3,cyclo-pentane-dicarboxylic acid
s.e.m.	standard error of the mean
SOM	somatostatin
TTX	tetrodotoxin
V	volts
VIP	vaso-intestinal-polypeptide
ω-cgtx	omega-conotoxin

CHAPTER ONE

1

General Introduction

This study is primarily concerned with inhibitory synaptic transmission mediated by the neurotransmitter, γ-amino butyric acid (GABA), in the hippocampus. The balance between excitation and inhibition is critical in the hippocampus *in vivo*. Disruption of the balance can lead to synchronous activation of the cellular network and excessive electrical discharges. The available literature presents a comprehensive characterisation of the excitatory receptor mechanisms has been made under physiological and pathological conditions both *in vitro* and *in vivo*.. Studies on inhibitory synaptic transmission have revealed a fast postsynaptic inhibition mediated by $GABA_A$ receptors, a slow postsynaptic inhibition mediated by $GABA_B$ receptors and a presynaptic inhibition mediated by $GABA_B$ receptors. There is a good understanding of the physiological role of the $GABA_A$ receptors in modulating synaptic transmission which is not as apparent for the pre and postsynaptic $GABA_B$ receptors.

At the time of commencing this work, most of the information available about the mechanisms of action of $GABA_B$ receptors was centred on the slow inhibition mediated by the postsynaptic $GABA_B$ receptor. Much less was known about the mechanisms of action of the presynaptic receptor and how it might differ from the postsynaptic receptor. Although GABA and baclofen had been shown to block the release of GABA and to depress excitatory transmission, a physiological role for the involvement of presynaptic $GABA_B$ receptors in the modulation of excitatory synaptic transmission had not been established. The heterogeneity of the pre and postsynaptic receptors was still very much in debate and there were no pre or post selective antagonists or agonists available.

The original aim of this study was to use the available $GABA_B$ antagonist 2-hydroxy-saclofen, to investigate the role of pre and postsynaptic $GABA_B$ receptors in the slow inhibition and the modulation of excitatory synaptic transmission at both the network (extracellular recording) and the single cell (patch clamp) level.

In order to understand the inhibitory mechanisms, it is of importance to have an idea of the structure and connectivity of the cells in which the inhibition is occurring. The following chapter is aimed at providing an insight into the nature of the anatomy and circuitry of synaptic transmission in the hippocampus, in particular the CA1 area.

1.1 Gross Anatomy And Circuitry

Evolution and Structure

The hippocampal formation was one of the first areas of the wall of the forebrain to become differentiated in primitive vertebrates. Its development was intermediate between the olfactory paleocortex and the neocortex. During evolution, as the forebrain expanded, the hippocampus was pushed and dragged around until it became attached to the temporal lobe, finally moving completely within it. In primates, the hippocampus can be found slightly behind and medial to the amygdala. It belongs to the limbic cortex, part of the limbic system and through adjacent structures and their projections, it is able to receive and send many projections, allowing it to influence activity in a large number of brain regions.

The three dimensional shape of the hippocampal formation is relatively complex (**Figure 1.1**). In the rat, it has a slightly twisted and curved septotemporal axis. In a horizontal section of rat brain, the subiculum, parasubiculum and entorhinal cortex (also known collectively as the parahippocampal region) are located behind the hippocampal formation. The name itself was derived by early histologists who likened the shape of the hippocampus to that of a sea-horse (hippocampus). The hippocampus proper (CA1-CA4) is sometimes referred to as Ammon's Horn, for its resemblance to a ram's horn.

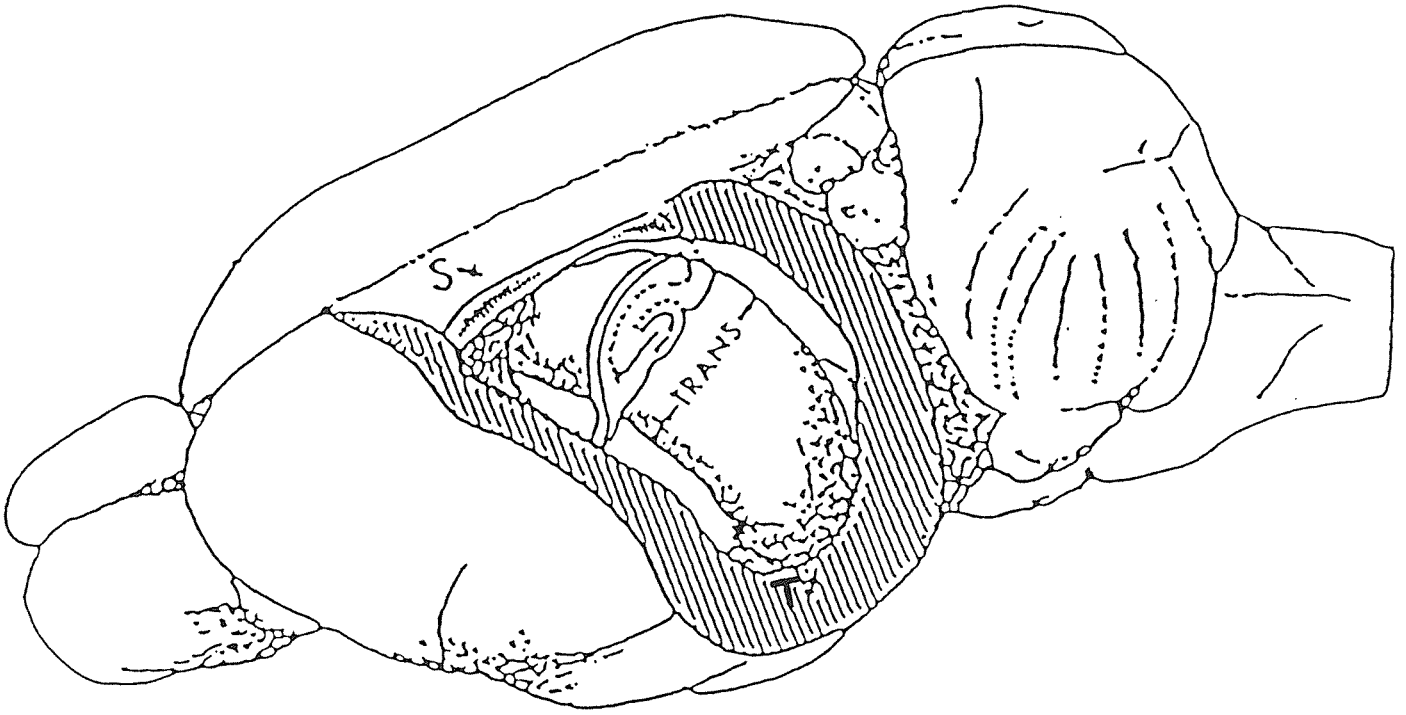


FIGURE 1.1

Taken from Amaral & Witter, (Amaral & Witter, 1989)

The position of the hippocampal formation in the rat brain is shown in this drawing of a preparation in which the cortical surface has been removed. The hippocampus is an elongated C-shaped structure with the long or septotemporal axis running from the septal nuclei rostrodorsally (S) to the temporal cortex (T) ventrocaudally. The short or transverse axis (TRANS) is orientated perpendicular to the septotemporal axis. The major fields of the hippocampal formation (except for the entorhinal cortex) are found in slices taken approximately midway along the septotemporal axis.

Functions

The highly organised structure of the hippocampal formation has attracted scientists to study a large number of aspects of it's physiology and pharmacology. It's primordial connections to the hypothalamus would appear to suggest a close involvement and role in functions such as endocrine control and expression of emotional states. It receives and integrates information from each of the sensory modalities and projects back to complex association areas, influencing, at the cortical level, somatomotor, visceral, motivational, affective and cognitive mechanisms. The plasticity of its connections also provide an important role for it in learning and memory in humans and rats (O'Keefe & Nadel, 1988; Scoville & Milner, 1957). A widely known and studied dysfunction of central hippocampal neurones is epilepsy, where many spontaneous seizures begin (Lothman,Bertram, & Stringer, 1991).

1.2 Gross Structure and Connectivity

The hippocampal formation consists of four regions: 1) the dentate gyrus; 2) the hippocampus proper (areas CA4, CA3, CA2, CA1); 3) the subicular complex (subiculum, presubiculum, parasubiculum); 4) and the entorhinal cortex (medial, lateral). The latter two regions are often grouped together and referred to as the parahippocampal region. The hippocampal cortex has also been subdivided into a number of layers, namely the alveus, stratum oriens, -pyramidale, -radiatum, -lucidum, -lacunosum and -moleculare (Ramon y Cajal, 1893). The division between the last two is not well defined in the rodent and is therefore more commonly referred to as the stratum lacunosum-moleculare (**Figure 1.2**). There are two morphologically and physiologically distinct groups of cells in the hippocampus, the pyramidal and non-pyramidal cells. (Lorente de No, 1934). The pyramidal cell bodies are found arranged in a thin sheet around 300μm below the ventricular surface in the stratum pyramidale of areas CA1,2,3 and 4, being about 20-40μm diameter across at the base and 40-60μm in height. There is a gradation in size from area CA1 to CA3, the latter having larger cell bodies and shorter, stouter dendrites. The largest cells in area CA3 are known as giant pyramidal cells. The pyramidal cells have tufted basal dendritic trunks which extend to the stratum lacunosum-moleculare and receive most of the excitatory contacts (Andersen,Blackstad, & Lomo, 1966). Myelinated axons from CA1 cells form the alveus where they can connect with and activate other pyramidal cells and interneurones, while outputs from CA3 form the fimbria. From there they form the fornix through which they pass in a long arc anteriorally and then ventrally to their projection sites deep in the brain.

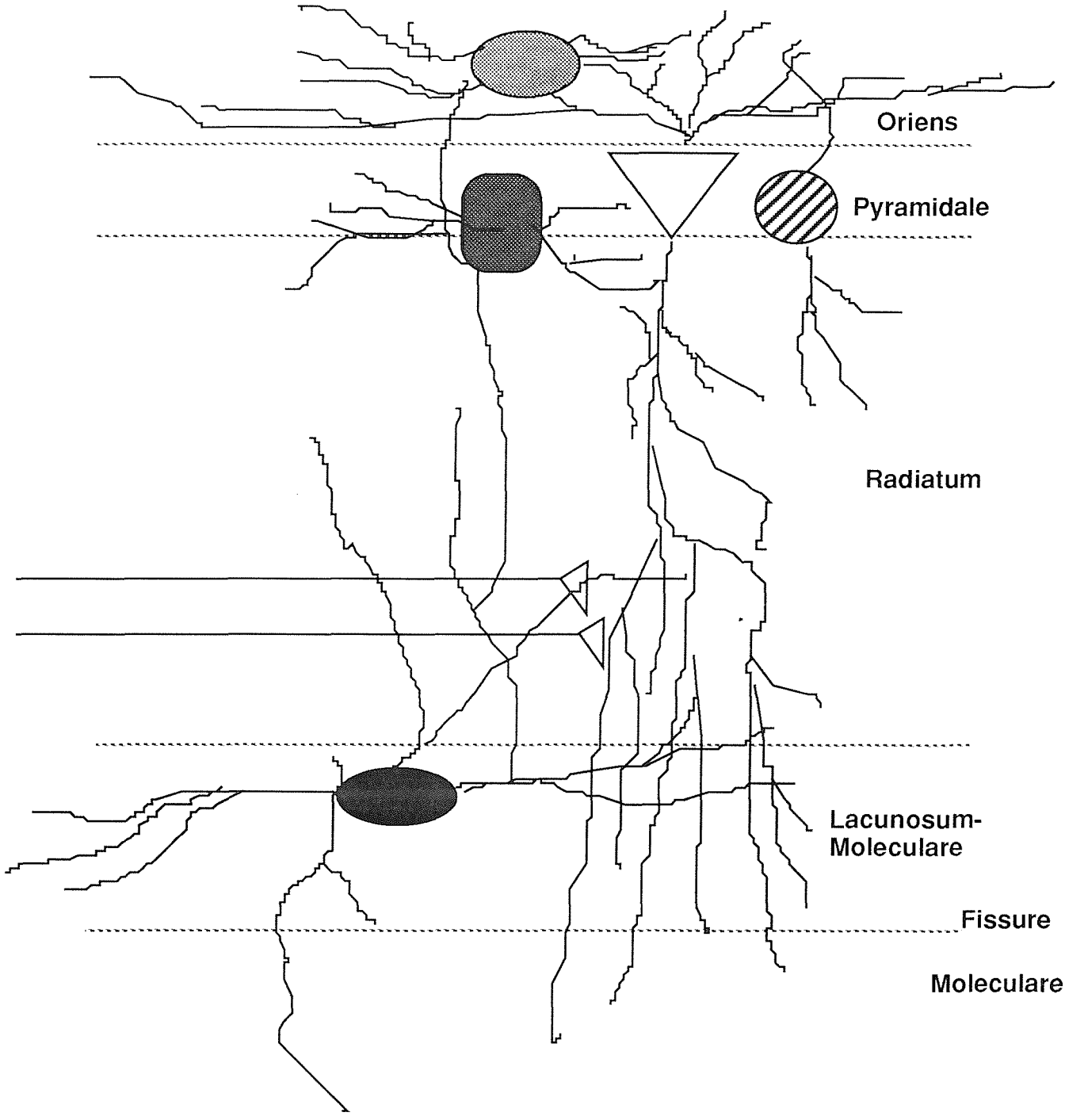


Figure 1.2 Layering in the CA1 region of the hippocampal slice.
The pyramidal cell ○ and a number of different interneurones are shown in the locations in which they have been identified. These include the basket cells ● the L/M cells ● axoaxonic cells ⊘ and oriens/alveus cells ◍

The basic connections of the hippocampus were elucidated by the classic Golgi studies of Ramon y Cajal and Lorente de No (Ramon y Cajal, 1893; Lorente de No, 1934) and by a number of degeneration studies (Blackstad, 1956; Blackstad, 1958; Raismann,Cowan, & Powell, 1965; Blackstad,Brink,Hern, & Jeune, 1970). The progression of activity is manifested as a trisynaptic pathway, where each of the fields are linked by unique and largely unidirectional connections, providing a natural sequence in these regions as seen below:

Trisynaptic Pathway (1,2,3 = synapse)

Entorhinal Cortex-- (1) --Dentate Gyrus-- (2) --CA3-- (3) --CA1--Subiculum--E.Cortex

The hypothesis of a lamellar organisation proposed by Andersen & colleagues (Andersen,Bliss, & Skrede, 1971b), suggested that the principal excitatory pathways of the hippocampal formation were organised in a lamellar fashion. In the light of new techniques available, this hypothesis has been challenged by Amaral & Witter (Amaral & Witter, 1989). It would appear from their experiments that aside, perhaps, from mossy fibres for which a lamellar organisation has been demonstrated (Blackstad et al., 1970), none of the intrinsic connections are organised in a true lamellar fashion. The previous view was thought to be oversimplified, not taking into account the longitudinal pathways which run at right angles to the trisynaptic pathway and extend out of the transverse plane.

Beginning in the entorhinal cortex the perforant fibres forming the temporodentate pathway traverse the surrounding cortex and terminate in the dentate gyrus thus giving rise to the first synapse in the trisynaptic pathway. The granule cells of this area project their mossy fibres to the CA3 field of the hippocampus proper forming the second synapse in the pathway. Cells of the CA3 field give rise to axons which travel out into the fimbria and form commisural connections with CA1 pyramidal cells in the contralateral hippocampus (Andersen, 1959; Raismann et al., 1965). Myelinated (Schaffer) collaterals arising from ipsilateral CA3 neurones are sent via the stratum radiatum to form *en passage* synapses with the distal portion of the CA1 pyramidal cell apical dendrites forming the third synapse. Axons of the CA1 field run in the alveus to join the fimbria which projects in turn to the septum, hypothalamus and via the subiculum to the entorhinal cortex of the contralateral hippocampus.

Entorhinal Cortex

The entorhinal cortex is divided into two areas, medial and lateral and consists of six layers. The divisions are based upon cytoarchitectonic differences and variations in the efferent and afferent projections (Lorente de No, 1933; Ramon y Cajal, 1955; Amaral, Insausti, & Cowan, 1987). The fibres, which primarily arise from layers II and III (Ruth,Collier, & Routtenberg, 1965; Steward & Scoville, 1976) exit the cortex, enter the underlying white matter and angular bundle and then traverse the pyramidal cell layer of the subiculum (Lorente de No, 1934; Ramon y Cajal, 1968; Witter,Groenewegen,Lopes Da Silva, & Lothman, 1989) thus either crossing the hippocampal fissure to the dentate gyrus, constituting the perforant path or temporodentate tract, or distributing to the molecular layer of the subiculum and CA3/CA1 forming the temporoammonic tract.

The fibres of the perforant path (mainly layer II) branch along the septotemporal axis and synapse on the outer two thirds of the dentate gyrus. Fibres from the medial area synapse upon the outer third and fibres from the lateral area upon the middle third (Steward, 1976; Witter et al., 1989). The main source of fibres synapsing in the molecular layer of areas CA1 and CA3 arise from layer III. In area CA3, fibres from the lateral area terminate on the outer third and fibres from the medial area on the middle third (Steward, 1976; Witter & Groenewegen, 1984). In CA1, lateral area fibres terminate at more distal areas close to the subiculum whereas medial area fibres synapse in the proximal area close to CA3. The entorhinal cortex sends projections on the ipsilateral side of the cortex to many areas, including the piriform cortex, prefrontal cortex and cingulate gyrus (Van Hoesen & Pandya, 1975b; Wyss, 1981; Sorensen, 1985). Less dense projections are sent to the contralateral side. It is also reciprocally connected to the piriform cortex, septal nuclei, amygdala, cingulate gyrus and inferior temporal cortex (Van Hoesen & Pandya, 1975a; Van Hoesen & Pandya, 1975b; Haberley & Price, 1978; Alonso & Kohler, 1984; Insausti, Amaral, & Cowan, 1987a).

Subicular Complex

The subicular complex consists of the subiculum, presubiculum and parasubiculum. It receives most of the output from the hippocampus and in turn projects back to the association neocortex directly and via thalamic relays (Rosene & Van Hoesen, 1977). The subiculum begins at the end of area CA1 and is characterised in the rodent by the rapid flaring of the pyramidal cells. The pre- and parasubiculum can be found intercalated between the subiculum and entorhinal cortex (Chronister & White, 1975). These two regions can be distinguished by variations in their neuronal populations (Ramon y Cajal,

1955). Fibres from the entorhinal cortex terminate in the outer two thirds of the molecular layer of the subiculum (Witter et al., 1989) and are thought to be excitatory (Van Groen,Van Haren,Witter, & Groenewegen, 1986a; Van Groen & Lopes da Silva, 1986b). Reciprocal projections also exist between the perirhinal cortex and the subiculum (Kosel,Van Hoesen, & Rosene, 1983). Projections are sent to the pre- and parasubiculum which in turn send their fibres to layers I, II & III of the entorhinal cortex (Van Groen & Wyss, 1990). Extrinsic connections are sent to the lateral and posterior septal nuclei, the bed nucleus of the stria terminalis, the amygdala, the midline and anterior thalamic nuclei and the mammiliary bodies (Swanson & Cowan, 1977; Van Groen & Wyss, 1990). It also receives fibres from the medial septal nucleus as part of the hippocampal septal system (Swanson,Kohler, & Bjorklund, 1987).

Dentate Gyrus

The dentate gyrus is the major target of the perforant path projection arising from the entorhinal cortex and forms the first synapse of the trisynaptic pathway (Lorente de No, 1934; Ramon y Cajal, 1968). It can be distinguished by a relatively small band of densely packed granule cells which are the principal cell type in this area. There are three layers in the dentate area: 1) the molecular layer, containing the granule cell dendrites where the perforant path terminates; 2) the granule cell layer; 3) the polymorphic layer, containing granule cell axons and other neurones. In the rat, two 'blades' of granule cells can be seen which meet at an apex and form a 'V' shape, between which lies the dentate hilus containing the mossy fibre axons. The granule cells give rise to the mossy fibres which enter the CA3 field, forming *en passant* synapses on the proximal apical dendrites of the pyramidal cells (Blackstad et al., 1970; Gaarskjaer, 1978; Claiborne,Amaral, & Cowan, 1986). The mossy fibre projection appears to promote the strongest evidence for a lamellar organisation (Amaral & Witter, 1989). Hilar mossy cells found in the polymorphic layer give rise to connections known as associational or commisural connections on the basis of whether they terminate in the dentate either ipsi- or contralaterally respectively, (Zimmer, 1971; Hjorth-Simonsen & Lauberg, 1977; Laurberg, 1979; Laurberg & Sorensen, 1981). The ipsilateral projection is thought to be organised to promote integration of information along the long axis of the dentate gyrus (Amaral & Witter, 1989). It has been suggested that the dentate gyrus may serve to integrate activity along the trisynaptic pathway via mossy fibres and at right angles to this along the associational pathway (Lothman et al., 1991). The neurotransmitter at the mossy fibre / CA3 synapse is thought to be glutamate (Ottersen & Storm-Mathisen, 1985). Immunocytochemistry has revealed cells containing GABA, cholecystokinin (CCK), vasoactive intestinal polypeptide (VIP), somatostatin

(SOM) and neuropeptide Y (NPY) (Bakst,Morrisen, & Amaral, 1985) and glutamate (Sloviter & Nilaver, 1987) .

The Hippocampus Proper (Ammon's Horn)

This comprises areas CA4, CA3, CA2 & CA1. The most likely candidates for the role of excitatory neurotransmitter in Ammon's horn CA3/CA1 synapse are glutamate and aspartate (Storm-Mathisen, 1977a; Cotman & Nadler, 1981; Storm-Mathisen, Leknes, Bore, Vaaland, Edminson, Haug, et al., 1983; Ottersen & Storm-Mathisen, 1985).

The CA3 field forms part of the hippocampus proper and is quite often mixed with area CA2, although CA2 neurones can be distinguished by their lack of dendritic spines. CA3 pyramidal cells receive large mossy fibre input from dentate granule cells on their proximal apical dendrites and projections from the entorhinal cortex. They in turn send out projections, the Schaffer collaterals, commisural afferents and the longitudinal association bundle (Schaffer, 1892; Ramon y Cajal, 1893; Lorente de No, 1934; Swanson, Sawchenko, & Cowan, 1980; Swanson, 1982). The Schaffer collaterals form the last part of the trisynaptic pathway and project to pyramidal cells in the CA1 field. Proximal CA3 neurones project to CA1 more septally while those nearer CA1 project to a more temporal level (Ishizuka,Weber, & Amaral, 1990). The projections travel both contra- and ipsilaterally to pyramidal cells and interneurones and are extensive along the entire septotemporal axis. Commisural connections are sent along the ventral hippocampal commisure to contralateral CA3 and CA1 areas, synapsing with pyramidal cells and interneurones. The longitudinal association bundle consists of CA3 fibres which remain in the CA3 field. Few fibres are seen entering the subiculum and no projections are found to the entorhinal cortex. Some CA3 and CA2 projections have been observed to the hilus and dentate areas (Ishizuka et al., 1990).

The CA1 field extends to the subiculum, with easily identifiable, densely packed, pyramidal cells. Basal dendrites of the CA1 pyramidal cells extend to the stratum oriens while apical dendrites extend throughout stratum lucidum, radiatum and lacunosum-moleculare, depending on their branching and synaptic input. The CA1 area is thought to form the major output area for the hippocampus, sending axons via the fornix and fimbria. It receives a large input from CA3 both ipsi- and contralaterally as well as projections of the temporoammonic tract from the entorhinal cortex. It, in turn, sends efferents to the ipsilateral subiculum (Finch & Babb, 1981) and the entorhinal cortex (Finch,Wong,Derian, & Babb, 1986).

CA2 field is often grouped with CA3 field and they cannot always be distinguished. Both associational and schaffer collateral projections also arise from CA2. The pattern of connections is thought to resemble most closely the distal part of CA3 (Ishizuka et al., 1990). The CA4 field is located in or just outside the dentate hilus and leads into CA3. In the rodent there is very little distinction between the polymorphic layer of the dentate and the CA4 neurones.

Extrinsic Connections

There is a massive projection from the hippocampus proper to the lateral septal nucleus (Swanson,Sawchenko, & Cowan, 1981) which forms the first part of a feedback loop between the hippocampus and the septum, as follows: Hippocampus -Lateral Septum - Medial Septum - Hippocampus (Milner & Amaral, 1984). Many biochemical studies indicate the cholinergic nature of the septohippocampal projections (Fonnum, 1970; Nadler,Cotman, & Lynch, 1974; Amaral & Kurtz, 1985) although GABAergic innervation is quite prominent (Kohler,Chan-Palay, & Wu, 1984; Schwerdtfeger, 1986; Freund & Antal, 1988; Gulyas,Seress,Toth,Ascady,Antal, & Freund, 1991) and substance P fibres are also present (Baisden,Woodruff, & Hoover, 1984). Septal fibres act in an excitatory manner to generate rhythmic, slow, theta activity (Swanson,Teyler, & Thomson, 1982; Stewart & Fox, 1989).

The hippocampus has been shown to send excitatory afferents to the amygdala (Ottersen, 1982; Mello,Tan, & Finch, 1992). Projections can also be found from the subiculum to the thalamus and hypothalamus (Swanson & Cowan, 1977). Connections from the hypothalamus to all areas of the hippocampus have been shown (Haglund,Swanson, & Kohler, 1984) as well as projections from the thalamus to the stratum lacunosum of area CA1 (Herkenham, 1978).

Projections from the serotonin containing cells of the raphe nuclei to the hippocampus have been studied (Azmitia & Segal, 1978) and it is thought that they provide serotonergic modulation of granule cells by feed forward inhibition (Assaf & Miller, 1978; Winson, 1980). There are also extensive septal and raphe inputs onto the GABAergic cells of the hippocampus (Miettinen & Freund, 1992).
Catecholaminergic fibres enter the hippocampus from the locus coerulus through the fimbria-fornix and are widely distributed throughout (Blackstad,Fuxe, & Hokfelt, 1967; Storm-Mathisen, 1977a). The transmitter used is noradrenaline, which has excitatory actions in the dentate gyrus and inhibitory actions in CA1, CA3.

1.3 Excitatory Synaptic Transmission In The Hippocampus

Pyramidal cells in CA1 receive a number of excitatory afferents as described in the previous pages, the most prominent being those arising from the ipsi- and contralateral CA3 neurones. Many of the excitatory contacts are found on the dendritic spines (Andersen,Blackstad, & Lomo, 1966). Glutamate and aspartate are generally held to be the main excitatory transmitters in the central nervous system and at the synapses on CA1 neurones (Watkins & Evans, 1981; Monoghan,Bridges, & Cotman, 1989; Lodge & Collingridge, 1990). Activation of presynaptic terminals releases glutamate which activates excitatory amino acid receptors and leads to an influx of sodium or calcium which depolarises the dendrites and results in an excitatory postsynaptic potential (EPSP). If the cell is depolarised above a certain threshold, an action potential will fire and activate other pyramidal and non-pyramidal cells in the rest of the hippocampus, as well as in other areas of the brain.

Excitatory transmission can be modulated by a number of factors including inhibitory cell activity, voltage changes, other neurotransmitters and pharmacological agents which modify the receptors and associated ion channels and second messenger pathways.

There are five classes of excitatory amino acid receptors which have been well characterised with agonists and antagonists. A recent review details the molecular biology of the first two classes, the NMDA and AMPA receptors (Seeburg, 1993). The **NMDA** receptor (N Methyl- D Aspartate) has been the most well defined to date and appears to be widespread throughout the central nervous system with the highest density in the hippocampus (Greenamyre, Olson, Penney, & Young, 1985; Cotman & Iverson, 1987). It is responsible for the slow component of the EPSP in electrophysiological recordings. Selective antagonists include 2-amino-5 phosphonovavaleric acid (D-APV), 3-((R)-Carboxy piperizine-4-yl) -propyl-1 phosphinic acid (CPP), phencyclidine (PCP), MK801, and 7-Chloro-Kynurenate. The receptor is a ligand gated ion channel with several identified modulatory sites, that has been recently cloned in the rat brain (Moriyoshi, Masu, Ishii, Shigemoto, Mizuno, & Nakanishi, 1991). and a number of subtypes of this receptor exist (Kutsuwada, Kashiwabuchi, Mori, Sakimura, Kushiya, Avaki, et al., 1992).

The ion channel associated with the receptor is blocked by magnesium in a voltage dependent manner (Nowak, Bregestorski, Ascher, Herbet, & Prochiantz, 1984; Mayer, MacDermott, Westbrook, Smith, & Barker, 1987). This block is relieved when the membrane is depolarised beyond threshold for activation of the cell (Mayer et al., 1987).

Modulation of the NMDA receptor is also carried out by zinc and glycine (Wong, Kemp, Priestly, Knight, Woodruff, & Wersen, 1986; Johnson & Ascher, 1987; Peters,Koh, & Choi, 1987; Westbrook & Mayer, 1987). Occupancy of the glycine site appears to be necessary for channel activation by glutamate or NMDA. The Ca^{2+} permeability of the NMDA receptor is very important and its entry appears to trigger the main effects of receptor activation, including activation of K^+ conductance's which have long term effects on cellular excitability (Zorumski,Yang, & Fischbach, 1989). Ca^{2+} entry is probably the main cause of excitotoxicity of NMDA agonists (Meldrum & Garthwaite, 1990).

The **AMPA** receptor is thought to mediate fast excitatory neurotransmission through activation of a ligand gated cation channel (Young & Fagg, 1990) and both AMPA and NMDA receptors appear to be required for synaptic transmission. There have been 4 subtypes described to date, GluR 1-4, (Boulter, Hollman, O'Shea-Greenfield, Hartley, Deneris, Maron, et al., 1990) which each have the ability to exist in two forms, flip and flop, as a result of alternate splicing (Monyer,Seeburg, & Wisden, 1991). The distribution of each of the variants appear to become altered after kindling induced seizure activity (Kamphuis,Gorter,Wadman, & Lopes da Silva, 1992). An antagonist at the AMPA receptor is 6-cyano -7-nitroquinoxaline-2,3-dione (CNQX).

The **KAINATE** receptor may or may not be a unique receptor, studies show that kainate which is a potent excitotoxin, may exert its effects at AMPA receptors (Watkins, Krogsgaard-Larsen, & Honore, 1990).

The **L-AP4** receptor is a presynaptic autoreceptor activated by glutamate which decreases transmitter release (Baskys & Malenka, 1991). There is some evidence that L-AP4 may activate a type of metabotropic receptor that is negatively coupled to adenylate cyclase (Thomsen, Kristensen, Mulvihill, Haldemann, Suzdak, 1992).

The **METABOTROPIC** receptor is coupled to phosphoinositol metabolism and is potently stimulated by glutamate and the antagonist trans ACPD (Sladecczek,Recasens, & Bockhaert, 1988; Monoghan et al., 1989; Schoepp & Johnson, 1989; Schoepp,Bockaert, & Sladeczeck, 1990). Since their discovery they have been intensely studied and cloned (Masu,Tanabe,Truchida,Shigemoto, & Nakanishi, 1991). Activation of these receptors inhibits the slow after-hyperpolarisation, depresses calcium currents and broadens the action potential (Stratton,Worley, & Baraban, 1989; Lester & Jahr, 1990; Hu & Storm, 1991). They are postulated to have a role in long term potentiation (LTP) at some sites and are thought to be necessary for the maintenance of LTP mediated by NMDA receptors (Aniksztejn,Otani, & Ben-Ari, 1992; Zheng & Gallager, 1992)

Interactions with other neurotransmitters

Excitatory synaptic transmission, as mentioned above, is modulated by a number of other neurotransmitters which are present in the hippocampus. Transmitters which include: noradrenaline, histamine, acetylcholine, dopamine, serotonin and adenosine all appear to have a net inhibitory effect on cellular excitability. **Noradrenaline** appears to decrease population spike amplitude and increase the frequency of the inhibitory post-synaptic potentials (IPSP's) (Mueller,Kirk,Hoffer, & Dunwiddie, 1982; Mynlief & Dunwiddie, 1988) as well as playing a part in the fast IPSP (Andreasen & Lambert, 1991) possibly via a direct innervation of the GABAergic interneurones (Milner & Bacon, 1989).
Histamine depresses most hippocampal neurones via H_2 receptors (Biscoe & Straughan, 1966; Haas & Wolf, 1977; Haas, 1981b) but a facilitatory action in CA1 and CA3 has also been observed (Segal, 1981; Haas & Greene, 1986). **Acetylcholine** is thought to depress excitability via a presynaptic mechanism (Dutar & Nicoll, 1988c; Williams & Johnston, 1990) by increasing the excitability of GABAergic interneurones (Pitler & Alger, 1992). Cholinergic disinhibition in CA1 appears to arise from a decrease in excitation of interneurones and not a direct action on the interneurones themselves (Lambert & Teyler, 1991).

Dopamine is known to reduce excitability (Herrling, 1981) and a modulatory role has been suggested in elevating the threshold for spike discharge (Stanzione, Calabrese, Mercuri, & Bernardi, 1984), possibly decreasing the incidence of frequency of epileptogenic activity (Suppes, Kriegstein, & Prince, 1985). It is also thought to enhance the release of CCK, an inhibitory peptide, thus effecting another modulatory role (Brog & Beinfeld, 1992). **Serotonin** generally acts via second messenger systems in CA1 resulting in a modification of g-protein linked K^+ conductances which lead to a depression of neuronal activity (Andrade,Malenka, & Nicoll, 1986; Andrade & Nicoll, 1987; Colino & Halliwell, 1987). Recent findings suggest that serotonin acts to excite GABAergic interneurones via $5HT_3$ receptors thereby decreasing pyramidal cell activity (Ropert & Guy, 1991).

Adenosine is known to inhibit synaptically evoked transmission via A1 receptors (Nishimura,Mohri,Okada, & Mori, 1990; Alzheimer,Kargl, & Bruggencate, 1991) with pre-and postsynaptic actions (Dunwiddie & Fredholm, 1989; Gerber,Greene, & Haas, 1989; Scholz & Miller, 1991a; Thompson,Haas, & Gahwiler, 1992). It may also protect against prolonged periods of hypoxia (Boissard,Lindner, & Gribkoff, 1992). However, at low doses, it appears to have an excitatory effect due to increase of transmitter release (Okada,Sakuri, & Mori, 1992). **Taurine** causes hyperpolarisation of CA1 neurones and inhibition of evoked action potentials and may have a neuroprotective role (Taber, Linc,

Liu,Thalmann, & Wu, 1986; Magnusson, Koerner, Larson, Simullin, Skilling, & Beitz, 1991).

Neuropeptides

There are a number of peptides which also modulate excitability. The **opioids** are believed to increase excitability by acting directly at receptors on interneurones to reduce GABAergic inhibition of the cells (Lee,Dunwiddie, & Hoffer, 1980; Nicoll,Alger, & Jahr, 1980; Madison & Nicoll, 1988; Pang & Rose, 1989; Lambert,Harrison, & Teyler, 1991a; Lupica & Dunwiddie, 1991; Wimpey & Charkin, 1991). **Cholecystokinin** (CCK) is an inhibitory peptide which has been localised to interneurones in the hippocampus (Greenwood,Godar,Reaves, & Hayward, 1981; Somogyi,Hodgson,Smith,Nunzi,Gorio, & Wu, 1984; Kosaka,Kosaka,Tateshi,Hamaoka,Yanaihara,Wu, et al., 1985; Sloviter & Nilaver, 1987) and may be able to block the effects of kainic acid in CA3 neurones (Aitken,Jaffe, & Nadler, 1991). **Vasoactive intestinal polypeptide** (VIP) appears to excite pyramidal cells and reduce membrane input resistance although the exact function of this is not known at present (Dodd,Kelly, & Said, 1979). **Somatostatin** (SOM) appears to be exclusive to the interneurones of the hippocampus proper and dentate gyrus (Sloviter & Nilaver, 1987) and has both excitatory and inhibitory effects (Matsuoka,Kaneko, & Satoh, 1991; Scharfmann & Schwartzkroin, 1989). **Neuropeptide Y** (NPY) inhibits excitatory transmission in CA1 via a presynaptic action in stratum radiatum which may be due to an inhibition of a Ca^{2+} current at the terminal (Colmers,Lukowiak, & Pittman, 1985; Colmers,Lukowiak, & Pittman, 1987; Haas,Hermann,Greene, & Chan-Palay, 1987; Colmers,Lukowiak, & Pitmann, 1988). It may also have a modulatory role at the NMDA receptor complex (Roman,Pascaud,Duffy, & Lunien, 1991). **Angiotensin II** produces an increase in excitability and a reduction in inhibition (Haas,Felix,Celio, & Inagami, 1980). **Vasopressin** is also thought to increase excitability by increasing the firing rate of cells (Muhletaler,Dreifuss, & Gahwiler, 1982).

The most prominent and immediate modulation of cellular excitability is provided by the inhibitory interneurones containing gamma-amino butyric acid (GABA). Under normal conditions the pyramidal cells will fire a single action potential in response to the stimulation of an excitatory postsynaptic potential (EPSP) and further firing is prevented by means of feedforward and feedback inhibitory mechanisms. If these mechanisms are not operational then the pyramidal cells will continue to fire and become synchronised into bursts, characteristic of epileptiform seizure activity (Schwartzkroin & Prince, 1980; Malouf,Robbins, & Schwartzkroin, 1990). Therefore the study of the inhibitory mechanisms is very important to our knowledge of how to control cellular excitability in the hippocampus.

1.4 Inhibitory Synaptic Transmission In The Hippocampus

GABAergic inhibition and its role in governing pyramidal cell excitability has been the focus of much attention during the last few years. Interneurones are thought to be generated prenatally (Bayer, 1980; Amaral & Kurtz, 1985; Lubbers,Wolff, & Frotscher, 1985) and at the fifth postnatal day they establish symmetric GABAergic synapses on projecting neurones (Seress,Frotscher, & Ribak, 1989). Inhibition appears to develop postnatally after the development of excitatory transmission (Michelson & Lothman, 1989; Swann, Brady, & Martin, 1989; Fedorov, 1990). Evidence indicates a sequential neurochemical and morphological maturation of non pyramidal neurones that may be related to differences in the maturation of inhibition during hippocampal development between CA3 and CA1 areas (Swann et al., 1989; Bergmann, Nitsch, & Frotscher, 1991).

The following deals primarily with the CA1 pyramidal area of the hippocampus proper where all the work in this study was carried out.

Interneurones

Interneurones appear to play an important role in the hippocampus by opposing excitatory inputs to the pyramidal cell population. A physiological feature of interneurones is their repetitive and high frequency firing in response to afferent stimulation (Andersen, 1975; Schwartzkroin & Mathers, 1978). The spike duration is generally short, around 1.2ms intracellularly (Knowles & Schwartzkroin, 1981; Turner, 1990). A knowledge of the connectivity and functional architecture of the different interneurones is essential to understand the role of hippocampal inhibitory currents. A number of interneurones have been identified in the CA1 region of the hippocampus. In the adult, different sub-populations of interneurones can be identified by their location, cellular contents and physiological properties. There isn't a great deal known about the functional diversity of interneurones in the hippocampus. They are morphologically heterogeneous, express different transmitters and receptors and are presumably functionally heterogeneous. Their sparse distribution and relatively low numbers make direct study of their electrophysiology difficult.

Basket cells, found in the strata oriens and pyramidale, appear to be most prevalent of the interneurones in the hippocampus (Schwartzkroin & Mathers, 1978; Ashwood,Lancaster, & Wheal, 1984; Schwartzkroin & Kunkel, 1985; Kawaguchi &

Hama, 1987; Kawaguchi & Hama, 1988). They have dendrites which extend to the strata radiatum, lacunosum-moleculare and oriens, with extrinsic afferents synapsing onto them (Frotscher & Zimmer, 1983; Frotscher,Leranth,Lubbers, & Oertel, 1984; Schwartzkroin & Kunkel, 1985). This, along with other studies indicates that as well as being involved in feedback inhibition (Andersen,Eccles, & Loyning, 1964a; Andersen,Eccles, & Loyning, 1964b; Knowles & Schwartzkroin, 1981) they may also play a role in feedforward inhibition (Buszaki & Eidelberg, 1981; Buszaki & Eidelberg, 1982; Alger & Nicoll, 1982a; Ashwood et al., 1984; Schwartzkroin & Kunkel, 1985). The basket cells appear to be primarily GABAergic in CA1 (Ribak & Anderson, 1980; Frotscher et al., 1984; Sloviter & Nilaver, 1987) as do the other groups of interneurones. However, a variety of other neuroactive substances have been observed co-localised with GABA, including CCK (Greenwood, Godar, Reaves, & Hayward, 1981; Handelmann,Mayer,Beinfeld, & Oertel, 1981; Harris, Marshall, & Londis, 1985), VIP (Loren, Emson, Fahrenkrug, Bjorklund, Alumets, Hakanson, et al., 1979; Kohler, 1982), Substance P (Mclean,Skirboll, & Pert, 1983) SOM (Kunkel,Schwartzkroin, & Hendrilkson, 1983) acetylcholine (Vijayan, 1979; Kimura,McGear,Peng, & McGeer, 1980; Kimura,McGeer,Peng, & McGeer, 1981) and NPY (Milner & Veznedaroglu, 1992).

A second group of interneurones are the **Axo-axonic** (chandelier) cells which make synapses on axon initial segments of target neurones, efficiently blocking transmission of action potentials at the site at which they are generated. These cells have been seen in the neocortex and hippocampus proper (Peters, 1984) and in the dentate fascia (Soriano & Frotscher, 1989; Soriano,Nitsch, & Frotscher, 1990). They can be recognised in Golgi preparations by their characteristic axonal arborisation which comprises a large number of vertically orientated terminal bouton rows giving the appearance of a chandelier. Identification of GABA as a transmitter in these cells has also been carried out both in the hippocampus and visual cortex (Somogyi,freund,Hodgson,Somogyi,Beroukas, & Chubb, 1985; Soriano & Frotscher, 1989; Soriano et al., 1990). This would suggest they play an important role in GABA mediated inhibition but as yet there is little information as to their physiological characteristics. Studies on human epileptic temporal cortex suggest they may be involved in either the generation of epileptic activity or the control of its propagation (Kisvarday,Adams, & Smith, 1986). Because one axo-axonic cell can synapse onto the initial segment of many pyramidal cells, disruption of a few could result in a major loss of inhibitory control of excitatory transmission.

A third group of interneurones are the **Lacunosum-Moleculare** (L/M) interneurones (Lacaille & Schwartzkroin, 1988a; Lacaille & Schwartzkroin, 1988b). These were identified and found to be generally fusiform or multipolar, with aspinous, beaded dendrites that projected to the strata lacunosum-moleculare, radiatum and oriens. The axons

branched in stratum radiatum and coursed towards stratum pyramidale. Some axons and dendrites also appeared to be directed to the stratum moleculare of the dentate area and were sometimes found crossing the hippocampal fissure. As well as receiving afferents from fibres in CA1, they may also receive afferents from the dentate gyrus, the raphe area and inputs from the medial septum (Freund & Antal, 1988; Freund,Gulyas,Ascady,Gorcs, & Toth, 1990). Experiments showed that they received both excitatory and inhibitory inputs. It is suggested that they mediate solely feedforward inhibition and contribute to the late, slow component of the IPSP. They stain positive for glutamic acid decarboxylase (GAD) (Ribak,Vaughan, & Saito, 1978; Kosaka,Kosaka,Tateshi,Hamaoka,Yanaihara,Wu, et al., 1985) and form symmetric synapses upon hippocampal dendrites (Kunkel,Lacaille, & Schwartzkroin, 1988). Simultaneous intracellular recordings have provided an indication of their inhibitory nature (Lacaille & Schwartzkroin, 1988b). Recent intracellular studies have shown the presence of low threshold Ca^{2+} currents in these cells which are thought to be important in the generation of hippocampal theta activity (Lacaille & Schwartzkroin, 1988a; Lacaille & Schwartzkroin, 1988b; Fraser & MacVicar, 1991).

A fourth group are the **Oriens/Alveus** (O/A) interneurones (Lacaille,Mueller,Kunkel, & Schwartzkroin, 1987; Lacaille & Williams, 1990). These appear on the oriens/alveus border in the CA1 region and possess axons which branch profusely in stratum oriens and pyramidale. They are directly excited by pyramidal cells and major hippocampal afferents (Lacaille et al., 1987). This suggests that they may mediate both feedforward and feedback inhibition (Lacaille et al., 1987; Lacaille & Williams, 1990). Recently a second type of interneurone has been suggested in this region on the basis of electrophysiological studies (Williams & Lacaille, 1992).

The location and connectivity of all of these cells can be seen in **Figure 1.3**

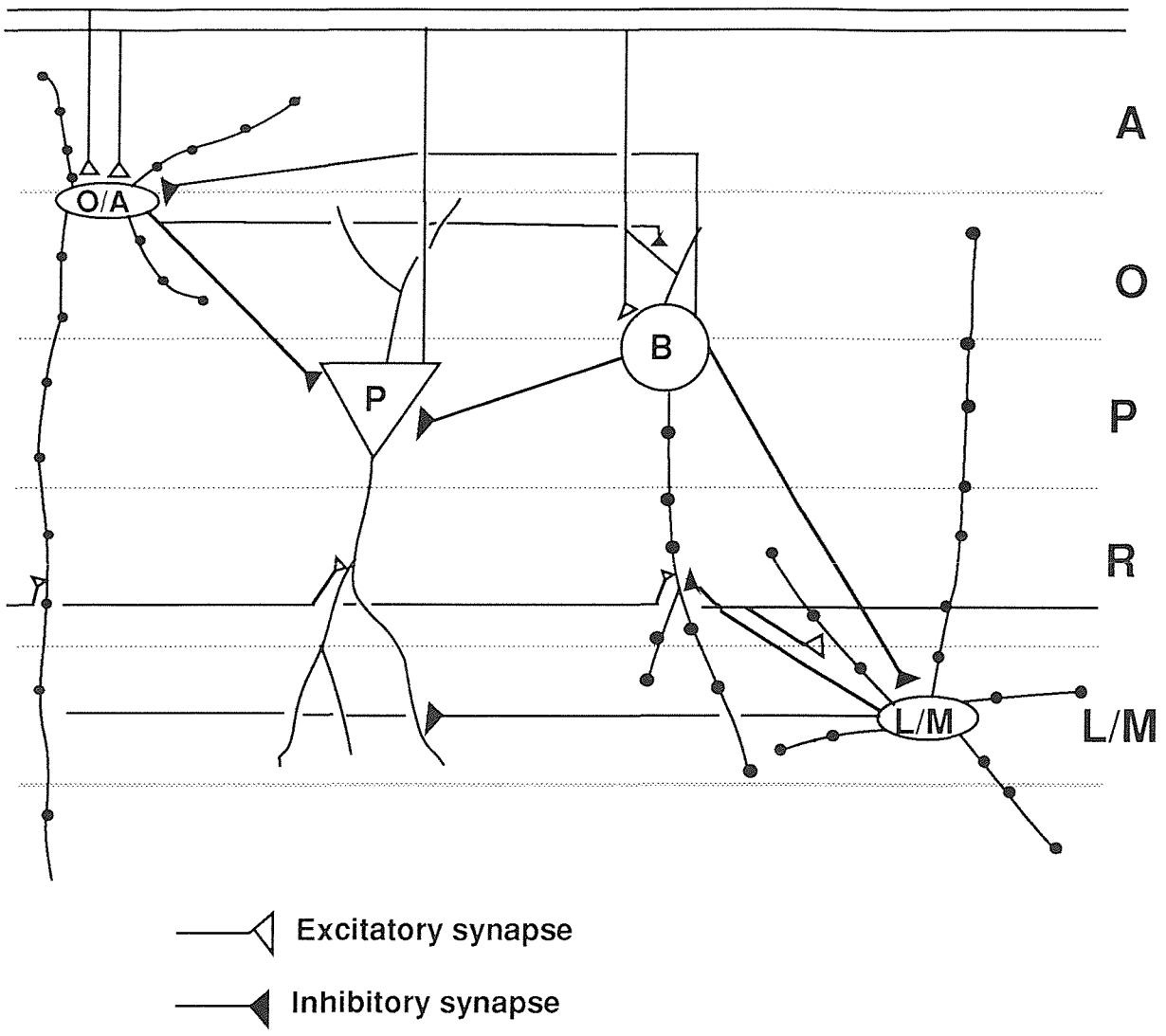


Figure 1.3 Model of inhibitory circuitry in the CA1 region of the rat hippocampus.

Afferents in stratum radiatum make excitatory synapses on apical dendrites of pyramidal cells, basket cells, O/A cells and L/M cells. Pyramidal cells send recurrent excitatory collaterals to pyramidal cell somata and O/A cells. Basket cells send inhibitory collaterals to pyramidal cell somata, L/M cells and O/A cells. O/A cells send inhibitory collaterals to the pyramidal cell somata and basket cell dendrites. L/M interneurones send inhibitory collaterals to the apical dendritic regions of pyramidal cells, basket cells and O/A cells.

Inhibition

The most important physiological function that has been assigned to interneurones is that of GABA mediated inhibition of pyramidal and non-pyramidal cells. GAD, the synthesising enzyme for GABA is used as a marker for GABAergic interneurones and the majority of interneurones in the hippocampus are thought to utilise GABA as a neurotransmitter (Babb,Pretovius,Kupfer, & Brown, 1988; Woodson,Neteki, & Ben-Ari, 1989). Inhibitory synaptic transmission is now appreciated to play a role that is on a par with excitatory transmission in the functioning of the nervous system.

GABA was demonstrated to be an inhibitory transmitter in work carried out by Biscoe & Straughan (Biscoe & Straughan, 1966). where GABA-induced depression of spontaneous and glutamate evoked firing of hippocampal neurones *in vivo* was observed. GABA has been localised using a number of techniques. GAD, the GABA synthesising enzyme has been localised (Ribak et al., 1978; Somogyi,Smith,Nunzi,Gorio,Takagi, & Wu, 1983a) along with GABA-transaminase (Nagai,McGeer, & McGeer, 1983) and GABA itself (Storm-Mathisen,Leknes,Bore,Vaaland,Edminson,Haug, et al., 1983).

GABAergic terminals appear to form a dense plexus around the somata of pyramidal cells in CA1 and CA3 and around granule cells in the dentate gyrus (Somogyi et al., 1983a; Storm-Mathisen et al., 1983; Misgeld & Frotscher, 1986; Woodson et al., 1989). GABAergic cells represent around 11% of the total population of hippocampal neurones (Woodson et al, 1989). Although most of the terminals are intrinsic there are some GABAergic afferents arising from the entorhinal cortex via the perforant path (Germroth,Schwertfeger, & Buhl, 1989) and some from the septum via the fimbria\fornix (Kohler,Chan-Palay, & Wu, 1984; Freund & Antal, 1988). The presence of GABAergic synapses in the hippocampus is reflected by the distribution of the $GABA_A$ and $GABA_B$ receptors (Sivilotti & Nistri, 1991).

For many years it was generally held that the prominent inhibition observed when recording from pyramidal cells in the hippocampus was **feedback (recurrent) inhibition** (Kandel & Spencer, 1961; Andersen,Eccles, & Loyning, 1963; Andersen et al., 1964a). In this model, excitatory afferents activate principal cells which send an excitatory recurrent axon collateral to the inhibitory cell which then discharges and inhibits the principal neurones, often including the one that activated it, therefore producing recurrent and lateral inhibition, the model being dependent on the firing of the principal cell. It was suggested that an afferent volley failed to activate the interneurones until it reached the level of threshold of population spike generation, corresponding to a synchronous discharge of many pyramidal neurones in the CA1 area (Andersen,Gross,Lomo, & Sveen,

1969; Andersen,Bliss, & Skrede, 1971a). While this model accounted for tonic inhibition and post activation suppression of principal cells, it became increasingly evident that it could not explain all the phenomena of inhibition.

Many lines of evidence have since become available that support both circuitry and physiological aspects of a direct **feedforward inhibition** in the hippocampus where an afferent volley directly excites the inhibitory interneurones which in turn induce IPSP's and suppress or prevent repetitive firing of the principal cells. These include: 1) the different shape of IPSP's recorded from cells, using antidromic and orthodromic stimuli (Alger & Nicoll, 1982a); 2) direct stimulation of interneurones with a rapid response (Ashwood et al., 1984; Freund & Antal, 1988); 3) voltage clamp analysis of inhibitory events (Brown & Johnston, 1983; Collingridge,Gage, & Robertson, 1984; Griffith,Brown, & Johnston, 1986); 4) direct two cell recordings of pyramidal cells and interneurones (Knowles & Schwartzkroin, 1981; Miles & Wong, 1984; Lacaille et al., 1987; Lacaille & Schwartzkroin, 1988b; Lacaille & Schwartzkroin, 1988a); 5) anatomical evidence (Buszaki, 1984; Frotscher et al., 1984; Seress & Ribak, 1984; Schwartzkroin & Kunkel, 1985) and 6) intracellular recordings of IPSP's (Turner, 1990). The first evidence for feedforward inhibition was provided by work carried out in the dentate gyrus, where commisural fibres were thought to directly excite the interneurones (Buszaki & Eidelberg, 1981; Buszaki & Eidelberg, 1982). Evidence was then provided for a commisural - interneurone synapse (Seress & Ribak, 1984). Anatomical data had also provided evidence for a direct commisural - interneurone synapse in Ammon's horn (Loy, 1978; Frotscher & Zimmer, 1983). Inhibition of hippocampal neurones also comes from extrinsic afferents which operate by feedforward and feedback mechanisms (Buszaki, 1984). Feedforward inhibition appears to be more widespread than feedback inhibition and may play a key role in the control of epilepsy (Traub & Wong, 1982) and long term activation of cellular excitability.

Recent studies of kainate lesioned hippocampi, which provide a model of temporal lobe epilepsy (TLE), have shown that interneurones in the CA1 area remain viable and the density and function of the GABA receptors on the CA1 pyramidal cells are approximately normal (Nakajinia,Franck,Bilkey, & Schwartzkroin, 1991). However, the frequency of occurrence of synaptic interactions between pyramidal cells and interneurones becomes lower after the lesion. These weak connections agree with the decreased inhibition seen. It was suggested that a functional uncoupling of the inhibitory interneurones and pyramidal cells may be responsible for the seizure like activity but this could be alleviated somewhat under conditions such as strong stimulus intensities which reduced the hyperexcitability

It would seem reasonable to suggest then, from the evidence available, that most input pathways directly excite both principal cells and interneurones and with this 'double' innervation and feedback inhibition, effective control of principal cells can be achieved under normal physiological conditions.

GABAergic Transmission in the Hippocampus: A General Overview

The first direct evidence for GABA acting as an inhibitory transmitter in the CNS was provided in the 1960's by Krnjevic and Schwarz (Krnjevic & Schwartz, 1967) who compared the properties of IPSP's and GABA induced membrane polarisation's. Further experiments using the plant alkaloid bicuculline revealed what is known now as the $GABA_A$ receptor (Curtis,Duggan,Felix,Johnston, & McLennon, 1971). This was accepted as the major GABA receptor for many years until the discovery of a receptor that was insensitive to bicuculline and other $GABA_A$ agonists and antagonists and was modulated by baclofen (Bowery,Hill,Hudson,Doble,Middlemiss,Shaw, et al., 1980; Dunlap, 1981; Bowery,Hudson, & Price, 1987). This became known as the $GABA_B$ receptor. A schematic diagram of a GABAergic synapse is given in **Figure 1.4.**

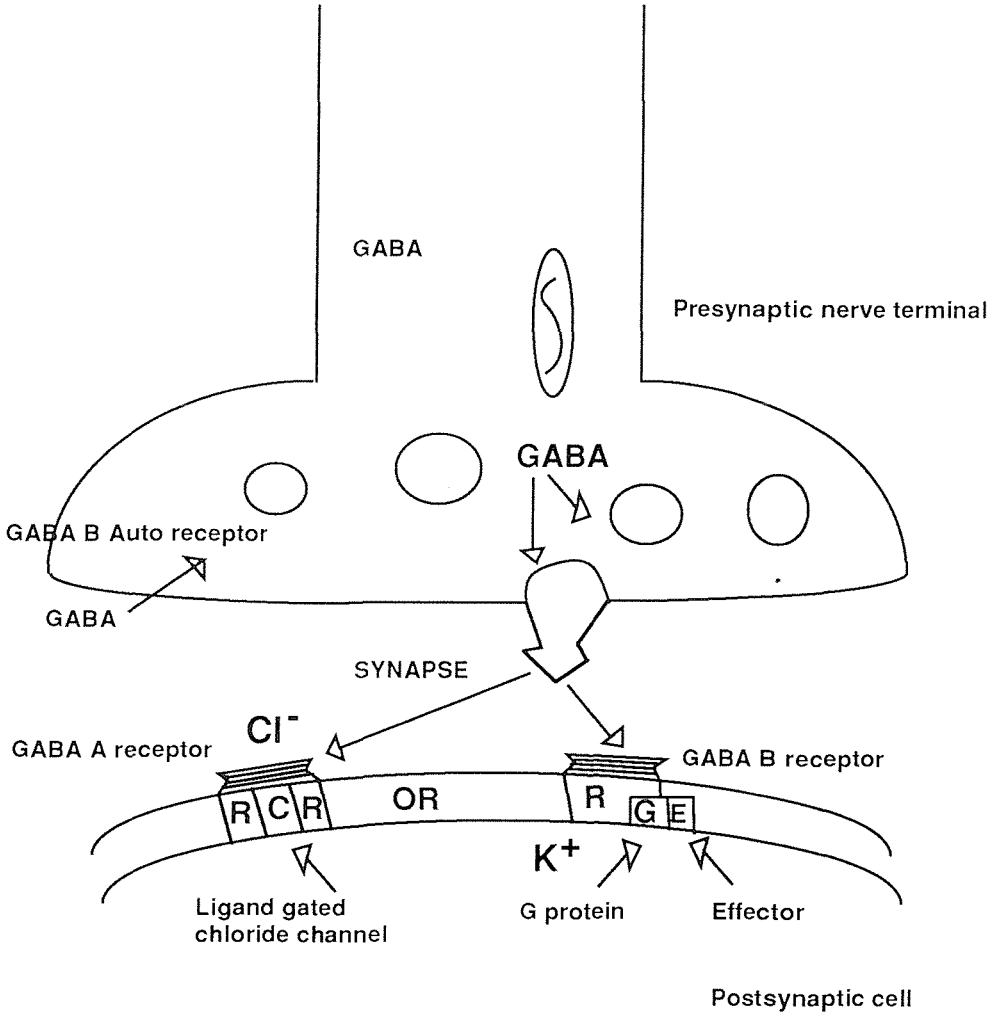


Figure 1.4 shows a schematic diagram of a GABA synapse. On the postsynaptic membrane the GABA A receptor is linked to a Cl^- channel and the GABA B receptor is linked to a G-protein which activates a K^+ conductance. On the presynaptic membrane, activation of the GABA B receptor depresses transmitter release.

$GABA_A$ receptors belong to the family of ligand gated ion channels and similarities in gene sequence and protein structure and function have been found between $GABA_A$ receptors, nicotinic receptors, acetylcholine receptors, strychnine sensitive glycine receptors and perhaps others (Schofield,Darlison,Fujita,Burt,Stephenson,Rodriguez, et al., 1987; Stephensen & Dolphin, 1989; Olsen & Tobin, 1990). Early studies suggested that the $GABA_A$ receptor complex consisted of 2 subunits α and ß but further work revealed γ, δ and ε, as well as subtypes of subunits e.g.. α1-4, ß1-3, γ1,γ2 (Tobin, 1991) The potential for $GABA_A$ receptor heterogeneity based on different subunit/subtype protein combinations is thus substantial.

More recent work on the expression of 13 $GABA_A$ receptor mRNA's showed a complex pattern in the hippocampus suggesting that it probably contains a large diversity of $GABA_A$ receptors. The α5 and β1 mRNA's generally co-localise and may encode predominantly hippocampal forms of the $GABA_A$ receptor (Wisden,Laurie,Monyer, & Seeburg, 1992). $GABA_A$ receptor function may be maintained by phosphorylation factors, the Cl^- current is regulated by Mg^+ATP and Ca^{2+} (Stelzer,Kay, & Wong, 1988; Chen,Stelzer,Kay, & Wong, 1990; Shirasaki,Aibara, & Akaike, 1992). The ß subunit of the receptor contains a sequence for phosphorylation by cAMP dependant protein kinase A (PKA) (Schofield et al., 1987). Protein kinase C (PKC) also appears to phosphorylate a ß subunit, but it may not be the same one (Browning,Bureau,Dudek, & Olsen, 1990). The significance of the phosphorylation by PKC is still unclear. Work on the development of $GABA_A$ receptors in the hippocampus has shown that $GABA_A$ mediated synaptic connections (inhibitory) are present in CA1 neurones at the earliest stages of postnatal life (Zhang,Spigelman, & Carlen, 1991) and they are thought to provide some of the excitatory drive and giant depolarising potentials in early postnatal life in CA3 neurones (Ben-Ari, Cherubini, Corradetti, & Gaiarsa, 1989; Ben-Ari & Cherubini, 1991).

The binding site for GABA which is thought to be located on the β subunit is recognised by structural analogues of GABA such as muscimol, isoguvacine, THIP (4,5,6,7-tetrahydro-iso-oxazolo(5,4-c)pyridin-3-ol) and DAVA (ß aminovaleric acid). The best studied competitive antagonist is bicuculline, whose actions were discovered over twenty years ago. Also many different classes of drugs are known to modulate the current flowing through the $GABA_A$ receptor, most of them interacting allosterically with the GABA binding site.

$GABA_B$ receptors, in contrast to $GABA_A$ receptors, have been less extensively studied and characterised with respect to structure and the protein has yet to be isolated , purified and cloned, largely due to a limited pharmacological repertoire. Antagonists that are both highly selective and potent are only now becoming available (Bittiger, Reymann, Froestl, & Mickel, 1992; Olpe, Karlsson, Pozza, Brugger, Steinmann, Rieza, et al., 1990; Olpe, Ferrat, Worner, Andre, & Steinemann, 1992). At present the $GABA_B$ receptor in the brain is thought likely to be an 80KDa protein which negatively couples to adenylate cyclase and phosphoinositide turnover systems (Kuriyama,Nakayasu,Mizutani,Hanai, & Kimura, 1992) via a G-protein thought to be Go (Dolphin,Sweeny,Pearson,Silver, & Menon-Johnson, 1992). Their distribution has been largely determined from receptor autoradiography (Bowery, Hill, & Hudson, 1983; Kato,Goto, & Fukuda, 1983; Majewska & Chuang, 1984; Hill, Bowery, & Hudson, 1984; Bowery, Hill, & Hudson, 1985; Asano, Ui, & Ogasawara, 1985; Bowery et al., 1987) and antibody labelling (Martinelli,Holstein,Pasik, & Cohen, 1992). Recent studies have produced an antagonist ligand 3H-CGP 54626 which labels high and low affinity states of the receptor or receptor subtypes which are only partially available to agonist ligands (Bittiger et al., 1992).

The possibility of $GABA_B$ receptor heterogeneity has and is being investigated and a number of lines of evidence support it (Dutar & Nicoll, 1988b; Colmers & Pittman, 1989; Lambert & Wilson, 1993). Recent findings suggest a number of subtypes of the $GABA_B$ receptor which regulate transmitter release (Raiteri, 1992). This hypothesis is largely based upon pharmacological studies and still requires further experiments/results before confirmation.

In hippocampal pyramidal cells, $GABA_B$ receptor activation increases the membrane conductance to K^+ ions (Newberry & Nicoll, 1984a; Gahwiler & Brown, 1985). Intracellular recordings from pyramidal cells in the hippocampus show that baclofen and GABA hyperpolarise these cells via an activation of K^+ channels (Ogata,Inoue, & Matsuo, 1987; Dutar & Nicoll, 1988a). The coupling of the postsynaptic receptor to K^+ channels appears to be through a pertussis toxin sensitive G-protein (Dutar & Nicoll, 1988a; Colmers & Williams, 1988). Phorbol esters, potent stimulators of PKC, reduce the GABA induced K^+ conductance (Andrade,Malaka, & Nicoll, 1986). The type of K^+ channel that is regulated by $GABA_B$ receptors is at present still uncertain and this will be dealt with in a later chapter. Detailed information about the activation of K^+ channels by $GABA_B$ receptors can be found in a recent review (Gage, 1992).

In the hippocampus, baclofen depresses excitatory and inhibitory transmission presynaptically ~~to reduce~~ **by reducing** transmitter release and depress subsequent firing (Bowery et al.,

1980; Ault & Nadler, 1983; Blaxter & Carlen, 1985; Newberry & Nicoll, 1985; Harrison,Lange, & Barker, 1988; Harrison, 1990). Baclofen acting on postsynaptic $GABA_B$ receptors was found to open voltage sensitive K^+ channels (Newberry & Nicoll, 1985). The IC_{50} for baclofen is around 10μM (Inoue,Matsuo, & Ogata, 1985a). Baclofen was found to depress GABA mediated inhibitory postsynaptic potentials by a presynaptic action at $GABA_B$ receptors on or near the terminals of inhibitory interneurones possibly at presynaptic autoreceptors (Harrison et al., 1988). The possibility that GABA and baclofen activate different receptors has also been suggested (Jarolimak,Bijak, & Misgeld, 1992). It would appear that baclofen is more potent in reducing the inhibitory transmission than increasing postsynaptic conductance and it is difficult to separate the pharmacological profile of the pre- and postsynaptic receptor (Yoon & Rothman, 1991; Thompson, Scanziani, Capogna, & Gawhiler, 1992). 3-APPA (3-aminopropylphosphinic acid) is a relatively recently described agonist which has been found to be more potent than baclofen and acts at both pre and post synaptic receptors in the hippocampus (Lovinger,Harrison, & Lambert, 1992). It has also been employed in a number of binding studies (Olpe et al., 1990).

The first compounds to be reported to have antagonist like actions at the $GABA_B$ receptor were 5-aminovaleric acid and 3-propanesulphonic acid. However, it became clear that these were not specific and effects were observed at the $GABA_A$ receptor (Muhyaddin,Roberts, & Woodruff, 1982; Giotti,Luzzi,Spagnesi, & Zilletti, 1983; Nakahiro,Saito,Yamada, & Yoshida, 1985). This effectively limited their use as $GABA_B$ antagonists. Following this, a number of compounds were synthesised and of these, phaclofen, saclofen and 2-hydroxy-saclofen (2-OH-S) appeared to be the most effective in displacing labelled ligand from $GABA_B$ binding sites and blocking $GABA_B$ responses (Kerr,Ong,Prager,Gynther, & Curtis, 1987; Kerr, Ong, Johnston, Abbenante, & Prager, 1988). More recently CGP 35348 was found to displace labelled 3-APPA binding to $GABA_B$ sites (Olpe et al., 1990) and became the most potent compound available. Currently two additional CGP compounds, CGP 36742 and CGP 54062 have become available which appear to be even more potent. The IC_{50} values are 35μM and 0.012μM respectively.

The lidocaine derivative QX-314 is believed to block the K^+ conductance linked to $GABA_B$ receptors in the hippocampus, effectively blocking the late slow inhibition (Nathan, Jensen, & Lambert, 1990; Andrade, 1991). Its use as a tool to separate the effect of activation of $GABA_B$ receptors on K^+ and Ca^{2+} conductances may be promising.

GABAergic inhibitory postsynaptic potentials (IPSP's) are evoked upon electrical stimulation of hippocampal afferent pathways. IPSP's recorded in CA1 pyramidal cells reveal a biphasic response. The first part is a rapid early IPSP mediated by $GABA_A$ receptors via an increase in Cl^- conductance, which rapidly curtails the EPSP (Andersen et al., 1964a; Andersen et al., 1964b; Ben Ari,Krnjevic,Reinhardt, & Ropert, 1981; Alger & Nicoll, 1982a; Alger & Nicoll, 1982b). The second part is a slow late IPSP mediated by $GABA_B$ receptors via an increase in K^+ conductance (Alger, 1984; Dutar & Nicoll, 1988a; Dutar & Nicoll, 1988b; Solis,Isaacson, & Nicoll, 1992b). In the CA1 area this biphasic IPSP may denote different interneurone types being responsible for each part by releasing GABA onto separate groups of receptors located on CA1 cells (Alger & Nicoll, 1982a; Alger & Nicoll, 1982b; Kunkel et al., 1988; Lacaille & Williams, 1990; Segal, 1990; Williams & Lacaille, 1992).

Early studies of GABAergic inhibition, revealed a depolarising response, which could be elicited upon orthodromic stimulation in the presence of barbiturates and iontophoresis of GABA onto the dendrites (Alger & Nicoll, 1979, Andersen, Dingledine, Gjerstad, Langmoen, & Mosfeldt-Laursen, 1980; Alger & Nicoll, 1982a; Alger & Nicoll, 1982b). The effects appeared to activate the response in the dendritic region, at a postsynaptic site and involved a change in Cl^- conductance. It was also suggested that it was mediated by spillover of GABA onto extrasynaptic receptors (Alger & Nicoll, 1982b).The mechanism and possible physiological role of the depolarising response was not apparent. More recently these depolarising responses to dendritic application of GABA have been shown to activate both inward and outward currents (Lambert,Borroni,Grover, & Teyler, 1991) and it is suggested that they might involve bicarbonate ions. The heterogeneity of the $GABA_A$ receptor may also play a part. However, all groups agree that under normal, physiological conditions only the hyperpolarising response is apparent and the role of the depolarising response is unclear.

GABAergic synapses have been located around the soma, axon hillock, initial segment, apical and basal dendrites of CA1 pyramidal cells (Ribak et al., 1978; Storm-Mathisen et al., 1983; Somogyi,Nunzi,Gorio, & Smith, 1983; Somogyi et al., 1983a; Misgeld & Frotscher, 1986; Woodson et al., 1989; Soriano & Frotscher, 1989). Regulation of the GABAergic system in the hippocampus can occur at a number of sites relative to the pyramidal cell including the postsynaptic site on the pyramidal cell itself and pre synaptic sites with respect to the pyramidal cell. This can take the form of autoregulation of IPSP's by GABA itself acting at GABA receptors presynaptically to inhibit release of GABA. A depression of the IPSP due to shifts in the ionic gradient can also occur. Phosphorylation factors and postsynaptic regulation by factors such as PKC may modulate inhibition (Alger, 1991).

Under normal physiological conditions in the *in vitro* slice preparation, only evoked release of GABA activates $GABA_B$ receptors while both evoked and spontaneous release will activate $GABA_A$ receptors. A continued barrage of slow IPSC's may be a critical factor in the regulation of excitation and action of several neuroactive compounds (Otis,Staley, & Mody, 1991; Otis & Mody, 1992). There is evidence to suggest that GABAergic interneurones in the hippocampus preserve their inhibitory potential in the period preceding delayed cell death of CA1 pyramidal cells under conditions of cerebral ischaemia and that benzodiazepine, GABA analogues and GABA uptake inhibitors may be useful in treatment of this condition (Johansen,Christensen,Jensen,Valente,Jensen,Nathan, et al., 1991).

It has been widely hypothesised that the generation of **epileptiform seizures** involves alterations in GABA mediated synaptic inhibition (Avoli, 1988; Taylor, 1988; Jeffreys, 1990) and an epileptiform burst in pyramidal cells may involve a change in GABAergic components (Karlsson & Olpe, 1989; McCormick, 1989; Malouf,Robbins, & Schwartzkroin, 1990). In fact, a relationship between GABA and seizure disorders has been suspected since the earliest studies of the function of GABA in the central nervous system (CNS) (Hayashi & Nagai, 1956; Killam, 1957). It has been suggested that the reduction of GABA-mediated inhibition is causal in epileptogenesis, yet this is in debate as a clear reduction has not been observed in animal models. It has also been proposed that GABA mediated inhibition is in fact increased and may be the synchronising mechanism (Tasker & Dudek, 1991).

Bicuculline, penicillin and picrotoxin, all of which block the $GABA_A$ **mechanisms** able to induce large paroxysmal depolarisation shifts (PDS) in hippocampal pyramidal cells. Initial effects are a depression of fast inhibition and an enhancement of excitatory potentials (Schwartzkroin & Prince, 1980). Long term effects include spontaneous and stimulus-evoked bursts and after-discharges. The bursts are superimposed upon large depolarisations that can inactivate somatic action potentials and these PDS's are thought to be due to compound giant EPSP's that would normally be counteracted by $GABA_A$ activation (Johnston & Brown, 1981; Dichter & Ayala, 1987).

A recent report suggested that the amplification of $GABA_B$ receptor mediated inhibition after partial disinhibition may possibly be an important endogenous compensatory mechanism preventing epileptogenesis (Scanziani,Gahwiler, & Thompson, 1991). Baclofen has been found to produce a selective disinhibitory effect in granule cells of the dentate gyrus by inhibiting activity of GABAergic neurones. At high concentrations it was found to produce a long lasting potentiation of the population response which was induced by a loss of inhibition (Burgard & Sarvey, 1991). This agrees with earlier reports that

baclofen suppressed recurrent inhibition of $GABA_B$ receptors on inhibitory interneurones giving a net proepileptic effect because the disinhibitory effect was greater than the suppressive effect on synaptic excitation (Mott,Bragdon,Lewis, & Wilson, 1989). Studies carried out with the $GABA_B$ antagonist CGP 35348 revealed a moderate increase in the frequency of extracellularly recorded spontaneous epileptiform burst discharges induced by penicillin, bicuculline and low Mg^{2+}. CGP 35348 also increased the duration of the paroxysmal depolarisation underlying an evoked epileptiform burst and reduced the early component of the after hyperpolarisation which followed. It was concluded that $GABA_B$ receptors appear to exert a suppressant effect on various kinds of epileptiform activity in the hippocampus in vitro. *In vivo* the role appears to be less prominent (Karlsson, Kolb, Hausdorf, Portet, Schmutz, & Olpe, 1992). It has also been indicated that $GABA_B$ receptors are of primary importance in a model of experimental absence epilepsy and that $GABA_B$ receptor antagonists may represent a new class of anti-absence drugs (Snead, 1992; Marescaux,Vergnes, & Bernasconi, 1992; Bernasconi, Lauber, Marescaux, Vergnes, Martin, Rubio, et al., 1992).

While fast IPSP's appear to limit the size of compound EPSP's and therefore prevent paroxysmal depolarising shifts, slow IPSP's appear to suppress recurrent synaptic excitation which can result in synchronised activity if left undampened (Malouf et al., 1990).

AIMS

As can be seen from the information given in the previous pages there is some evidence for the importance of GABAergic inhibition mediated by $GABA_B$ receptors in the hippocampus and many studies are being carried out to distinguish between the receptor types and their physiological properties. However, there is a great deal of conflicting evidence as to the nature of the pre- and postsynaptic effects of receptor activation and the idea of a physiological role in the control of excitatory synaptic transmission:

Are there separate receptor types?

Are they responsible for a particular component of the slow IPSP?

Are the receptors mediating the slow IPSP the same as those mediating the depression of the EPSP/C?

Can we distinguish between them using electrophysiological/pharmacological techniques?

This study set out to try and answer these questions and provide some more insight into the physiological role of pre and postsynaptic $GABA_B$ receptors in area CA1 of the hippocampus.

CHAPTER TWO

2

Materials and Methods - General

This chapter will deal with the general methods and materials used throughout the following four chapters. More specific and relevant details will be added to each individual chapter in turn.

2.1 Tissue Preparation

All experiments were carried out using male Wistar rats weighing approximately 200g. Animals were taken and anaesthetised under halothane and then immediately decapitated using a standard guillotine. The brain was rapidly removed and placed in oxygenated, cold (4^{o} C) artificial cerebrospinal fluid (ACSF) for at least four minutes prior to removing both hippocampi. These were covered in ACSF and transverse slices were prepared using a McIllwain tissue chopper. The slices were placed in a petri dish upon filter paper soaked in ACSF and transferred to a perspex holding chamber which was kept at room temperature and humidified with 95% O_2 / 5% CO_2.The composition of the standard ACSF was: (mM) NaCl 118; $NaHCO_3$ 26; KCl 3.3; KH_2PO_4 1.25; $MgSO_4.7H_2O$ 1.0; $CaCl_2$ 2.5; D-Glucose 10, at pH 7.4. A stock solution of concentrated ACSF was made up and refrigerated and then diluted to requirements each day. For the whole cell voltage clamp recordings the osmolarity of the solution was checked before the start of each experiment with an osmometer (Roebling, Camlab) and adjusted to approximately 325 milliosmoles using sucrose.

Slices were transferred as needed from the holding chamber after a delay period of about one hour, to an immersion bath using a fine sable brush. They were secured by means of two nylon nets and submerged under a constant flow of warmed, oxygenated ACSF (approx. 32^{o} C). They were left to equilibrate for 5-10 minutes before starting experiments.

2.2 Extracellular Recording

Extracellular voltage recordings were obtained from area CA1 using glass microelectrodes (Clark Electromedical, 150F) containing 3M NaCl with a tip resistance of 4-10MΩ and placed in the stratum pyramidale . Orthodromic stimuli (0-30V, 0.2msec) were delivered to the Schaffer collaterals / commisural afferents (excitatory afferents) with a twisted bipolar stimulating electrode connected to a digitimer stimulating box. Electrodes were lowered into the slice using a micromanipulator (Leitz), directly into the cell layer of the CA1 area while recordings were made using an Axoclamp 2A. These were filtered at 1KHz, digitally sampled at 10 KHz and stored to disc using a PC 286 (Dell). Subsequent analysis averaged six individual responses under each condition to reduce noise before measurement of the size of the population response amplitude and the field excitatory potential (epsp) slope. The population spike response was measured as the difference of the peak from the value of the underlying wave form linearly extrapolated from the values immediately before and after the spike. The field EPSP slope was calculated from a linear approximation of the initial rise of the wave form. The stimulus voltage was routinely set to achieve either half maximal stimulus voltage or half maximal response amplitude (see individual chapters).

2.3 Whole Cell Recording

Glass electrodes (GC 100F, Clark Electromedical) were pulled using a double pull protocol on a two stage puller (Narashige PB7). The electrodes produced were neither flame polished or coated with sylgar before use. They were accepted for use if the tip resistance's were between 5 and 10 MΩ when filled with a standard intracellular solution containing (mM): Gluconate 100; KCl 12.6; Hepes 40; EGTA 10; ATP 5; Leupeptin 0.1, adjusted to a pH of 7.2 with KOH (approximately 180mM). The electrode was then attached to a HL-1-12 electrode holder with an AgCl - coated silver wire and recordings were made using an Axopatch 1D (Axon instruments). Positive and negative pressure were applied to the electrode by means of an air filled syringe and suction tube.

Single cell recordings were obtained by lowering the electrode into the CA1 layer of the slice while under positive pressure using a Leitz manipulator. On entering solution the Axopatch was zeroed and switched to voltage clamp mode and a current evoked by a

0.2mV oscillation applied to the electrode was used to estimate the resistance while attempting to form a seal to the cell. Upon approaching a cell, a decrease in the size of the oscillations was seen due to an increase in electrode resistance from the tip of the electrode pressing against the membrane surface of the cell. The positive pressure was removed at this point and was followed by a further decrease in the size of the pulse. Gentle suction (negative pressure) produced a hundred-fold increase in the seal resistance producing a gigaohm seal. The suction was then removed, the pulses switched off and the seal left to stabilise while the software was activated to provide a holding potential of -60mV. Compensations for electrode capacitance were then made.

Whole cell recordings were achieved by breaking through the small patch of membrane under the tip of the electrode by gentle suction and success was apparent when a response to a depolarising voltage step of 10mV from -60 to -70 mV could be elicited which had capacitance transients at the beginning and end of the step. Measurements of the input resistance's of the cells were taken for 15 minutes until the cell was stable and compensations were made for the series resistance of the cell.

every 10 secs
Synaptic currents were evoked using a bipolar stimulating electrode placed in either the Schaffer collaterals/commisural afferents or directly to the lacunosum/moleculare. Data was filtered at 5KHz and sampled at 10KHz and collected by a PC 286 (Dell) and analysis was carried out using P Clamp 5.1 software (Axon Instruments).

2.4 Intracellular Staining

The correlation of electrophysiological and morphological features of an individual neurone provides a greater understanding of the functioning of that neurone. The use of the hippocampal slice ensures that the neurone stained is intact and living, providing a more accurate interpretation of the cell morphology than a fixed preparation.

The confocal scanning laser microscope (CSLM) was used to image the neurone seen in **Figure 2.1**. Images were taken throughout the depth of the slice, allowing a Z-series to be built up and three dimensional images created.

The dye used in this study was 5,6-carboxyfluorescein, a low molecular weight fluorescent dye (Molecular probes). A 5% solution of the dye was made up in 0.1M KAc, heated to 60^{o}C and titrated to pH 7.8 - 8.2 with NaOH 1M and filtered. Intracellular electrodes

(GC120F, Clark Electromedical) were pulled on a Flaming-Brown microelectrode puller. The electrode tip was placed into the dye solution allowing the dye to fill the tip by capillary action. The electrode was then backfilled with 4M KAc. A cell impalement was achieved by stepping the electrode through the slice and injecting hyperpolarising current to stabilise the cell and eject the dye (-0.3nA, 100msec pulses, 2s intervals, minutes). The electrode was then stepped out of the slice and the slice transferred to a slide which was transferred onto the microscope.

Figure 2.1 shows a fluorescent image of the whole cell as it was filled and a higher power magnification of the axonal output pathway with its characteristic 'beading'. Also shown is a high power view of an apical dendrite with swellings that are believed to be the spines where synaptic transmission takes place.

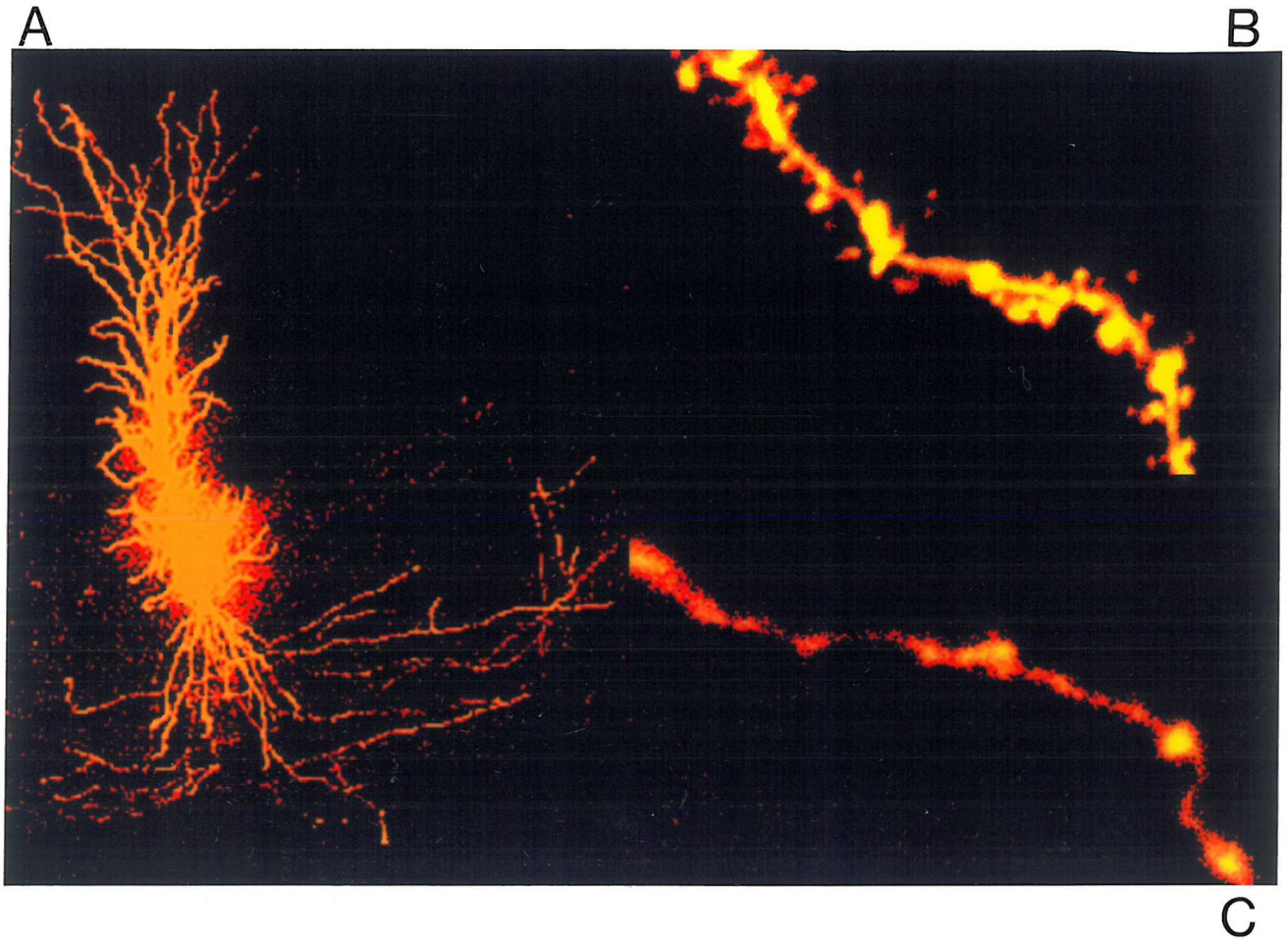


Figure 2.1 CA1 pyramidal cell filled with 5,6-carboxyfluorescein

A) The soma, basal and apical dendrites and axonal output pathways of a CA1 pyramidal cell are shown in false colour to enhance the image seen. The vertical scale (2cm = 50μm) gives an idea of the size of the cell and its connections.

B) A high power image of a single dendrite with swellings believed to be dendritic spines. This is where the majority of receptors and ion channels are thought to be situated.

C) A high power image of the axonal output pathway showing characteristic beading, thought to be points of synaptic contact.

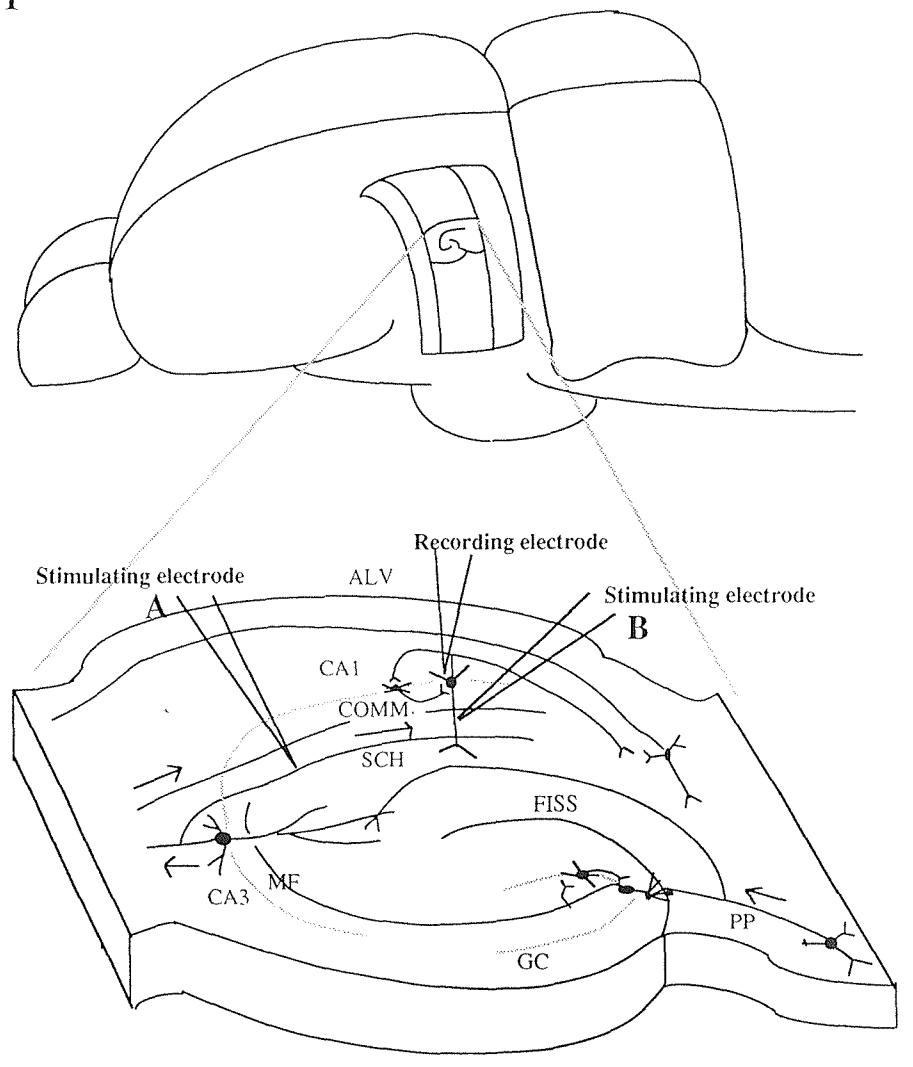
2.5 Pharmacology

All drugs used in this study were made up as concentrated stock solutions and frozen until required. Aliquots were taken and dissolved to concentration in ACSF. Drugs were introduced into the perfusion system from a conical flask (kept at the same temperature as the ACSF) by means of a three way tap. Control experiments showed no effect of swapping between the different ACSF reservoirs in the absence of drugs. Each of the individual chapters will list the source of the drugs used and the concentrations at which they were used.

Figure 2.2 shows the position of the stimulating and recording positions that were used in these experiments.

CHAPTER THREE

1



2

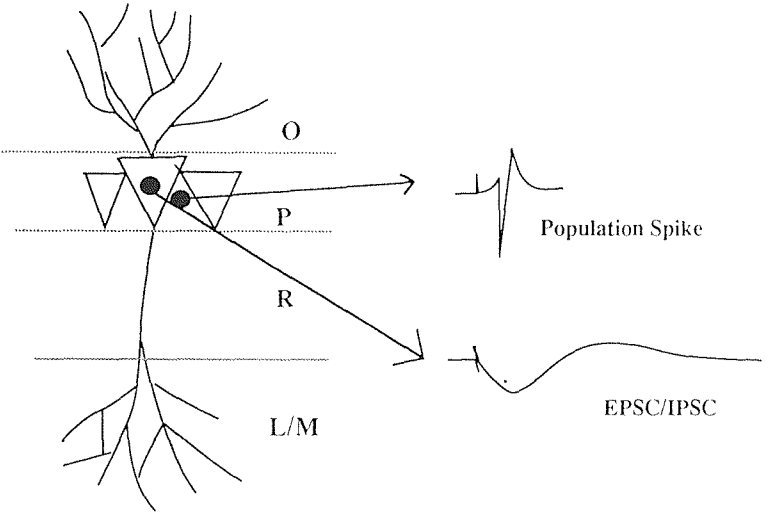


Figure 2.2

Diagram of a hippocampal slice showing the areas recorded from under extracellular and whole cell voltage clamp conditions .

1 A) Orthodromic stimuluation was given to the excitatory afferents in CA3 and recordings were taken from either individual cells or a population of cells.

1 B) Local orthodromic stimuli were given to the area where the L/M interneurones are belived to be in order to activate a smaller number of fibres and obtain a larger inhibitory response when recording from single cells.

2) A diagram of a pyramidal cell and the layers in CA1 showing the site of the electrodes used to record population spikes and single cell currents as indicated.

3

Identification of 2 Phases Of Synaptic Inhibition In Area CA1 Of The *In Vitro* Hippocampal Slice

3.1 Introduction

GABA is the major inhibitory transmitter in the CNS and its ability to modulate cellular excitability is well documented (Karlsson & Olpe, 1989; Sivilotti & Nistri, 1991). GABA mediated inhibition of CA1 pyramidal cells takes the form of two distinct IPSP components which have been separated on the basis of their pharmacology and time course. These are a fast IPSP mediated by $GABA_A$ receptors on the soma/initial segment and a slow IPSP mediated by $GABA_B$ receptors on the dendrites (Alger & Nicoll, 1980b; Hotson & Prince, 1980; Knowles & Schwartzkroin, 1981; Alger & Nicoll, 1982b; Miles & Wong, 1984; Newberry & Nicoll, 1984b; Dutar & Nicoll, 1988a; Solis & Nicoll, 1992a). Activation of $GABA_A$ receptors is known to cause an increase in membrane conductance to Cl^- and can be antagonised by the plant alkaloid bicuculline. Activation of $GABA_B$ receptors causes an increase in membrane conductance to K^+ via a G-protein. These can be distinguished from the $GABA_A$ receptor by the lack of effect of bicuculline and the sensitivity to phaclofen and its analogues (Bowery,Doble,Hill,Hudson, & Shaw, 1981; Kerr,Ong,Prager,Gynther, & Curtis, 1987; Kerr, Ong, Johnston, Abbenante, & Prager, 1988; Seabrook, Houser, & Lacey, 1990)

To investigate the inhibitory mechanisms, it is necessary to first be able to observe them within a given experimental preparation. Measurement of the extracellular response on CA1 provides information about how the population of neurones is behaving at any one time. While this may not provide direct information at the single cell level, it enables a good overview of the general mechanism that occurs under physiological conditions within the circuit in the slice preparation. The two phases of GABAergic inhibition have been

identified previously in this type of preparation (Gribkoff & Ashe, 1984; Karlsson & Olpe, 1989; Davies,Davies, & Collingridge, 1990).

When the pyramidal cells are stimulated above the threshold for firing, action potentials are induced and a negative population spike is recorded superimposed on the EPSP. The population spike is the field correlate of synchronous firing of a population of cells (Andersen,Bliss, & Skrede, 1971a). Throughout the experiments in this chapter, the electrodes were consistently placed in as in Figure 2.2 (see Methods).

In these experiments a homosynaptic paired pulse stimulus protocol was used to distinguish between $GABA_A$ and $GABA_B$ mediated inhibition. Two identical consecutive stimuli were applied to the excitatory afferents which resulted in a first, conditioning response and a second, test response. The respective amplitudes of the conditioning (C) response and test (T) responses, were measured, and inhibition was quantified as the ratio of the decrease in response of the test response to the conditioning response amplitude {(C-T)/C}.The rationale is that the first stimulus (conditioning) activates the inhibitory circuitry, which causes a depression of the response to the second (test) stimulus. The degree to which the second response is reduced in comparison to the first response, gives an indication of the amount of inhibition present in the slice. Several factors can affect the amount of inhibition seen and it is important to control for variations in stimulus intensity between experiments. This was set to produce a half maximal conditioning response for all experiments carried out in this study.

/excitation

3.2 Materials and Methods

Slices were prepared as described in chapter 2 and those from the septal end of the hippocampi were used preferentially, as septo-temporal gradients have been observed in paired pulse experiments, septal slices exhibiting stronger inhibition (Radpour & Wheal, 1987). Slices were transferred to the recording chamber and perfused with standard ACSF. This was maintained at approximately 32°C and oxygenated throughout the experiment. Drugs were diluted to the correct concentration in ACSF and perfused through the chamber until the recording of the population spike had stabilised.

A twisted bipolar electrode was placed in the stratum radiatum to activate the excitatory afferents. Unless stated, the stimulus was set to achieve a population response of half maximal amplitude. Extracellular recordings from the CA1 pyramidal cell layer and their subsequent analysis were obtained as set out in the methods section.

The drugs used were: Phaclofen 1mM (Tocris Neuramin) and Bicuculline 1μM (Sigma).

3.3 Results

These experiments set out to identify the two components of GABAergic inhibition in area CA1 of the hippocampus and to separate them on the basis of their pharmacology and time course, using extracellular recording methods.

Paired Pulse Stimulation of Excitatory Afferents Exposes Two Independent Phases of Inhibition in the Hippocampal Slice *in vitro*.

Paired, homosynaptic stimuli, were given to the excitatory afferents in CA3 in order to evoke population spikes in the pyramidal cells of CA1. Intervals of between 10 and 500msec were used to separate the stimuli. At intervals of 10-30msec and 200-400msec we could observe a reduction in the amplitude of the second, test (T) response compared to the first, conditioning (C) response, which was taken to reflect paired pulse inhibition. Typical control responses which reflect this are shown in **Figure 3.1A**. The reduction in amplitude of T at short intervals (10 -30msec) appeared as a fast, early component of inhibition, while the reduction in amplitude of T at longer intervals (200-400msec) was a slower and weaker component of inhibition.

We could also identify a small amount of paired pulse facilitation of the population spike at intervals of 50-100msec (50msec, facilitation up to 10%). This is quite important, as it may reflect the absence of inhibition accompanied by a presynaptic facilitation of the excitatory synapses (see later). The use of homosynaptic stimuli may have over-estimated the actual amount of facilitation by producing a presynaptic enhancement of synaptic strength, which would not be the case if using heterosynaptic stimulation of separate but overlapping fibres. However, it was the investigation of the inhibitory components that was of most interest at this point and it was decided that homosynaptic stimulation would be adequate for this purpose.

Quantification of paired pulse inhibition and facilitation (see introduction) yields a measure effectively independent of conditioning spike amplitude to a first approximation. Analysis of the dependence of paired pulse inhibition upon the sequence in which the interpulse interval was tested with no significant difference being observed (n=2). This is shown quantitatively in **Figure 3.1 B**. The early (10-30msec) and late (200-400msec) inhibitions can be seen clearly, separated by a region of paired pulse facilitation (negative inhibition).

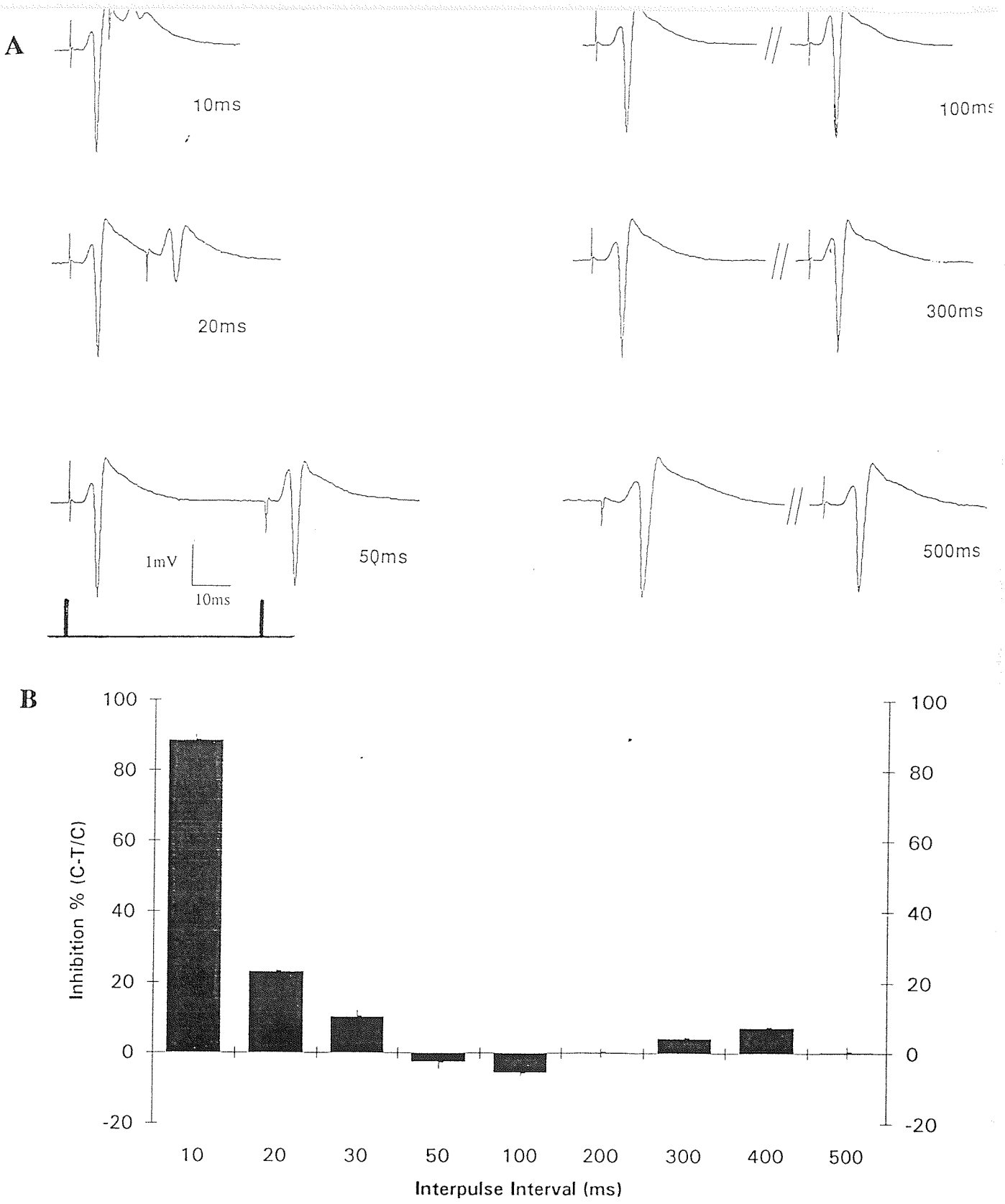


Figure 3.1 A typical example of extracellular field recordings under control conditions showing the conditioning (C) and test responses (T) after paired stimuli to excitatory afferents in CA3. **A)** From left to right for each time interval C and T are shown at different interpulse intervals (IPI's). Early inhibition is seen as a reduction in T with respect to C at IPI's of 10-20ms. Paired pulse facilitation can be observed as an increase in T compared to C at an IPI of 100ms. The late inhibition is seen as a reduction in T as compared to C at IPI's of 300-500ms. The stimulus protocol is indicated under the IPI for 50ms. **B)** The inhibition is quantified [(C-T)/C %] and represented graphically for the stimuli given at random IPI's, to indicate that the 2 phases of inhibition are not dependent on the order in which the IPI's are given.

Effect of Bicuculline and Phaclofen on Paired Pulse Inhibition Evoked in CA1 Pyramidal Cells.

Once it had been established that we could identify two phases of inhibition, which could be separated on the basis of their time course, we then proceeded to investigate the pharmacology of the individual components.

The fast, early component of inhibition (10-30msec) was first investigated and could be significantly blocked by the $GABA_A$ antagonist **Bicuculline** (1μM, n=4, control, 20msec 90.58 ± 3.16%; Bic 19.34 ± 9.06% , $p< 0.01$). There was a slight increase in the size of the conditioning response from 5.90±.06mV to 6.20±.09mV (**Figure 3.2**). There was no significant effect upon the late component of inhibition measured at 200-400msec (control 15.78±4.6%; Bic 10.68±1.69%). The inset in figure 3.2 shows the stimulus response curve taken from the control recordings before application of bicuculline (n=4).

Analysis of the dependence of paired pulse facilitation and inhibition of the population spike and the effect of bicuculline is shown (**Figure 3.3A**).The histogram here and in future figures represent the mean ± s.e.m. There appeared to be no facilitation of the population spike at any of the intervals in this set of experiments until after the addition of bicuculline, when there was a trend towards facilitation, seen with the removal of the inhibition at 100msec (inhibition, control, 20.99±9.37%; Bic, .54±2.58%). This effect was reversible. The reduction of the early inhibition can clearly be seen and this is reflected when plotting the individual experiments (**Figure 3.3B**).

These 'ribbon graphs' provide us with the results for each individual experiment on the same graph. The reason for plotting the data in this way is to provide an indication of any deviation from the mean given in the histogram. It would be immediately noticeable here, as each individual ribbon can be clearly seen. We would be able to observe any outlying results, and therefore our interpretation of the effects of any of the drugs used would ideally be more accurate.

Although the measurement of the population spike is very important, it can only really provide us with information on what is occurring at the postsynaptic sites of these cells. The change in the size of the initial slope of the field EPSP (mV/msec) was also measured and quantified (**Figure 3.3C**). This measurement reflects the presynaptic release of transmitter (Andersen,Blackstad, & Lomo, 1966) and its ability to activate the receptors on the postsynaptic cell. This is the way we can get an idea of any changes that are happening at a presynaptic loci from the extracellular data. However, because of the size of this event and the noise, this is only an approximate measurement.

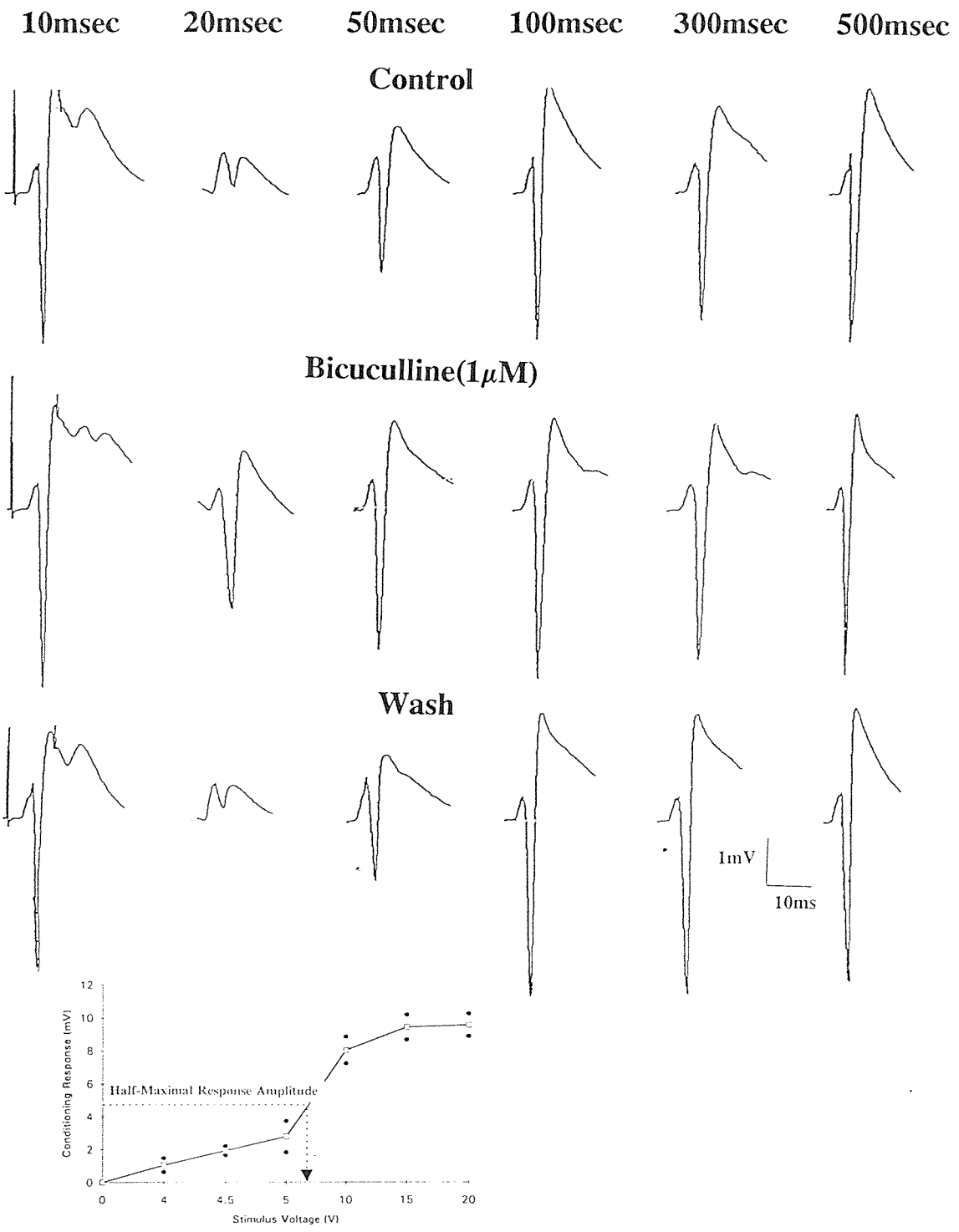


Figure 3.2 A typical example of extracellular field recordings under control conditions showing the conditioning (C) and test responses (T) after paired stimuli to excitatory afferents in CA3. From left to right in each row, C is shown with T for an IPI of 10ms, followed by only the test response for longer IPI's in the following columns. Early inhibition is seen as a reduction in T with respect to C at IPI's of 10-20ms. Bicuculline (1μM, n=4) increased the conditioning response and reversibly reduced the inhibition at short IPI's. There was no significant effect on inhibition at longer intervals. Bicuculline also appeared to reduce the inhibition observed at IPI's of 50-100ms. Inset: Stimulus response curve for these experiments.

Figure 3.3 (next page) The effect of bicuculline (1μM, n=4) on paired pulse inhibition of the population spike amplitude and the initial slope of the field EPSP. Inhibition was calculated as [(C-T)/C %].

A) The % inhibition (mean±s.e.m.) of the population spike amplitude is shown at each IPI for controls, bicuculline and wash. Bicuculline significantly and reversibly reduces the early inhibition. There is also some reduction of the inhibition at longer intervals.

B) The ribbon graph shows the % inhibition of the population spike amplitude at each IPI for each experiment, which produces the mean values shown in A.

C) The % inhibition (mean±s.e.m.) of the slope of the field EPSP for all IPI's. Bicuculline had no significant, reversible effect on the inhibition or facilitation of the slope.

D) The individual results show that a few measurements deviate from the mean values in C reflecting the difficulty in analysing this small event.

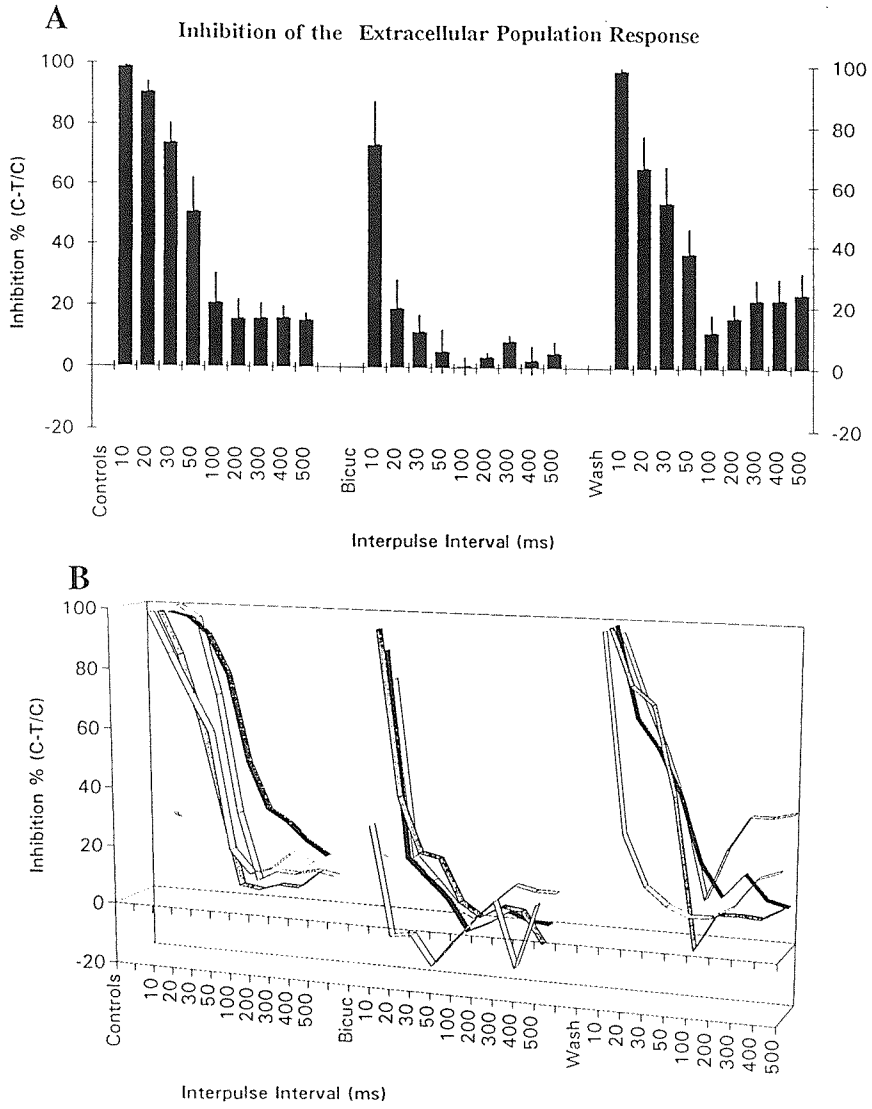


Figure 3.3 Bicuculline Effects On Paired Pulse Inhibition

Bicuculline did not appear to significantly affect the initial slope at any IPI of the components of inhibition, apart from revealing a small amount of facilitation at 300-500msec which did not appear to reverse on washing. This can also be seen from the scattering of the individual results (**Figure 3.3D**). These observations would appear to be consistent with a postsynaptic site of action for bicuculline.

The second, slower component of inhibition was investigated with the $GABA_B$ antagonist **Phaclofen** (1mM, n=8). Phaclofen had no significant effect on the early component of inhibition (**Figure 3.4**, control 55±12%; phaclofen 58±11%). However, it significantly reduced the late inhibition (control, 6.4 ± 3.6%; Phac, -0.7 ± 2%, $p< 0.01$, n=8). The inset shows the stimulus response amplitude for control cells (n=25). In this case the protocol used to elicit the response was half maximal stimulus voltage (n=8). Further experiments were carried out using a stimulus to achieve half maximal response amplitude (n=2). This proved of more benefit and revealed an increased amount of late inhibition (300msec, 6.4±3.6% to 28.61±1.04%). This is because when using half maximal stimulus voltage the stimulus is generally higher, activating the feedforward pathways and producing some disinhibition. This was also reduced by phaclofen (control 28.61±1.04%, phaclofen 10.04±3.6%;).

The dependence of paired pulse facilitation and inhibition for the means and the individual results (**Figure 3.5 A,B**) show a visible increase in the amount of inhibition at 20-30msec but this was not found to be significant. The decrease in the late inhibition can be clearly seen at 200-400msec. Paired pulse facilitation was observable at intervals of 30-200msec and this was depressed at intervals of 30-100msec by addition of phaclofen (control 100msec -8.8±7.02%; Phac,-1.9±2.6%).

The reduction in paired pulse facilitation of the population spike, was reflected in the apparent reduction of the facilitation of the initial slope of the field EPSP at the same intervals (50-100msec). However, these results were distorted by one particular cell which deviated considerably from the others. Also, the effects on the other cells were not reversible and occurred to a small extent at other IPI's. (**Figure 3.5 C,D**)

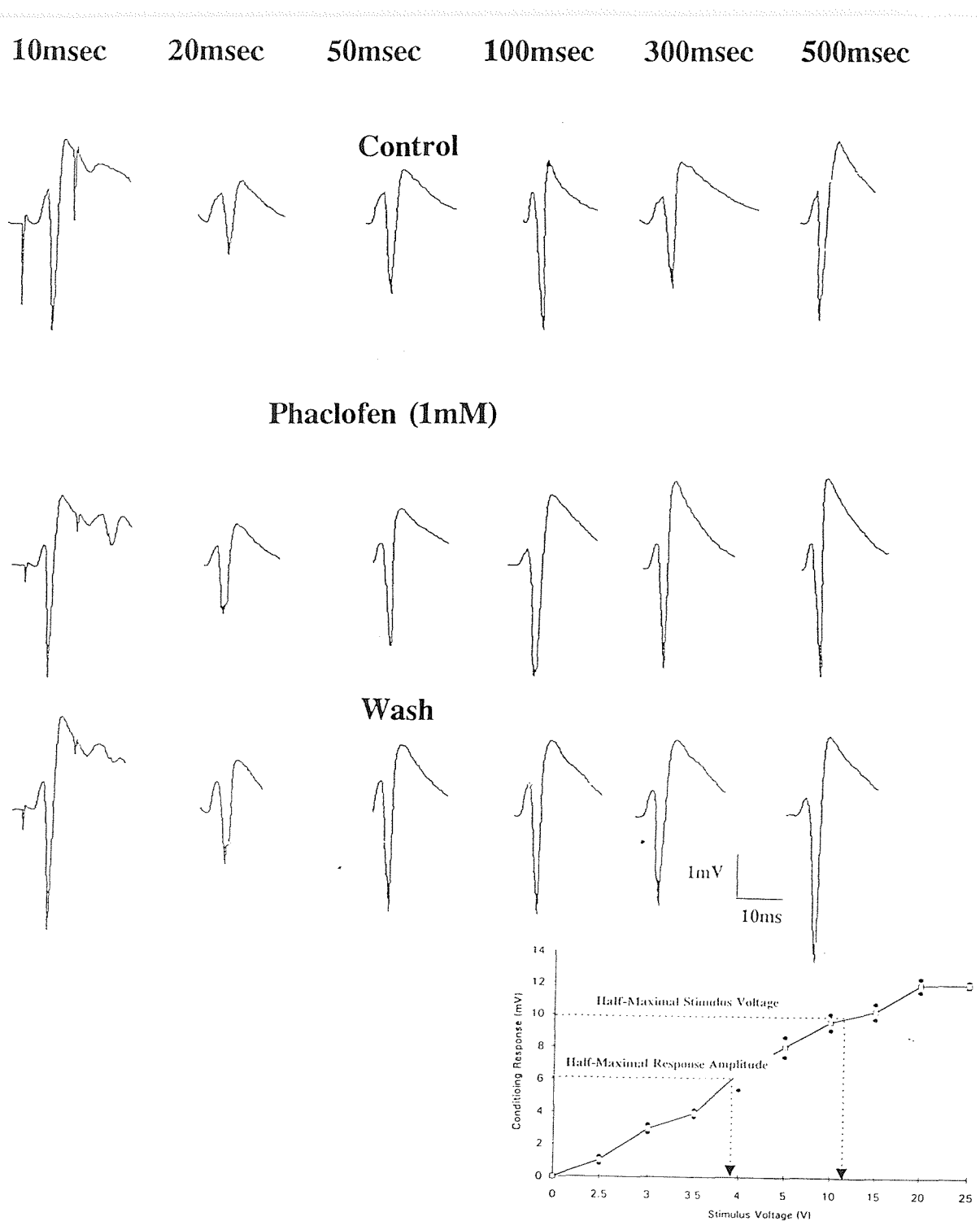


Figure 3.4 A typical example of extracellular field recordings under control conditions showing the conditioning (C) and test responses (T) after paired stimuli to excitatory afferents in CA3. From left to right in each row, C is shown with T for an IPI of 10ms. followed by only the test response for longer IPI's in the following columns. Early inhibition is seen as a reduction in T with respect to C at IPI's of 10-20ms. Phaclofen (1mM, n=8) reversibly reduced the inhibition at long IPI's. There was no significant effect on the C or the inhibition at short IPI's. Phaclofen also appeared to reduce the facilitation observed at IPI's of 50-100ms. Inset: Stimulus response curve for these experiments.

Figure 3.5 (next page) The effect of phaclofen (1mM, n=8) on paired pulse inhibition of the population spike amplitude and the initial slope of the field EPSP. Inhibition was calculated as [(C-T)/C %].

A) The % inhibition (mean±s.e.m.) of the population spike amplitude is shown at each IPI for controls, phaclofen and wash. Phaclofen significantly and reversibly reduces the late inhibition. There is also a reduction of the facilitation at 50-100msec.

B) The ribbon graph shows the % inhibition of the population spike amplitude at each IPI for each experiment, which produces the mean values shown in A.

C) The % inhibition (mean±s.e.m.) of the slope of the field EPSP for all IPI's. Phaclofen depressed the facilitation of the slope.

D) The individual results show that some measurements (recovery) deviate from the mean values in C reflecting the difficulty in analysing this small event.

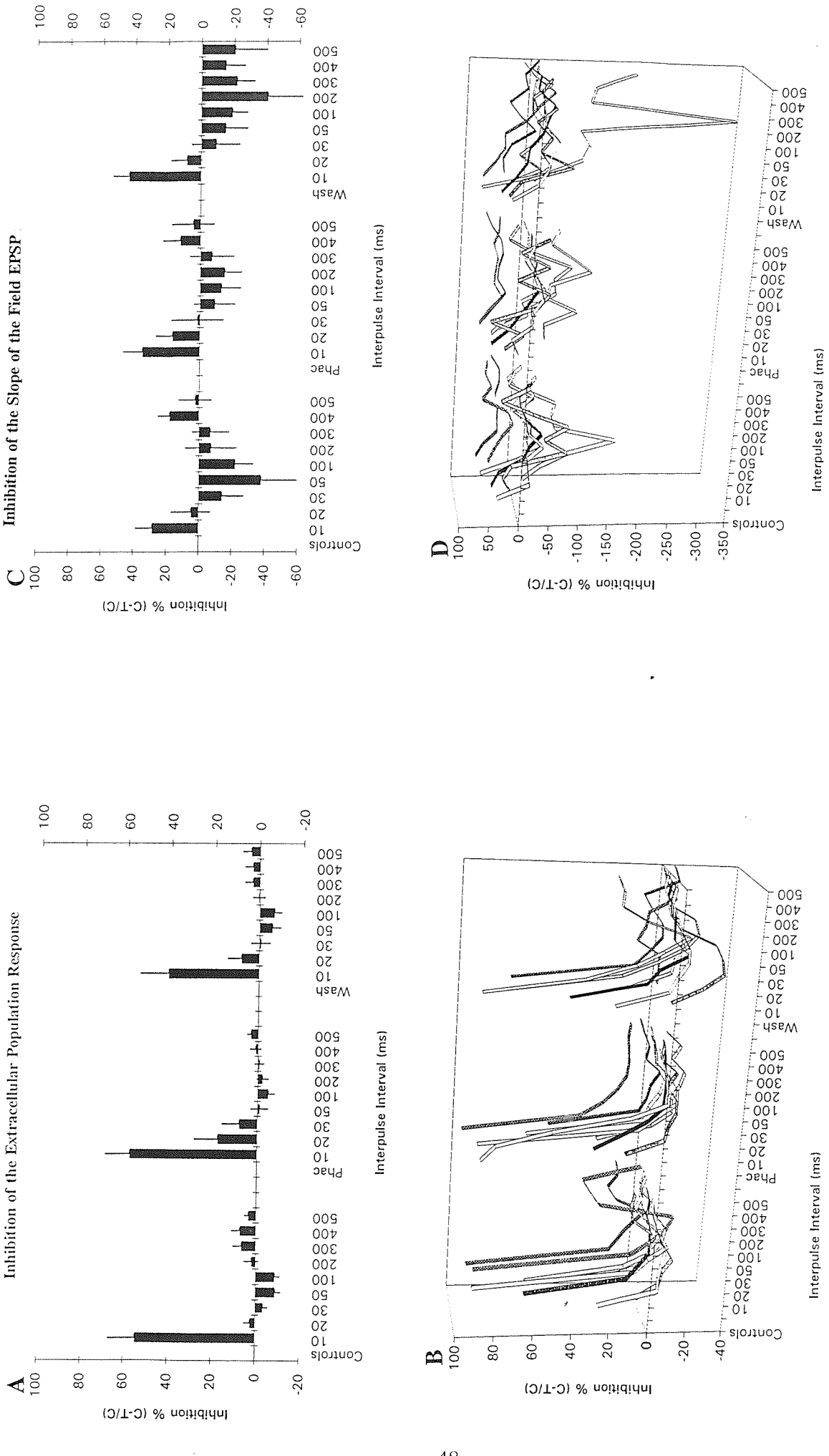


Figure 3.5 Phaclofen Effects on Paired Pulse Inhibition

3.4 Discussion

The extracellular population spike recordings from CA1 pyramidal neurones, presented here, reveal two distinct phases of GABAergic inhibition. These can be separated on the basis of their distinct time courses and pharmacology.

Paired Pulse Inhibition

The first, fast phase of inhibition which became apparent at interpulse intervals of 10-30msec was attenuated by the $GABA_A$ antagonist bicuculline and was therefore assumed to be $GABA_A$ mediated. The second, slow phase of inhibition appeared at intervals of 200-400msec and could be blocked by the $GABA_B$ antagonist phaclofen and was taken to be $GABA_B$ mediated. These results agree with previous studies (Gribkoff & Ashe, 1984; Karlsson & Olpe, 1989; Davies et al., 1990).and suggest that under these experimental conditions, inhibition mediated by GABA receptors can be studied.

The inhibition was clearly dependent on the interpulse interval and reflects the time course of activation of both $GABA_A$ and $GABA_B$ receptors. The order in which the interpulse intervals were given appeared to have no bearing on the facilitation and inhibition observed and indicated that the two phases of inhibition were not artefacts of a stimulation protocol. A recent study has shown that paired pulse depression of the field EPSP and the EPSC is maximal at 300msec and is believed to result from activation of presynaptic $GABA_B$ receptors on the excitatory terminals, that can be blocked by $GABA_B$ antagonists. Paired pulse facilitation of GABA release from inhibitory terminals at 50msec is thought to 'spillover' and activate GABAB receptors on the excitatory terminals to decrease glutamate release (Isaacson,Solis, & Nicoll, 1993). Although some idea of the presynaptic inhibitory effects could be seen from a measure of the initial slope of the population response, this is not accurate enough to make any definite claims about their role or mechanisms of action.

Paired Pulse Facilitation

In these experiments paired pulse facilitation was most apparent at IPI's of 30-100msec. Activation of the presynaptic $GABA_B$ receptors on inhibitory terminals, depresses the release of GABA and the activation of postsynaptic $GABA_A$ receptors. This has been shown to be largely responsible for the facilitation of the EPSC/P (Nathan,Jensen, & Lambert, 1990a; Nathan & Lambert, 1991). The results here indicate that the facilitation of

the population spike and the slope of the field EPSP observed at these intervals could be blocked by the $GABA_B$ antagonist phaclofen as would be expected if this were to prevent the activation of the presynaptic receptors by GABA.

In summary, bicuculline proved itself to be an effective tool in blocking the $GABA_A$ mediated inhibition. Phaclofen, however, had to be used in very high concentrations in these experiments (1mM) in order to reduce the $GABA_B$ mediated inhibition. It was decided therefore, to investigate $GABA_B$ mediated inhibition using the more potent ligand 2-hydroxy-saclofen (2-OH-S) which was available at that time.

N.B.

In this and the following chapter, the reduction in the test spike seen at 10ms is most probably due to the cells still being in their refractory state, unable to fire within the short time interval given. The inhibition due to activation of $GABA_A$ receptors is found later at 20-40ms.

CHAPTER FOUR

4

Extracellular Investigations Of $GABA_B$ Mediated Synaptic Inhibition In Area CA1 Of The *In Vitro* Hippocampal Slice

4.1 Introduction

2-hydroxy-saclofen (2-OH-S), a sulphonic derivative of baclofen, is known to be active at $GABA_B$ receptors in the mammalian CNS and appears to have a more potent effect on inhibition, than the existing ligand phaclofen (Kerr et al., 1988). Many groups found that it antagonised the actions of GABA and baclofen, both pre and postsynaptically (Kerr et al., 1988; Harrison,Lovinger,Lambert,Teyler,Prager,Ong, et al., 1990; Scholz & Miller, 1991b) and the $GABA_B$ IPSP (Morrisett, Mott, Lewis, Swartzwelder, & Wilson, 1991; Williams & Lacaille, 1992; Samulack & Lacaille, 1993). With this in mind, the actions of this drug on the late $GABA_B$ mediated component of inhibition were evaluated, using the experimental protocol tested in the previous chapter.

There is an increasing amount of evidence, which indicates that $GABA_B$ receptors at different sites when activated can mediate both inhibitory and disinhibitory effects. GABA is thought to be able to regulate it's own release locally, via its action on autoreceptors located on the presynaptic inhibitory terminals (McCarren & Alger, 1985; Deiz & Prince, 1989; Davies et al., 1990). Application of baclofen, a $GABA_B$ agonist, leads to a block of both excitatory and inhibitory synaptic transmission, via actions on presynaptic receptors (Bowery, Hill, Hudson, Doble, Middlemiss,Shaw, et al., 1980; Lanthorn & Cotman, 1981; Ault & Nadler, 1983; Inoue, Matsuo, & Ogata, 1985a; Conners, Malenka, & Silva, 1988; Dutar & Nicoll, 1988b; Thompson & Gahwiler, 1989b; Davies et al., 1990; Harrison, 1990). $GABA_B$ receptors on presynaptic terminals could have a significant pharmacological influence on synaptic processing in the brain. The location of these receptors on pre and postsynaptic terminals may enable a dual preventative/compensatory control of pathological processes such as epilepsy. Also, differences in their sensitivity,

pharmacology or transduction mechanisms could allow specific targeting of therapeutic drugs with, perhaps, fewer side effects.

However, even though the $GABA_B$ system provides a powerful control of the excitatory system there appears to be very little evidence for a physiological role for the suppression of glutamate release and inhibition of excitatory transmission via presynaptic $GABA_B$ receptors (Morrisett et al., 1991; Isaacson et al., 1993). Presynaptic inhibition of the release of endogenous GABA may well be physiological. Neurochemical techniques have established the presence of $GABA_B$ autoreceptors in brain tissue and they appear more abundant than presynaptic $GABA_A$ receptors (Pittaluga,Asaro,Pellegrini, & Raiteri, 1987; Waldmeier,Wicki,Feldtrauer, & Baumann, 1988; Waldmeier & Baumann, 1990). More recently an influence of $GABA_B$ receptors in the genesis of long term potentiation has been proposed by some groups (Olpe & Karlsson, 1990; Davies,Starkey, & Pozza, 1991; Mott & Lewis, 1991).

Investigation of the mechanisms of $GABA_B$ mediated inhibition will hopefully provide us with some answers about the functional role of these presynaptic receptors and their heterogeneity.

4.2 Materials and Methods

(See Chapter 2)
Briefly, the slice preparation was as described before, including the holding and transfer of slices to the recording chamber. Orthodromic stimuli (0-30v, 0.2msec) were applied as before and responses recorded from the CA1 pyramidal cell layer. Analysis averaged 6 individual responses to reduce noise before measurement of the population response and field EPSP slope as before. Paired pulse inhibition was assessed as in chapter 3.

Drugs used were: 2-Hydroxy-Saclofen 200μM (Tocris Neuramin); Bicuculline 1μM (Sigma) and Phaclofen 1mM (Tocris Neuramin).

4.3 Results

Effect of 2-Hydroxy-Saclofen on Homosynaptic Paired Pulse Inhibition Evoked in CA1 Pyramidal Cells.

Orthodromic stimulation of the excitatory afferents in CA3, evoked extracellular population responses in CA1 pyramidal cells (n=17). Following the delivery of two homosynaptic stimuli, the early and late phases of inhibition, described in the previous chapter, were revealed at intervals of (10-30msec) and (200-400msec). Our initial experiments (Chapter 3, Figures 3.1 to 3.5) were able to show that these can be blocked by bicuculline (1μM) and phaclofen (1mM) respectively and are mediated by $GABA_A$ and $GABA_B$ receptors. Also apparent at around 100msec was a small amount of facilitation (up to 10%, indicated by negative inhibition values) that was also seen in the previous experiments. These experiments were carried out with stimuli set to achieve half maximal stimulus response amplitude (see **Figure 3.4**). There were also experiments carried out using stimuli set to achieve half maximal stimulus response (2-OH-S 200μM, n=8) . The results obtained were comparable between the two, but the former were used for the reasons explained in the previous chapter.

The most potent $GABA_B$ antagonist available at that time, 2-OH-S (200μM, n=9), was tested upon the facilitation and inhibition of the population response under these conditions. As expected, 2-OH-S reversibly attenuated the late inhibition (300msec, control 12.3±2.6%; 2-OH-S -2.4±1.9%; $p< 0.01$). However, in contrast to the effects of phaclofen (**Figures 3.4 and 3.5**), 2-OH-S also depressed the conditioning response and attenuated the early inhibition (10msec, control 89 ± 2%; 2-OH-S 46±15%, $p< 0.01$). All these changes were reversible on washing. The raw data traces from a representative experiment are shown in **Figure 4.1**.

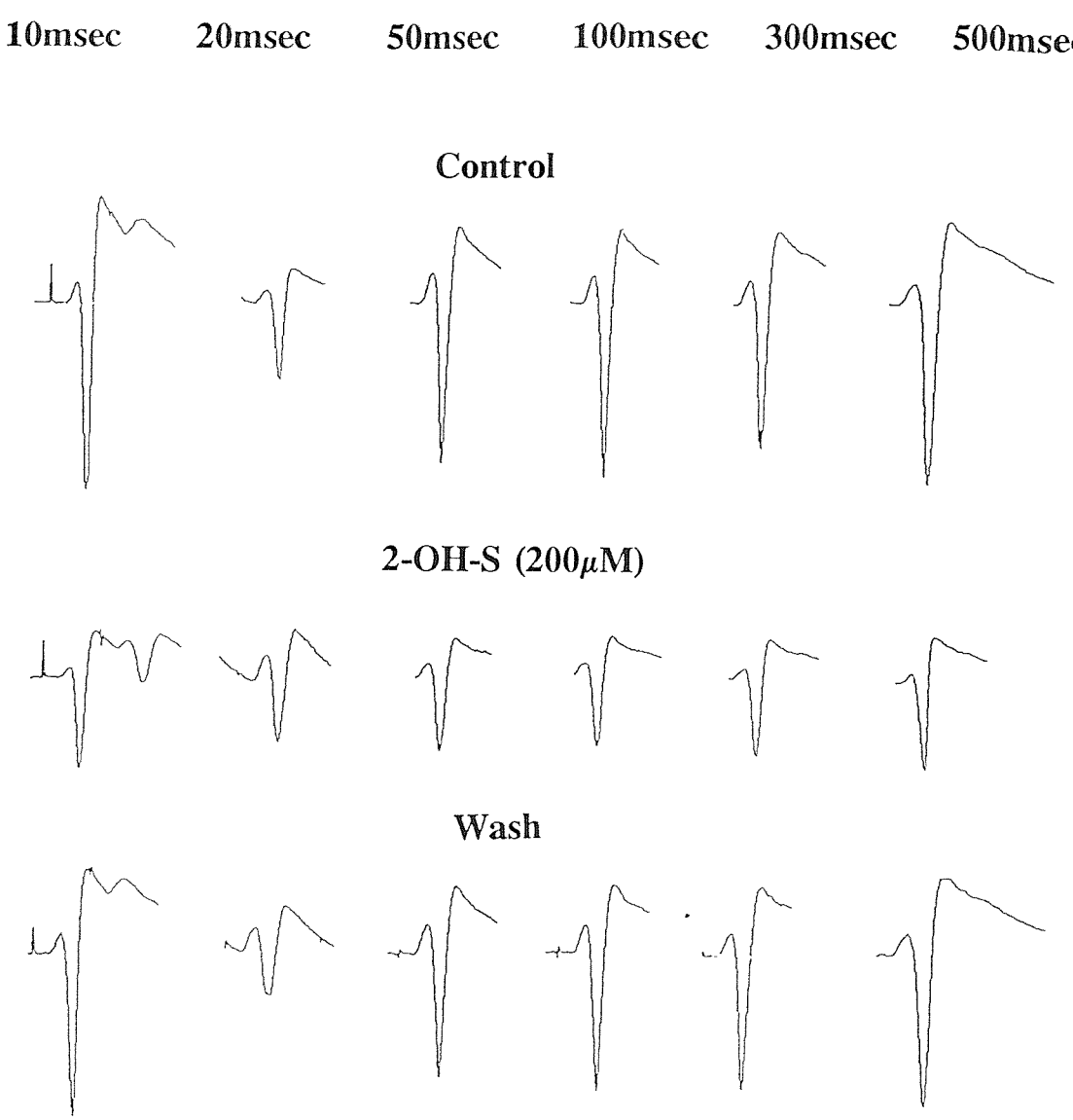


Figure 4.1 A typical example of extracellular field recordings under control conditions showing the conditioning (C) and test responses (T) after paired stimuli to excitatory afferents in CA3. From left to right in each row, C is shown with T for an IPI of 10ms, followed by only the test response for longer IPI's in the following columns. Early inhibition is seen as a reduction in T with respect to C at IPI's of 10-20ms and late inhibition is seen as a reduction in T at IPI's of 200-400ms. 2-OH-S (200μM, n=9) depressed the conditioning response and reversibly reduced the inhibition at short and long IPI's.

Analysis of the dependence of paired pulse inhibition on IPI and the effect of 2-OH-S at each IPI, is shown (**Figure 4.2**, n=9). 2-OH-S also increased the amount of facilitation at 100msec (control, -7.5±2.9%; 2-OH-S, -17.5±4.9% , n=8; Control, 2.45±3.47%; 2-OH-S, -18.48±2.3%, n=9). This was in contrast to phaclofen, which decreased the amount of facilitation at this time interval. The ribbon graphs which were also used in chapter 3, are used again here, to show the results of the individual experiments. There was one individual which produced a huge facilitation of the paired pulse inhibition in response to 2-OH-S. The reason for this was unknown. This meant that the mean ± s.e.m. values for the facilitation were over-estimated. However, when this value was removed the significance of the above results remained the same.

Analysis of the changes in initial slope of the field EPSP, which are used to provide evidence for a presynaptic change, show that the increase in the facilitation of the slope matches the increase in the facilitation of the population spike for IPI's of 30-200msec (**Figure 4.2 C**). The inhibition of the slope at 300msec also reflected the inhibition of the spike.

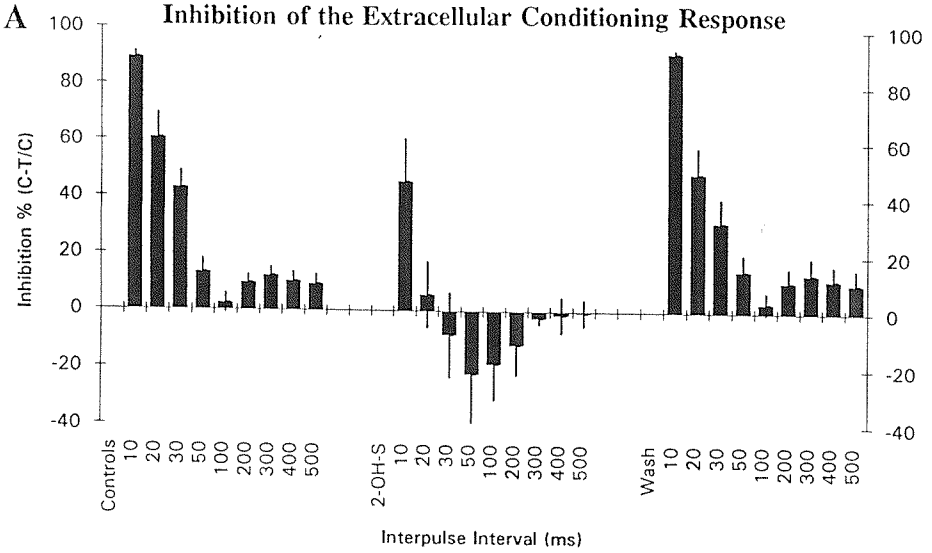
Figure 4.2 (next page) The effect of 2-OH-S (200μM, n=9) on paired pulse inhibition of the population spike amplitude and the initial slope of the field EPSP. Inhibition was calculated as [(C-T)/C %].

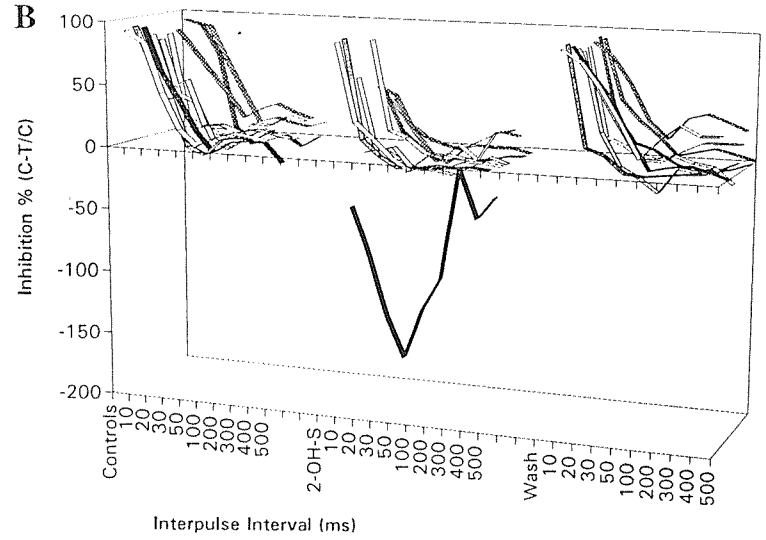
A) The % inhibition (mean±s.e.m.) of the population spike amplitude is shown at each IPI for controls, 2-OH-S and wash. 2-OH-S significantly and reversibly reduces the conditioning response, the early (10-30msec) inhibition and the late inhibition (200-500ms). There is also an increase in facilitation at 30-300msec.

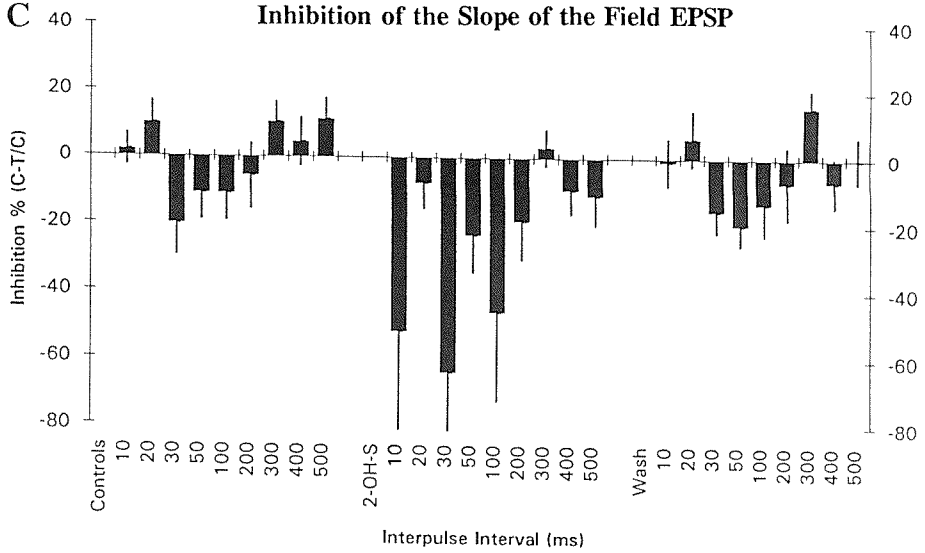
B) The ribbon graph shows the % inhibition of the population spike amplitude at each IPI for each experiment, which reflects the mean values shown in A. One experiment deviates considerably from the rest, which has increased the value of the facilitation seen in the mean.

C) The % inhibition (mean±s.e.m.) of the slope of the field EPSP for all IPI's. 2-OH-S enhanced the facilitation of the slope at 30-100msec and reduced the inhibition of the slope at 300-500ms.

D) The individual results show the variability in the measurements of this small event.







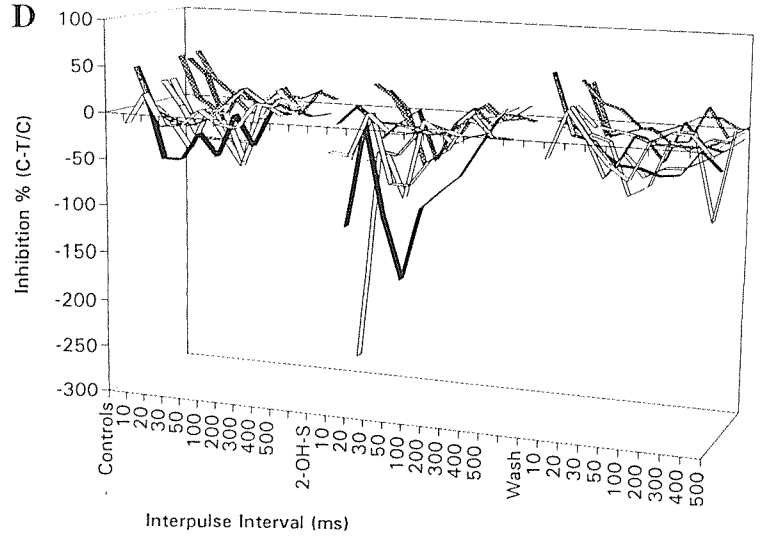


Figure 4.2 2-OH-S Effects on Paired Pulse Inhibition

Effect of 2-Hydroxy-Saclofen on the Conditioning and Test Responses and the Stimulus-Response Curve.

The depression of the conditioning response by 2-OH-S in the paired pulse experiments was quite marked, so the reduction in peak amplitudes of the conditioning response and the test response were analysed more closely (**Figure 4.3**). It could well be the case that this reduction in size was largely responsible for the decrease in the ratio of inhibition. 2-OH-S depressed the conditioning response in all experiments, at each IPI (**A**, n=9, controls 5.2mV ± .03mV; 2-OH-S 3.3mV ± .04mV, $p<0.05$). However, test responses, rather than being reduced at all IPI's as well, appeared to be enhanced at intervals of 10-30msec (**B**, control, 20msec, 1.74±.68mV; 2-OH-S 2.63±.53mV) and reduced at intervals of 50-500msec (control 4.73±.07mV: 2-OH-S 3.37±.08mV). This may suggest perhaps, that the attenuation of the early inhibition was primarily due to the depression of the conditioning response and attenuation of feedback inhibition and the apparent reduction in the late inhibition was due to a reduction in the ratio and not the test spike. The effect of 2-OH-S (200μM, n=3) on the stimulus response curve of the conditioning response was also tested to show any stimulus dependant effects (**Figure 4.4**). A reduction in both the amplitude of the population spike and the slope of the field EPSP was seen at all stimulus intensities. These are represented on the same graph and also on separate graphs for clarity.

It was obvious from the results so far, that 2-OH-S was having quite a different effect on the responses than phaclofen. In order to establish that 2-OH-S was indeed acting at $GABA_B$ receptors and not $GABA_A$ receptors, it was necessary to investigate the effects of bicuculline and phaclofen against the depression of the response evoked by 2-OH-S.

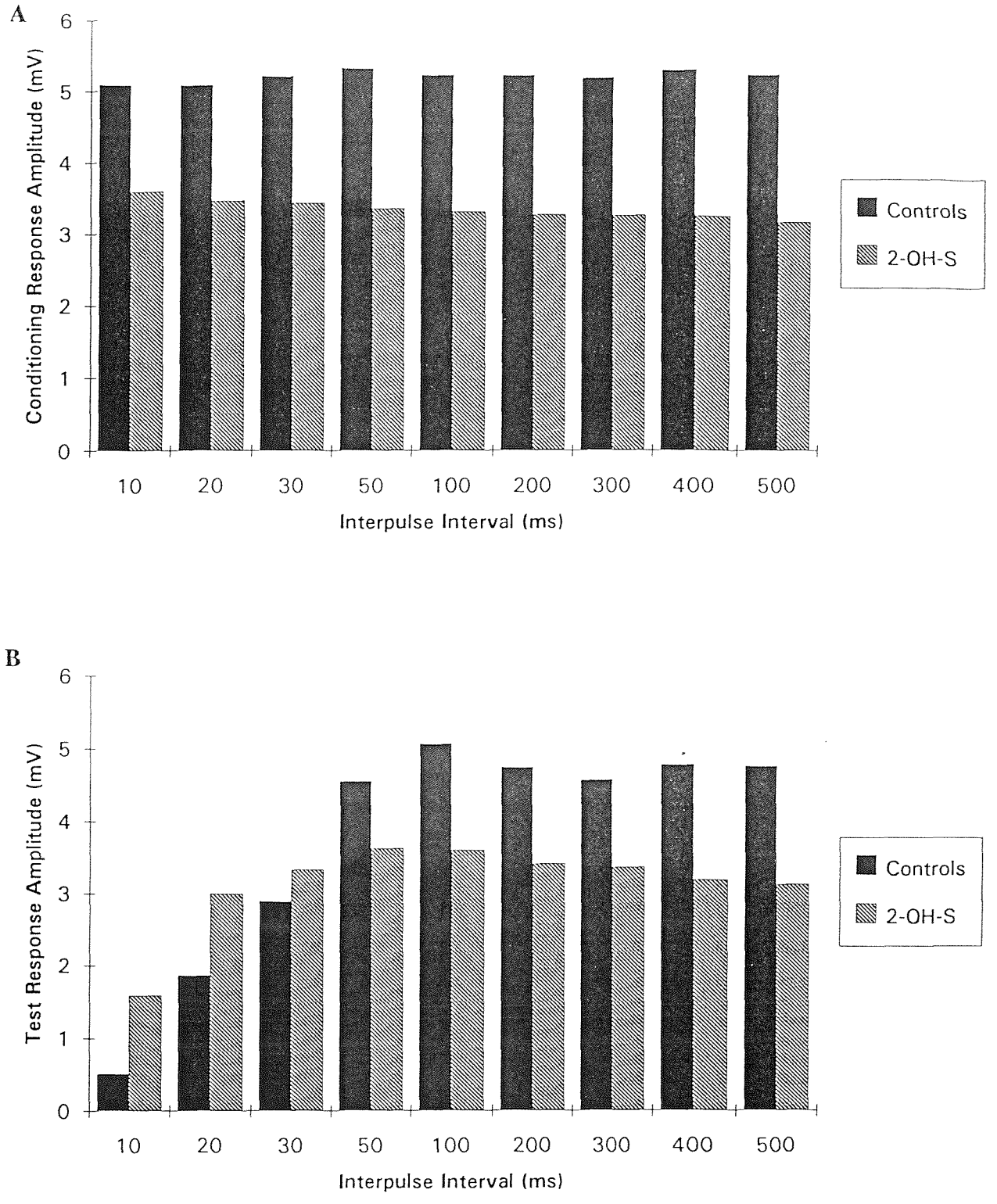
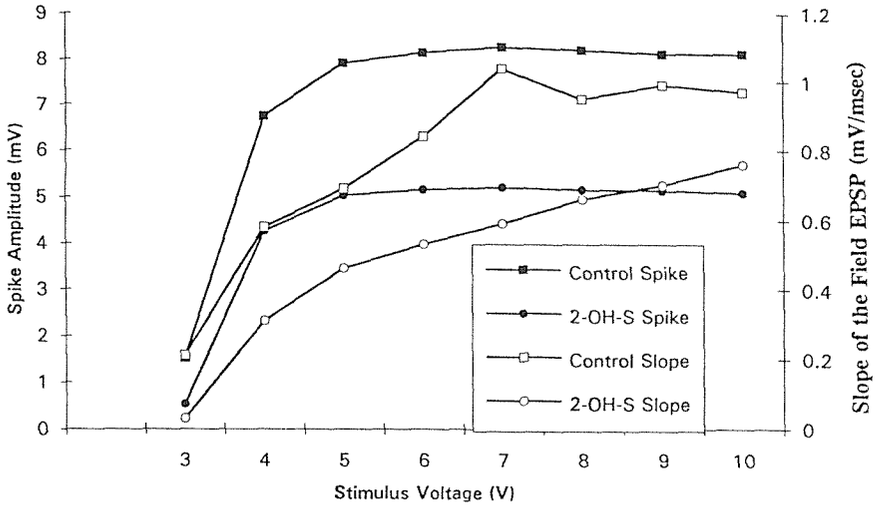
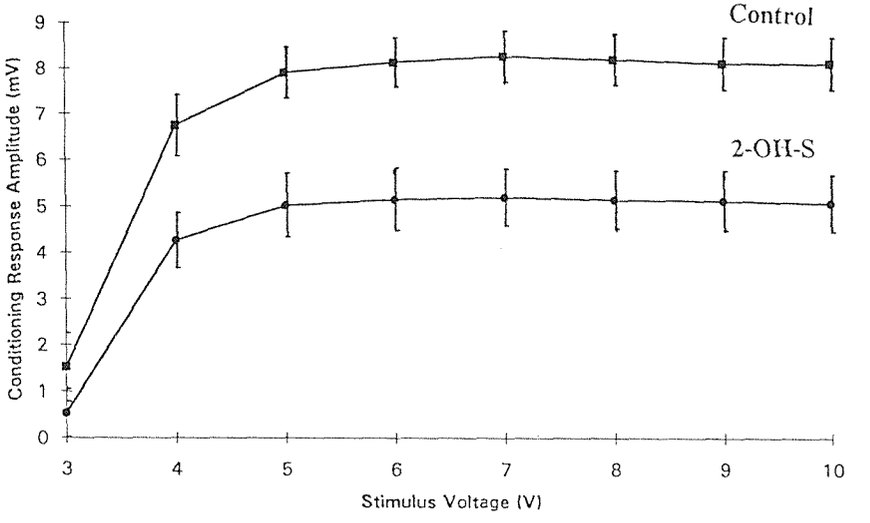


Figure 4.3 The reduction in the conditioning and test responses are shown for controls and in the presence of 2-OH-S. **A**) The conditioning response is significantly depressed by 2-OH-S (200μM, n=9, $p<0.01$). This is apparant and of a similar magnitude at all IPI's. **B**) Under the same conditions, the test response at early IPI's (10-30msec) was enhanced by 2-OH-S, while the test response at the other IPI's (50-500msec) was depressed.





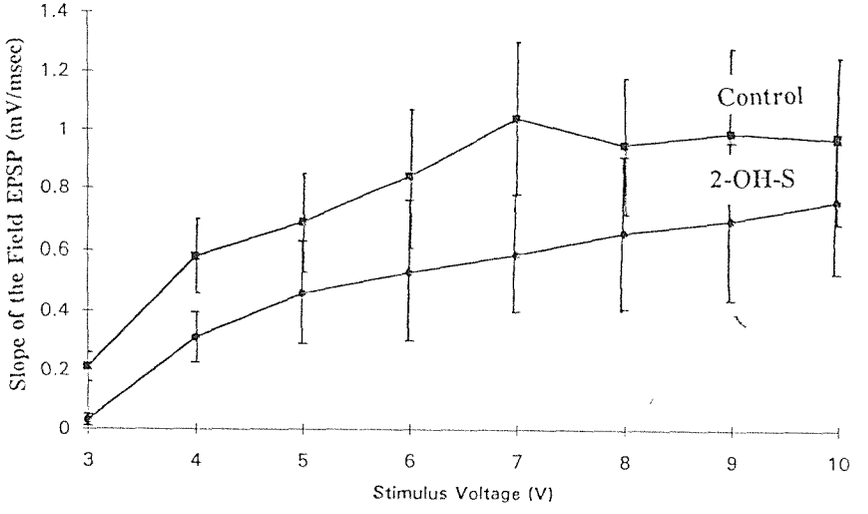


Figure 4.4 The stimulus-response curves for the conditioning response and the slope of the field EPSP are shown under control conditions and upon addition of 2-OH-S (200μM, n=3). **A)** 2-OH-S reduces both the conditioning response amplitude and the field EPSP slope at all stimulus voltages. The scale bar on the left side refers to the measurement of the spike amplitude and the scale bar on the right refers to the amplitude of the slope. **B)** The plot of the reduction in spike amplitude is shown seperately as mean $\pm$ s.e.m. as is **C)** the plot of the reduction in the slope of the field EPSP.

Effect of 2-Hydroxy-Saclofen, Bicuculline and Phaclofen on the Conditioning Response Amplitude.

The reduction in the conditioing response may have been due to a reduction in excitation or activation of GABAA receptors on the postsynaptic membrane. In order to determine whether 2-OH-S had a direct effect on $GABA_A$ receptors the effects of bicuculline and phaclofen were tested against the 2-OH-S induced depression of the conditioning response. It was hoped that one or other of these drugs, which were selective for the $GABA_A$ and $GABA_B$ receptors, would antagonise the effects of 2-OH-S and provide us with an answer to the question.

The depression of the conditioning response by 2-OH-S was investigated in more detail in a series of experiments (n=10) in which sets of individual stimuli (5 at 10s intervals) were repeated every 5 minutes, whilst drugs were perfused through the recording chamber. Recordings were averaged to give a single amplitude value at each time point for each experiment and normalised to the average value during the control period. The spike amplitude data is presented as the normalised average ± sem through time.

In the first set of experiments (n=5), under control conditions, the spike amplitudes were steady, and upon addition of 2-OH-S (200μM) there was a depression of the spike amplitude (control, 6.25±3.1mV; 2-OH-S, 5.17±.3mV; Bic, 5.28±.5mV). The further addition of bicuculline (1μM), in the continued presence of 2-OH-S, did not appear to elicit any other change in the spike amplitude in either direction (**Figure 4.5 A**) The histograms in this and the following set of experiments are the mean ±s.e.m. Representative population responses from one experiment are shown above the first histogram indicating the effects of 2-OH-S and bicuculline (**Figure 4.5**). Removal of both compounds led to a return of the spike amplitude towards control levels.

Analysis of the initial slope of the field EPSP showed a reduction of the response by 2-OH-S, which continued in the presence of bicuculline and reversed after washing (**Figure 4.5C**). Individual data for the population spike and the initial slope of the field EPSP are presented in the form of ribbon graphs (**Figure 4.5B,D**). The population responses for each experiment appeared to follow the same trend, as did those for the slope values.

In the next set of experiments (n=5) the same procedure was repeated, showing the depression of conditioning spike amplitude produced by 2-OH-S (control 30m 6.13±.24mV; 2-OH-S 60m 4.98±.41mV; p, 0.02), but testing the effect of the further addition of phaclofen (1mM, **Figure 4.6A**). Representative population responses from one experiment are shown above the first histogram indicating the effects of 2-OH-S and

phaclofen (**Figure 4.6**). As the phaclofen/2-OH-S was perfused through the chamber the amplitude of the spike returned toward control levels (6.11±.24mV at 90m) and no further change was observed on washing (6.15±.24mV at 120m).

Analysis of the initial slope of the field EPSP showed a reduction of the response by 2-OH-S, which reversed after addition of phaclofen. (**Figure 4.6C**). Individual data for the population spike and the initial slope of the field EPSP are presented in the form of ribbon graphs (**Figure 4.6B,D**). The population responses and slope values for the individual experiments appeared to follow the same trend.

In addition to looking at the depression of the conditioning response amplitude, the paired pulse inhibition was also measured at the end of the half hour period of each drug application. In the first set of experiments, the early inhibition was reduced by 2-OH-S (control, 20msec, 1.45±.43%; 2-OH-S, 35.57±3.59%) and further attenuated by bicuculline (Bic, 75.04±5.51%). The late inhibition was also reduced by 2-OH-S (control, 300msec, 75.75±5.82%; 2-OH-S, 93.07±2.14%) which was further reduced slightly upon addition of bicuculline (Bic, 102.07±3.01%). The latter result was probably due to the ongoing application of 2-OH-S, as opposed to any effect of bicuculline.

Figure 4.5 (next page) The effect of bicuculline (1μM) on the reduction in spike amplitude caused by 2-OH-S (200μM, n=5). The histogram represents mean±s.e.m.. Data is normalised to the average values obtained during controls. representative recordings from one experiment at the times 30, 60, 90 and 120 minutes are shown above the first histogram.

A) 2-OH-S significantly ($p<0.01$) and reversibly reduces the population spike amplitude. Further addition of bicuculline elicits no other significant change in the spike amplitude.

B) The ribbon graph shows the reduction of the spike amplitude for the individual experiments which reflects the mean values in A.

C) The depression of the slope of the field EPSP. 2-OH-S depressed the slope similar to the depression of the spike and this continued after addition of bicuculline, i.e. it was not reversed.

D) The individual results suggest a scatter in some of the experiments but the trend still remains the same.

Figure 4.5 2-OH-S & Bicuculline Effects on the Conditioning Response

In the second set of experiments, 2-OH-S again reduced the early inhibition (control, 20msec, 53.23±13.3%; 2-OH-S, 66.83±13.45%) with phaclofen causing very little change (Phac, 64.13±7.7%). However, the reduction of the late inhibition (control, 300msec, 88.15±3.11%; 2-OH-S, 102.01±1.26%) was partially reversed by phaclofen (Phac, 99.97±2.38%).

Figure 4.6 (next page) The effect of phaclofen (1mM) on the reduction in spike amplitude caused by 2-OH-S (200μM, n=5). The histogram represents mean±s.e.m.. Data is normalised to the average values obtained during controls. Representative recordings from one experiment at the times 30, 60, 90 and 120 minutes are shown above the first histogram.

A) 2-OH-S significantly ($p<0.01$) and reversibly reduces the population spike amplitude. Further addition of phaclofen significantly reverses the depression of the spike amplitude which recovers fully on washing.

B) The ribbon graph shows the reduction of the spike amplitude for the individual experiments which produces the mean values in A.

C) The depression of the slope of the field EPSP. 2-OH-S depressed the slope similar to the depression of the spike and this continued until 10 minutes after the addition of phaclofen, when. it was reversed.

D) The individual results suggest a scatter in some of the experiments but again the trend still remains the same.

Figure 4.6 2-OH-S & Phaclofen Effects on the Conditioning Response

4.4 Discussion

The experiments presented here reveal a qualitative difference in the actions of the $GABA_B$ ligands phaclofen and 2-OH-S, distinct from the quantitative differences in attenuation of the late inhibition mediated by $GABA_B$ receptors as described previously (Dutar & Nicoll, 1988a; Dutar & Nicoll, 1988b; Kerr et al., 1988; Davies et al., 1990).

2-OH-S has been shown to have direct effects on the amplitude of the population spike responses of the CA1 neurones to orthodromic stimuli, distinguishing it's activity from phaclofen which had no direct actions. Assuming selectivity for $GABA_B$ receptors, antagonism of $GABA_B$ receptors could only account for these observations if they were located on inhibitory cells. For instance, this effect could be produced by the enhancement of a tonic inhibition by antagonism of presynaptic autoreceptors on basket cell synapses, leading to an increase in $GABA_A$ inhibition. In this case the reduction in population spike amplitude would be indirect and should be prevented by blockade of postsynaptic GABA A receptors. However, this is not the case. Bicuculline, at a dose sufficient to attenuate $GABA_A$ inhibition in our experiments, failed to reverse the effects of 2-OH-S.

Alternatively, 2-OH-S could have an agonist action on $GABA_B$ receptors to lead to a reduction in population spike amplitude, similar to baclofen (Nathan & Lambert, 1991), (Stanford & Chad, unpublished observations). The reduction in population spike amplitude could be conceivably produced by either pre- or postsynaptic agonism of $GABA_B$ receptors or a combination of both. In support of this, 2-OH-S appeared to increase the facilitation of the response at 50-100msec which is believed to reflect a presynaptic depression of the inhibitory response (Isaacson et al., 1993).

Measurements of the slope of the field EPSP provide a measurement of the magnitude of the inward current flow at the excitatory synapses, and thus provides an index of presynaptic activity (Andersen et al., 1966) independent of changes in excitability of the postsynaptic cell which would also affect the population spike amplitude. Application of 2-OH-S, but not phaclofen reduces the field EPSP slope, implying a reduction in presynaptic activity, which appears to account for a large component of the observed reduction in population spike amplitude.

Both the reduction of the field EPSP slope and population spike amplitude are prevented by co-administration of phaclofen. Thus an agonistic effect of 2-OH-S at presynaptic receptors of excitatory synapses could account for the observed changes. Presynaptic receptors are

known to be present in the hippocampus and appear to decrease neurotransmitter release when activated, at both inhibitory and excitatory terminals (Harrison et al., 1990; Thompson, Capogna, & Scanziani, 1993). Any postsynaptic actions would compound these effects.

Based on the evidence presented from these experiments and previously published observations of blockade of both the actions of baclofen (Lambert, Harrison, Kerr, Ong, Prager, & Teyler, 1989; Harrison et al., 1990) and the slow IPSP, we suggest that 2-OH-S is acting as a full or partial agonist at presynaptic $GABA_B$ receptors. This hypothesis can account for the anomalous observations that 2-OH-S reduces early, $GABA_A$ mediated inhibition and also that bicuculline fails to affect population spike amplitude in its presence. It is proposed that 2-OH-S can activate presynaptic $GABA_B$ receptors of inhibitory interneurones (as well of excitatory synapses), reducing their release of GABA and hence reducing early (and late?) inhibition. This would reduce tonic GABA release and hence prevent the action of bicuculline to increase population spike amplitude by antagonising this tonic effect.

In order to investigate these results in further detail, a more selective approach was needed to look at the mechanisms of inhibition in an individual cell. It was decided that the technique of whole cell voltage clamp should be used for this purpose, to enable inhibitory currents, in particular those mediated by $GABA_B$ receptors, to be analysed in isolation. These aims and results are set out in the following two chapters.

CHAPTER FIVE

5

Mechanisms Of GABAergic Inhibition In A Single Cell

The preceding two chapters dealt with the identification and separation of the two phases of GABAergic inhibition using extracellular recording techniques. The anomalous results obtained with the GABA B ligand 2-hydroxy-saclofen were investigated further at the single cell level using the whole cell patch clamp technique. It was hoped that by employing this method we would be able to isolate the components of inhibition without the global effect of the population of cells and the numerous synaptic contacts between them.

5.1 Introduction

The patch clamp technique was pioneered in order to isolate and record ionic conductances in cells of the CNS and a number of configurations have evolved, including whole cell patch, inside-out and outside-out patch (Neher & Sakmann, 1976; Neher, 1988; Edwards, Konnerth, Sakmann, & Takahashi, 1989; Konnerth, 1990; Madison, 1991).

When the technique was originally applied to the CNS, the cells were either isolated using enzymatic techniques, or cultured, in order to obtain cells with few or no processes (Hamill,Marty,Neher,Sakmann, & Sigworth, 1981; Sakman,Hamill, & Bormann, 1983). The resistance of the seal is of paramount importance, formation of a gigaohm seal minimises the leakage current and background noise. To facilitate this magnitude of seal, clean electrodes and gentle suction are used each time (Neher, 1988). In order to permit recordings from cells in intact synaptic circuits, a simple cleaning technique where the solution bathing the cells was streamed over the cell area to remove debris, has also been used (McCarren & Alger, 1987). In addition to this, high power optics can be used which allow visual identification of cells within thin slice preparations (200μm) and leave enough working distance to insert stimulating and recording electrodes (Edwards et al., 1989).

Further to this, it was discovered that it was not necessary to preclean the surface of the cells when using brain slice preparations and that a gigaohm (GΩ) seal could be obtained without flame polishing the tips of the recording electrodes as had been done previously (Randall,Schofield, & Collingridge, 1990). The pyramidal cells are densely packed in a relatively compact area of the hippocampal slice, so there was no need for special optics or cleaning techniques. The cells studied in this chapter and the next were obtained by this 'blind technique'.

Under whole cell patch clamp, once a seal has been formed, the membrane at the tip of the electrode is ruptured to allow low resistance electrical access to the interior of the cell, allowing the total membrane current to be recorded. A cell is held by means of the clamp at a known voltage and the current then measured to keep the cell at that potential is a direct reflection of ionic movements through the cell. Stepping the voltage positive or negative from the holding potential results in the activation of mixed inward and outward currents through voltage gated channels. The membrane conductance is directly proportional to the ion channel activity and by holding the membrane potential constant you can ensure that the current is linearly proportional to the conductance that is of interest.

For complete voltage clamping the cells need to be small and processes limited to avoid the problem of inadequate space clamp. This occurs as a result of not being able to exert voltage control over regions of the cell which are at a distance from the soma. Caution should be taken when recording currents from remote synapses such as those on the dendrites, as the current may be distorted when it arrives at the soma.

The advantage of using whole cell voltage clamp as opposed to conventional intracellular techniques is the reduction of damage to the cell and the ability to obtain good access allowing the contents of the electrode to dialyse with the contents of the cell. This enables ion channel blockers, fluorescent dyes and other drugs to be introduced via the electrode. The GΩ seal and reduced access resistance also means the signal to noise ratio is far better than conventional intracellular recordings. The damage caused by impalement with the sharp intracellular electrode reduces the input resistance and the time constant unlike whole cell electrodes. This means that very rapid events can be observed. The disadvantage may be that in trying to replace the cell contents one may lose important unknown factors which may alter the results obtained.

In the hippocampus, many groups have recorded EPSC's (Hestrin,Nicoll,Perkel, & Sah, 1990; Randall et al., 1990) and $IPSC_A$/$IPSC_B$'s (Davies et al., 1990; Nathan & Lambert, 1991; Solis, Isaacson, & Nicoll, 1992b; Roepstorff & Lambert, 1992; Thompson &

Gahwiler, 1992a; Lambert & Wilson, 1993; Mott, Xie, Wilson, Swartzwelder, & Lewis, 1993; Otis , DeKoninck, & Mody, 1993). Whole cell voltage clamp of the cells has allowed characterisation and manipulation of individual synaptic currents. The size of the current recorded is largely dependent on the voltage that the cell is held at and the ionic concentrations. In the above studies, the isolation of the IPSC's was achieved by pharmacological blockade of the EPSC with CNQX and D-APV.

Studies of postsynaptic currents from single cells have enabled characterisation of the EPSC. The contribution of the EAA receptor subtypes to the EPSC has been well charaterised. The major portion of the current at hyperpolarised potentials is mediated by non-NMDA receptors (Hestrin et al, 1990; Lambert & Jones, 1990). At more depolarised potentials a slow NMDA component becomes apparant. The kinetic properties which have been described (Hestrin et al, 1990) can be more easily characterised as intracellular blockers can be injected in to the cell via the electrode. However, none of the studies in which the EPSC was present could reveal clearly the fast and slow IPSC's under the standard physiological slice conditions.Removing the complications of the temporal overlap of the EPSC/IPSC by activating local interneurones directly has allowed the observation and study of monosynaptic inhibitory currents. Many of the above groups have investigated the disinhibition of inhibitory and excitatory currents in this way.

The main advantages of using this recording technique in the in the slice preparation above conventional intracellular techniques is the reduction in the noise levels recorded. Local stimulation of the afferents can be employed which allows very small synaptic potentials to be recorded such as quantal events from single spines. Also it is usually easier than trying to impale the cell with a sharp electrode and less damage results in healthier cells.

As stated at the beginning, the aim of this section of work was to try and obtain good whole cell recordings of excitatory and inhibitory currents in order to investigate GABA mediated inhibition. Initially, we did not block any of the excitatory currents and we stimulated the excitatory afferents in the CA3 as in the extracellular studies to try and obtain stable recordings EPSC's and IPSC's from the CA1 pyramidal cells under relatively physiological conditions.

5.2 Methods

The hippocampi were prepared as indicated in chapter 2 and the ACSF concentration was kept the same as that used for extracellular recordings. The conditions for holding and transferring the slices were also the same.

The methods for obtaining whole cell recordings were detailed in the main methods section in chapter 2 and the solution in the recording electrodes was as follows: (mM) Hepes 40; Gluconate 100; KCl 16; EGTA 10; ATP 5;. Leupeptin 0.1. This was corrected to pH 7.2 with KOH (approx. 170mM). The electrode tips had resistances of 4-10MΩ.

Synaptic currents were measured in response to stimulation of the excitatory afferents (Schaffer/Commisurals) using a bipolar stimulating electrode (80/20 Ni/Cr).identical to that used for the extracellular recordings.

The drugs used were: D-APV (50μM) Tocris Neuramin and Bicuculline (1μM) Sigma.

5.3 Results

Compound EPSC's and IPSC's were recorded from individual pyramidal cells in CA1 of the rat hippocampal slice, upon stimulation of the Schaffer collaterals/commisural afferents, under normal (physiological) conditions. Perfusion with the NMDA antagonist D-APV (50μM) and the $GABA_A$ antagonist bicuculline (1μM) partially reduced the slow EPSC and the fast IPSC respectively, but was not sufficient to isolate the individual components of GABAergic inhibition in order to investigate the effects of 2-hydroxy-saclofen on pre and postsynaptic $GABA_B$ receptors.

Whole Cell Patch Clamp Recordings from CA1 Pyramidal Cells - General properties.

Individual CA1 pyramidal cells were obtained and held at -60mV. The input resistance (IR) was measured every 5 minutes for 15 minutes until the cell had stabilised (**Figure 5.1A**, n=13). This was done by stepping the cell from -80mV to -40mV in 10mV steps. The resulting current was plotted against the voltage and the IR was measured as the the slope of the linear regression line. Capacitance transients (arrow) were observed at the start and finish of the voltage steps and were due to the charging of the cell membrane. These were compensated for on line as far as was possible to avoid any contamination. The IR gave us a good indication of the general health of the cell and those in which IR was below 100MΩ were rejected.

Once the cells had stabilised, the series resistance, which is a measure of the electrode access resistance, was compensated for on line. The values for this compensation were between 10 and 30 MΩ. Cells which needed any greater compensation were rejected because the membranes had become too leaky and it was generally a sign that the cell was unstable.

In standard ACSF, EPSC's were evoked with a maximal stimulus (without breakthrough sodium spikes) and recorded as inward currents under control conditions (**Figure 5.2A**). These displayed a mean amplitude of -235 ± 47.85pA measured at the peak of the response and a latency to peak of 7.3±1.8msec (n=8). This was followed immediately by a compound IPSC which had a mean amplitude of 63.97±15.96pA measured at the peak of the compound response and a latency to peak of 19.98±6.75msec. This latency to peak corresponded with that of the early inhibition measured in the extracellular paired pulse

studies (10-30msec) and suggested that it was mainly the fast IPSC mediated by $GABA_A$ receptors which would correspond with the fast, early inhibition seen at intervals of 10-20msec under extracellular paired pulse recordings.

The reversal potential for the EPSC was measured by stepping the cell from its holding potential of -60mV to voltage steps from -100mV to -50mV, with evoked EPSC during the step. The amplitude of each EPSC was measured at the peak value and plotted against the corresponding voltage (**Figure 5.1B**). The reversal potential was calculated as the intercept of the linear regression line at the X-axis. In most cases, no actual reversal was observed and the value was extrapolated. This is because the reversal potential for NMDA and non-NMDA receptors is nearer 0mV (Mayer & Westbrook, 1987). In addition to this, the lack of Na^+ in the recording electrode would alter the measured reversal potential. The extrapolated reversal potential for the EPSC was -27±5.1mV (mean±s.e.m.,n=8).

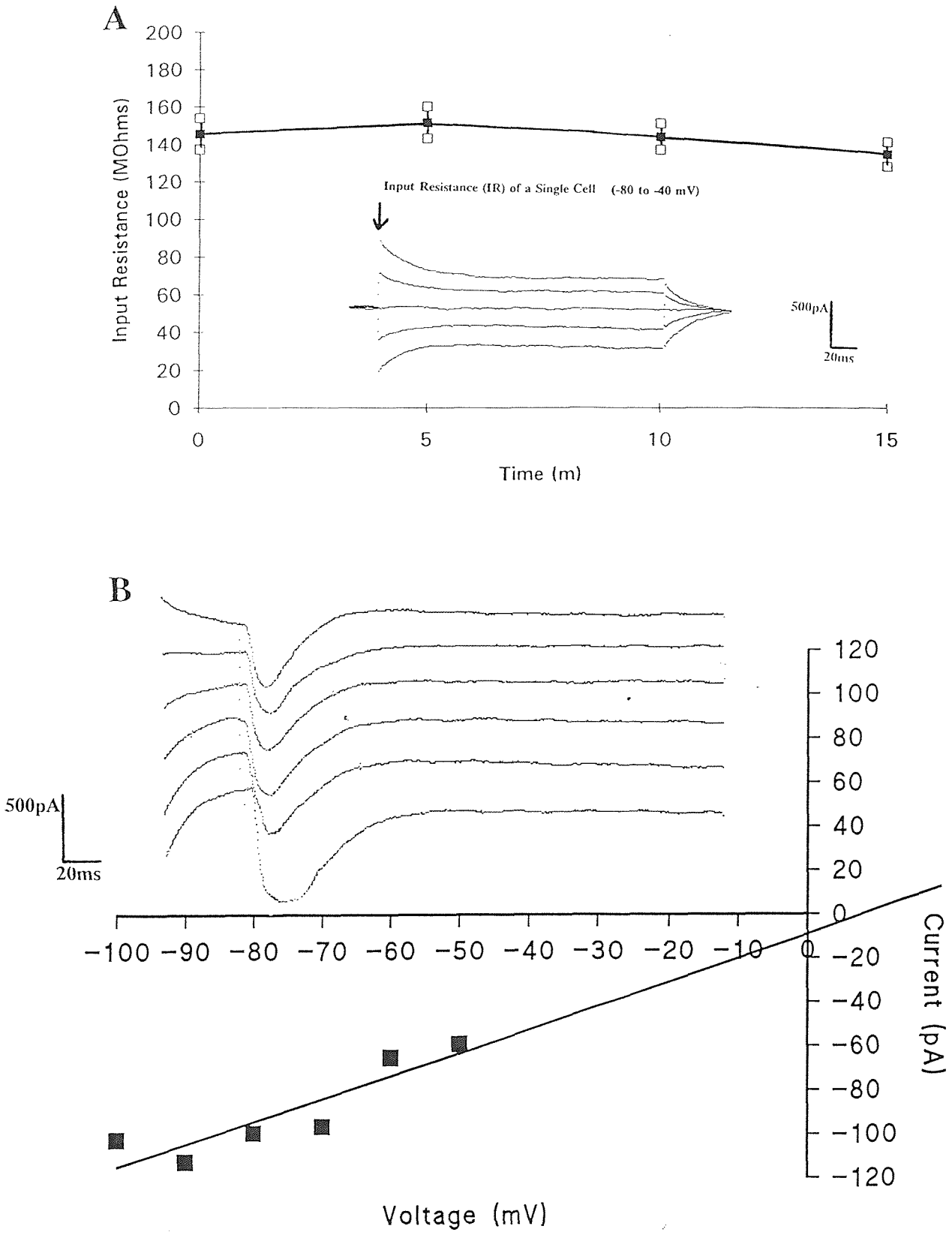


Figure 5.1 A) Cells were held at -60mV and the input resistances were measured over a 15 minute period by stepping the voltage of the cell from -80mV to -40mV. Cells with an input resistance of <100 MOhms were discarded. Inset: typical currents evoked by the voltage steps applied to measure input resistance. B) The cell was stepped from -100mV to -50mV to measure the reversal potential of the EPSC and IPSC. Inset: the typical currents evoked by the voltage steps applied to measure the reversal potential shown with the evoked EPSC. The line indicates the time point at which the peak current was measured.

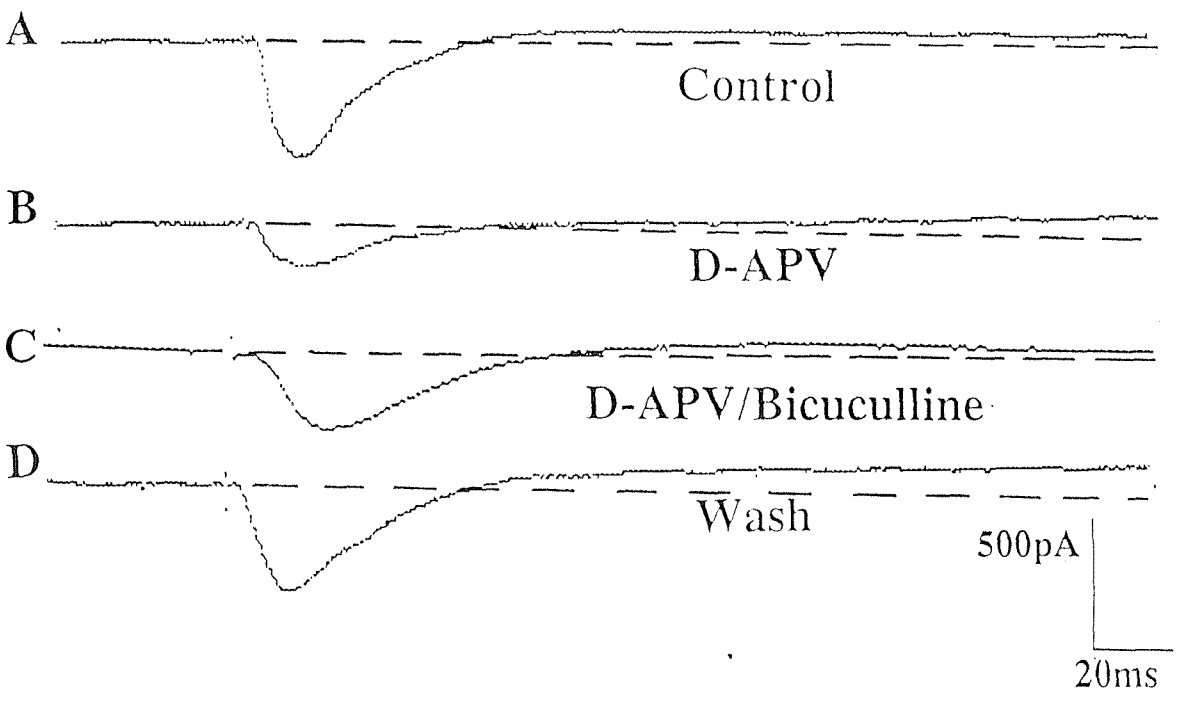


Figure 5.2 The EPSC evoked by orthodromic stimulation of the excitatory afferents under control, drug and wash are shown. A) a single EPSC was evoked using a stimulus set to achieve a maximal response without any breakthrough sodium spikes (n=8). Under these conditions it was sometimes possible to see a compound IPSC (n=5) (A,B) B) application of D-APV (50μM) reduced the NMDA component of the EPSC. c) additional application of bicuculline (1μM) had a small enhancing effect in some cells. D) full recovery was obtained on washing. (n=8). The broken line (0) is used to show the compound outward IPSC. (ECl- = -60mV)

The Effect of D-APV and Bicuculline upon the EPSC and IPSC.

The NMDA receptor antagonist D-APV (50μM) was used to block the slow component of the EPSC in the hope of isolating the IPSC (n=8). D-APV significantly reduced the peak amplitude of the EPSC by 52% (control -235.85±47.85pA; D-APV -111.57±26.69pA, $p<0.02$) and the amplitude of the IPSC measured at 20msec by 33% (control 63.96±15.96pA; D-APV 42.67±13.97pA). (**Figure 5.2B**).

Concurrent addition of the $GABA_A$ antagonist bicuculline (1μM) resulted in an increase in the peak amplitude of the EPSC (D-APV -111.57±26.69pA; D-APV/Bic -193.70±39.22pA, $p<0.02$) in 7 out of the 8 cells tested (**Figure 5.2C**). There was no significant change in the peak amplitude of the compound IPSC (20msec, D-APV 42.67±13.97pA; D-APV/Bic 42.27±17.46pA) except for one cell where there was a reduction of the amplitude by 82%. This may suggest that a large part of the IPSC remaining after D-APV was either mediated by $GABA_B$ receptors or that the concentration of bicuculline was insufficient for a full block of the $GABA_A$ receptors. Most of the changes were reversible upon washing (**Figure 5.2D**).

These results were quantified and the effect of the application of D-APV and bicuculline on the peak amplitudes of the EPSC and IPSC is shown in **Figure 5.3A,C**. The histograms represent mean ±s.e.m. Ribbon graphs are used as in the previous two chapters, to show the results for each of the individual cells (**Figure 5.3B,D**). There is a larger degree of scatter between the results for the IPSC and no recovery was apparent, which may reflect the difficulty in recording this event with the stimulus and pharmacological parameters used. It was only possible to measure an IPSC in 5 out of the 8 cells which were recorded from.

Figure 5.3 (next page) The effect of D-APV and bicuculline on the peak amplitude of the evoked EPSC and IPSC.

A) D-APV (50μM) reduced the peak amplitude of the EPSC. This was then slightly reversed by addition of bicuculline (1μM) and fully recovered on washing (n=8).

B) The ribbon graph shows the responses of the individual cells in A. Although there is some scatter, the overall trend of the response is the same.

C) D-APV and bicuculline reduced the IPSC without any recovery.

D) The lack of individual data was due to a lack of recovery of the IPSC.

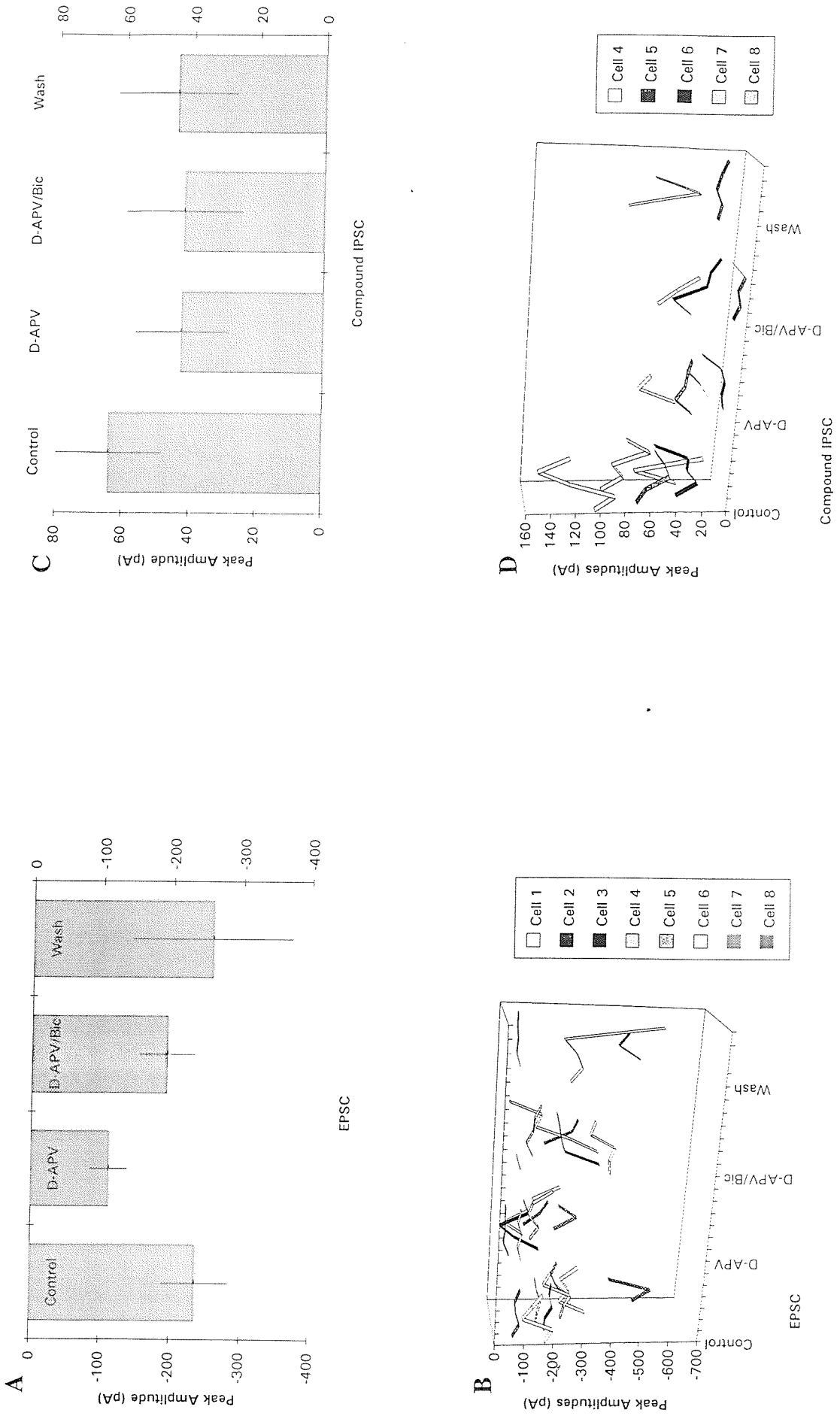


Figure 5.3 EPSC/IPSC. Effects of D-APV and Bicuculline

The Effect of D-APV and Bicuculline on the Reversal Potentials of the EPSC and IPSC

The raw data for the effect of the compounds on the EPSC/IPSC at different voltages is shown for short and longer time courses with the arrow indicating the point at which the cell was stimulated (**Figure 5.4 1&2**). Because the use of the original time course didn't reveal a period of slow inhibition a longer time course was employed where the EPSC was evoked prior to stepping the voltage of the cell. However, this also did not reveal a clear, late inhibitory event.

The reversal potentials of the $IPSC_A$ (-74.75±4.81mV) and $IPSC_B$ (-87.84±11.82mV) were measured by taking the amplitudes at the times corresponding to $GABA_A$ (20msec) and $GABA_B$ (300msec) receptor mediated inhibition in the extracellular studies. This was because they could not be identified clearly under the conditions employed. Although a reversal of the $IPSC_A$ could usually be seen, a clear reversal of the $IPSC_B$ could not always be seen and therefore the values were extrapolated from the regression line. The size of the event being measured and the nature of trying to clamp a cell with long processes may lead to the errors in the values measured for the reversal potentials.

The mean reversal potential for the EPSC was -27.51±5.1mV. There was a small change in the reversal potential of the EPSC toward a more negative value after application of D-APV which was reversed and then made more positive by the additional application of bicuculline. This could be reversed to control values on washing.(control, -27.51±5.1mV; D-APV,-48.14±14.4mV; D-APV/Bic, -17±8.6mV; Wash, -40.80±9.6mV). Both the $IPSC_A$ and $IPSC_B$ showed a small positive change with bicuculline. Recovery was obtained on washing ($IPSC_A$, control,-76.75±4.8mV; D-APV/Bic,-35.76±3.8mV; Wash,-67.44±4.6mV; $IPSC_B$,control,-87.84±11.8mV; D-APV/Bic, -34.21±8.3mV; Wash,-51.04±2.3mV). The mean±s.e.m. for the reversal potentials and individual plots are shown in **Figure 5.5**. None of these changes to the absolute or approximated reversal potentials were significant under any drug condition.

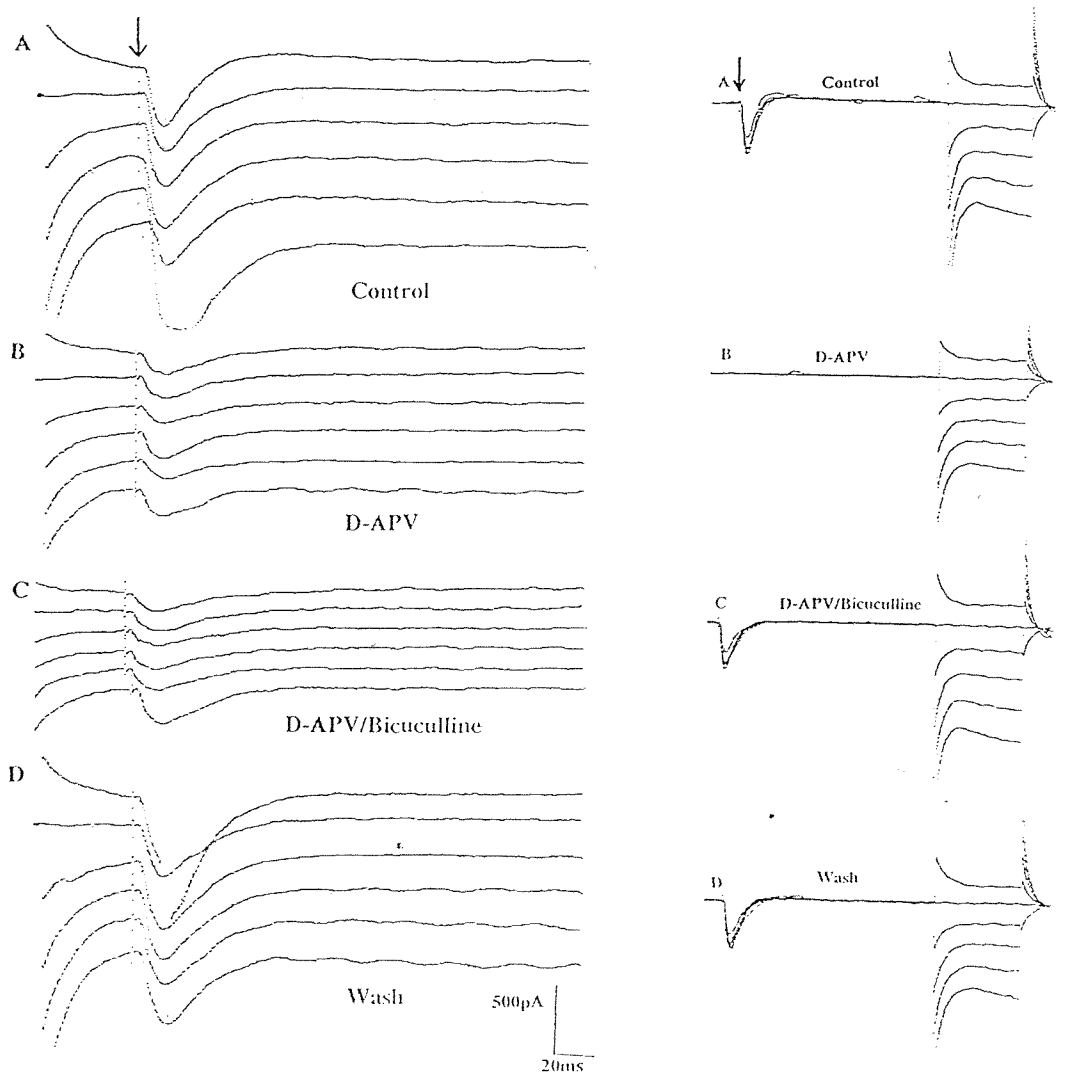


Figure 5.4 Representative current traces obtained when stepping the cell from a holding potential of -60mV. Steps of -100mV to -50mV (bottom in each case = -100mV) were used with an evoked EPSC/IPSC during the step. These are shown under each experimental condition. The left hand side (A-D) show EPSC's/IPSC's evoked during a voltage step. The right hand trace (A-D) shows the voltage steps elicited after evoked EPSC's/IPSC's, which were used to observe any change in the IPSC B at a later time course. The arrow indicates the point at which the cell was stimulated.

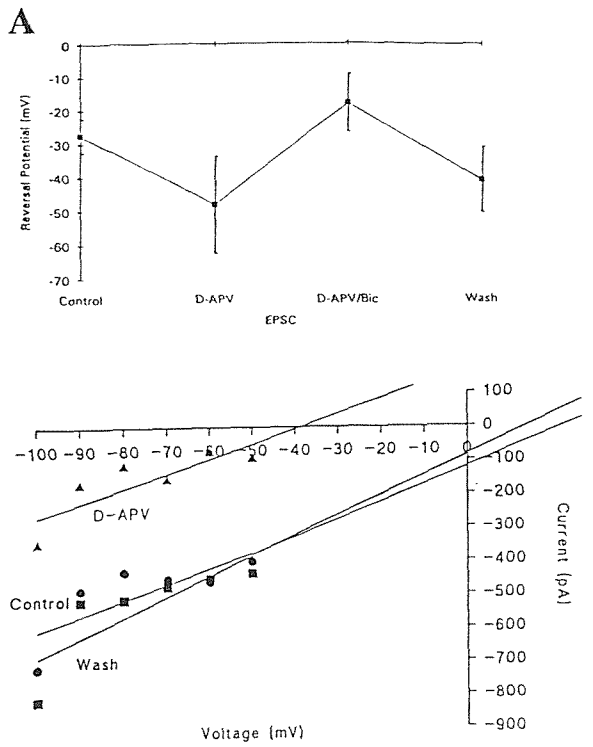
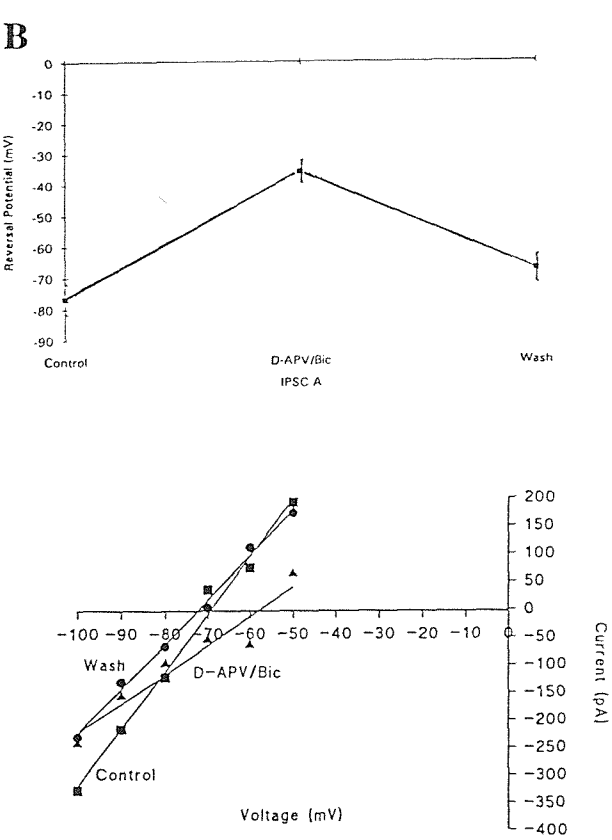
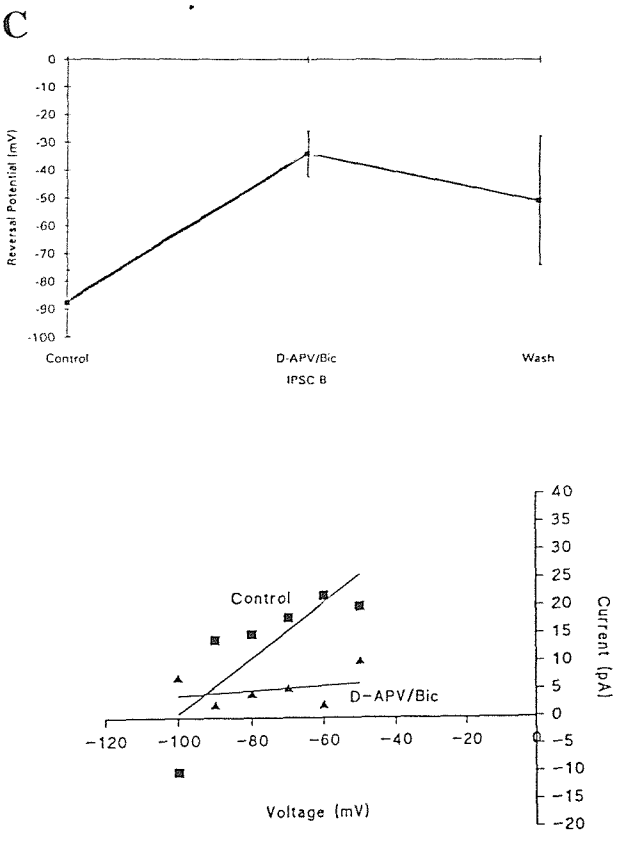


Figure 5.5 The average reversal potentials for the EPSC, IPSC A and IPSC B (A,B,C) are shown and in each case accompanied by a representative graph of a current/voltage plot for one cell (n=8 under each drug condition).
A) D-APV (50μM) appeared to shift the reversal potential of the EPSC to a more negative value which was reversed by bicuculline (6μM) and partially recovered on washing.
B) Bicuculline shifted the reversal potential of the IPSC A to a more positive value and recovered on washing.
C) Bicuculline again shifted the reversal potential of the IPSC B to a more positive value and this partially recovered on washing. None of these changes were significant. The values for the reversal potentials of the IPSCB and EPSC are approximations and the lines have been extrapolated to indicate the point at which they should intercept the line.





5.4 Discussion

Whole cell synaptic currents were successfully recorded from individual CA1 pyramidal cells in the hippocampal slice using the whole cell patch clamp technique. Measurement of the input resistances indicated that good, high resistance seals had been formed and that the cells were stable for periods of up to about 90 minutes. A compound (fast & slow) EPSC was observed with similar amplitude and time course to that observed by other groups in hippocampal slices (Hestrin et al., 1990; Randall et al., 1990), dissociated cells (Yoon & Rothman, 1991b) and cultures (Scanziani,Capogna,Gahwiler, & Thompson, 1992). A compound IPSC immediately followed the EPSC. From measuring the latency to peak of the IPSC (approx. 20msec) it was assumed that it was largely $GABA_A$ mediated.

Application of the NMDA antagonist D-APV reduced the amplitude of the EPSC although it did not appear to significantly affect the latency to peak, which may be expected to shorten, having removed the slow component of the EPSC. The major portion of evoked synaptic currents at hyperpolarised holding potentials has been shown to be the non-NMDA EPSC (Hestrin et al, 1990; Lambert and Jones, 1990). The $GABA_A$ antagonist bicuculline, only partly blocked the compound IPSC, suggesting that there may have been a $GABA_B$ component, or that there was insufficient bicuculline to block the event. The latency of the inhibition measured (20-30msec) corresponded most closely with that measured for the $GABA_A$ inhibition under extracellular conditions, suggesting that the latter explanation might be more appropriate. Neither D-APV nor bicuculline caused any significant changes in the reversal potential of any of the currents, which would be expected as we did not alter the concentrations of any of the ions responsible for these currents.

In these experiments, it was not possible to clearly identify a slow $GABA_B$ IPSC which would correspond with the later period of inhibition mediated by $GABA_B$ receptors that was seen at around 300msec under extracellular conditions. Because of this it was not possible to separate any pre/post-synaptic events and carry out any further investigations of the effects of 2-OH-S as detailed in the previous chapter.

Although it was not possible to isolate the components of GABAergic inhibition with the manipulations employed here, these experiments were able to show that whole cell synaptic currents can be successfully recorded from CA1 pyramidal cells in this preparation and can be manipulated with pharmacological tools. It then became necessary to employ different methods in order to obtain isolated inhibitory currents and these are demonstrated in the next chapter.

CHAPTER SIX

6

GABA$_B$ Mediated Inhibition In A Single Cell: Evaluation Of Pre- And Post-Synaptic Mechanisms And The Actions of 2-Hydroxy-Saclofen

The temporal overlap of the EPSC/IPSC components presented obvious difficulties for the investigation of GABAergic inhibition in the slice preparation. The main problem in the previous section was the lack of a clearly defined GABA$_B$ response. It was therefore necessary to isolate the constituent responses, the GABA$_A$ IPSC (IPSC$_A$) and the GABA$_B$ IPSC (IPSC$_B$), in order to be able to manipulate them individually and subsequently determine the effects of 2-hydroxy-saclofen on GABAergic inhibition. This would then enable us to look more closely at the pre/post -synaptic mechanisms involved in the inhibition mediated by GABA$_B$ receptors and its role in the control of cellular excitability.

6.1 Introduction

Recent studies have shown that it is possible to isolate the individual inhibitory potentials which comprise the evoked EPSC/IPSC observed in hippocampal pyramidal cells by blocking the EPSC with antagonists of NMDA and non-NMDA receptors, D-APV and CNQX. The fast IPSC$_A$ can then be blocked with either picrotoxin or bicuculline leaving the IPSC$_B$ which is then more amenable to direct study (Davies et al., 1990; Solis et al., 1992b). The presynaptic GABA$_B$ effects on the postsynaptic cell events can also be looked at in isolation by blocking the K^+ conductance activated by the postsynaptic GABA$_B$ receptor with QX-314 and Cs^+ions in the intracellular solution (Nathan,Jensen, & Lambert, 1990b; Andrade, 1991; Nunez & Buno, 1992)

The focus of a considerable amount of research over the past few years has been the question of heterogeneity of the pre and postsynaptic GABA$_B$ receptors, whether they operate via the same mechanisms and whether they can be distinguished pharmacologically (Wojcik & Holpainen, 1992; Bowery, 1993; Thompson et al., 1993). There is also the question of whether the presynaptic receptors on excitatory terminals are different and can

be separated from those on inhibitory terminals. Then of course, there arises the question of the functional significance of pre and postsynaptic $GABA_B$ receptor activation in the hippocampus and their role in the physiological processes evident in this area, such as learning and memory, or their involvement, causal or compensatory in pathological processes such as epilepsy, ischaemia, depression and others (Bowery, 1993).

Postsynaptic $GABA_B$ Receptors

It is now widely accepted that activation of postsynaptic $GABA_B$ receptors leads to an increase in K^+ conductance via a pertussis toxin (PTX)-sensitive G-protein, and mediates a hyperpolarisation, which is thought to underlie the late, slow inhibitory potential, enhancing the inhibitory control of excitability already provided by the $GABA_A$ receptors (Gahwiler & Brown, 1985; Dutar & Nicoll, 1988a; Solis et al., 1992b). In addition $GABA_B$ receptor activation has been seen to inhibit ωCgTX-sensitive and dihydropyridine sensitive Ca^{2+} channels in the soma of cultured hippocampal neurones (Scholz & Miller, 1991b). Postsynaptic $GABA_B$ receptors also play a part in regulating NMDA-mediated synaptic responses (Morrisett et al., 1991). Hyperpolarisation of the membrane results in an enhanced block of the NMDA channels by Mg^{2+} and prevents the induction of LTP. By this mechanism postsynaptic $GABA_B$ receptors could play an important part in the NMDA receptor mediated processes involved in neuronal plasticity.

Presynaptic $GABA_B$ Receptors: Heterogeneity and Mechanism of Action

The mechanism of action of presynaptic $GABA_B$ receptors and the question of heterogeneity between pre-and post- has still to be fully elucidated. Whilst some groups originally believed that they may be pharmacologically distinct (Dutar & Nicoll, 1988b; Harrison, 1990) others now disagree (Yoon & Rothman, 1991b; Thompson & Gahwiler, 1992a; Lovinger,Harrison, & Lambert, 1992). It may be possible however, that they are separable on the basis of their transduction mechanisms. Presynaptic $GABA_B$ receptors may work via Ca^{2+} or K^+ conductances and may affect an as yet unknown component of vesicle release downstream from the channels (Thompson et al., 1993; Bowery, 1993).

Baclofen, an agonist at presynaptic $GABA_B$ receptors, has been shown to decrease Ca^{2+} currents (N and L type) which may or may not be responsible for the presynaptic inhibition (Scholz & Miller, 1991b). It has also been suggested that the baclofen activation of the presynaptic receptors is insensitive to Ba^{2+}, which blocks the postsynaptic K^+ conductance (Lambert,Harrison, & Teyler, 1991b). In some cases they do not appear to

work via the same PTX-sensitive G-protein that is linked to the postsynaptic $GABA_B$ receptor (Dutar & Nicoll, 1988b; Colmers & Williams, 1988; Colmers & Pittman, 1989; Harrison, 1990). However, other groups suggest that activation of the presynaptic receptor is PTX sensitive (Scholz & Miller, 1991b; Yoon & Rothman, 1991a) but that it may be more difficult to access than the postsynaptic receptor.

It may also be the case that different subtypes of $GABA_B$ receptors exist on nerve terminals with different transduction mechanisms. The presynaptic receptors located on inhibitory terminals appear to be PTX-sensitive (Scanziani et al., 1992; Thompson & Gahwiler, 1992a). The effect of baclofen on the IPSP is sensitive to Ba^{2+} in area CA3 (Scanziani et al., 1992; Thompson & Gahwiler, 1992a), suggesting activation of a K^+ conductance, although this may not be true of area CA1 (Lambert et al., 1991b). Ba^{2+} resistant actions may be mediated by decreases in Ca^{2+} current (Alford & Grillner, 1991).

If presynaptic inhibition was indeed mediated by a decrease in Ca^{2+} conductance, it could be predicted that agonists at presynaptic receptors would have a similar effect to non specific Ca^{2+} channel blockers. Studies on the frequency and amplitude of miniature synaptic currents in the presence of TTX (tetrodotoxin) which blocks action potential dependant release, can provide information about the pre- and postsynaptic changes respectively (Thompson & Gahwiler, 1992a) demonstrated that the non specific blocker of Ca^{2+} channels, Cd^{2+} had no effect on the frequency or amplitude of miniature EPSC's/IPSC's (mEPSC / mIPSC) and concluded that voltage dependant Ca^{2+} channels can only be opened by action potentials and do not contribute significantly to Ca^{2+} permeability at rest. A number of GABAergic and non-GABAergic agonists do decrease the frequency of miniature currents but not the amplitudes confirming their effects at presynaptic sites (Scanziani et al., 1992). it was suggested that activation of these receptors leads to an inhibition of transmitter release by interfering with the release process and not by altering presynaptic conductances. However, this does not exclude the possibility that activation of presynaptic $GABA_B$ receptors does in fact decrease Ca^{2+} influx directly.

The receptors on excitatory terminals appear to consist of both PTX-sensitive and PTX-insensitive types (Scanziani et al., 1992; Thompson & Gahwiler, 1992a; Potier & Dutar, 1993).The effect of baclofen upon the inhibition of the EPSP is Ba^{2+} insensitive (Dutar & Nicoll, 1988b; Scanziani et al., 1992; Thompson & Gahwiler, 1992a) and appears to be independent of (ω-CgTx) sensitive, N-type Ca^{2+} channels (Potier & Dutar, 1993). New studies indicate that, if the presynaptic receptors worked via a K^+ conductance, it would have to be substantially different from the Ba^{2+} activated K^+ conductance that is responsible for the postsynaptic effects (Otis et al., 1993).

It has previously been shown that GABA and baclofen acting presynaptically, remove inactivation of the K^+ 'A' current by evoking a large reversible positive shift in the voltage dependence of the inactivation of the current, leading to a decrease in the amplitude and duration of the action potential, a reduction in the influx of Ca^{2+} and a depression in the release of transmitter (Saint,Thomas, & Gage, 1990; Gage, 1992). In support of this, recent evidence also suggests that in CA3 pyramidal cells, while postsynaptic receptors are sensitive to THA (tetrahydroaminoacridine) another K^+ channel blocker, presynaptic receptors are insensitive to its effects (Lambert & Wilson, 1993).

The pre and post synaptic actions of agonists on the IPSP/C and EPSP/C also appears to be inhibited by phorbol ester treatment which stimulates PKC leading to a phosphorylation process which ultimately renders the G-proteins inactive. In addition, it may phosphorylate other unknown effector components in the transduction pathway (Andrade,Malenka, & Nicoll, 1986; Dutar & Nicoll, 1988b; Lambert et al., 1991b; Thompson & Gahwiler, 1992a; Thompson et al., 1993).

A summary of the effects of presynaptic inhibition on EPSP's and IPSP's and postsynaptic inhibition is shown in **Table 6.1. below**: A (+) indicates a sensitivity to the treatment and a - indicates no effect by the treatment. References for these findings are given in the text.

$GABA_B$ Receptors	Presynaptic		Postsynaptic
	IPSP/C	EPSP/C	IPSP/C
PTX	+	-	+
Ba2+	-	-	+
THA	-	-	+
Phorbol Esters	+	+	+

Presynaptic $GABA_B$ Receptors and their Role in Disinhibition and LTP.

Many groups have shown that activation of $GABA_B$ receptors on presynaptic terminals leads to a decrease in the amount of transmitter released from the terminal on which they are situated (McCarren & Alger, 1985; Thompson & Gahwiler, 1989a; Thompson & Gahwiler, 1989b; Thompson & Gahwiler, 1989c; Deiz & Prince, 1989; Davies et al., 1990; Brucato,Morrisett,Wilson, & Swartzwelder, 1992; Mott et al., 1993).This is believed to underlie GABAergic disinhibition. However, while evidence to support the

inhibition of EPSC/P's (Dutar & Nicoll, 1988b; Thompson & Gahwiler, 1992a) and the $IPSC_A$ (Davies et al., 1990; Harrison, 1990; Otis & Mody, 1992) has been obtained using selective antagonists and agonists for $GABA_B$ receptors, this cannot be used to look at the inhibition of the $IPSC_B$ because the involvement of the postsynaptic current and desensitisation is difficult to rule out. Recent evidence obtained from granule cells of the dentate gyrus, looking directly at the kinetics of the postsynaptic $GABA_B$ current and comparing them with the kinetics of paired pulse depression, suggests that the depression of the $GABA_B$ and $GABA_A$ components are similar and due to presynaptic mechanisms (Otis et al., 1993). In contrast to all this evidence though, a recent paper suggested that the depression of the slow $GABA_B$ IPSP arises from at least two components, an increase in the depolarising GABA response due to Cl^- accumulation and inhibition mediated by activation of $GABA_A$ receptors on the interneurones (Bernardo, 1993).

Disinhibition has may play an important part in the modulation of the NMDA response and the induction and facilitation of LTP in a number of areas of the hippocampus (Mott,Bragdon, & Lewis, 1990; Olpe & Karlsson, 1990; Burgard & Sarrey, 1991; Davies et al., 1991; Mott & Lewis, 1991; Davies & Collingridge, 1992; Mott, Xie, Wilson, Swartzwelder, & Lewis, 1992; Mott & Lewis, 1992; Olpe, Ferrat, Worner, Andre, & Steinemann, 1992). Ordinarily the $GABA_A$ hyperpolarisation is very large and suppresses the NMDA mediated current. When the former is depressed by the activation of the presynaptic $GABA_B$ autoreceptors, the result is a net increase in the NMDA current which then enhances the induction of LTP. This therefore provides an important point of control in the synaptic process of learning and memory. The frequency at which the disinhibition facilitates LTP is also important and appears to correlate with the endogenous hippocampal theta rhythm (Mott et al., 1993). At higher frequencies, $GABA_B$ antagonists appear to facilitate, rather than block, the induction of LTP (Olpe & Karlsson, 1990).

With this array of evidence in hand, the effects of 2-OH-S upon GABAergic inhibition in area CA1 of the hippocampal slice were studied and compared with the effects of CGP 36742 and (-)baclofen. The effects of paired pulse inhibition on the EPSC and the underlying GABAergic mechanisms was also investigated.

6.2 Methods

The slice preparation and ACSF were as described previously (chapter 2). The osmolarity of the ACSF was routinely adjusted to approx. 325mOsM using either distilled water or sucrose. The acquisition and analysis of the data was as detailed in chapter 2.

Stimulus Protocol and Intracellular Solutions

In these experiments the stimulating electrode was placed in the stratum lacunosum/moleculare in order to activate a local population of interneurones (Figure 2.2, chapter 2). A small number of excitatory fibres however, were also activated due to the size of the stimulating electrode and the small area to be worked on. For the paired pulse experiments on the EPSC the electrode was placed as for the extracellular experiments to activate the schaffer collateral's/commisural afferents. The recording electrode contained (mM) Gluconate 100; Hepes 40; KCl 12.6; EGTA 10; ATP 5; Leupeptin 0.1; adjusted to a pH of 7.2 with KOH (approx. 170mM). The osmolarity was adjusted to approx. 295mOSM using either distilled water or sucrose. For the experiments looking at presynaptic $GABA_B$ mechanisms the KOH was replaced with CsOH and QX-314 10mM (Alamone Labs) was included in the intracellular solution.

Pharmacology

Drugs use were: D-APV 50μM; CNQX 20μM; 2-hydroxy-saclofen 200μM (all Tocris Neuramin), Bicuculline 6μM (Sigma); (-) Baclofen 10μM (Lioresal, a gift from CIBA GEIGY); CGP 36742 100μM (a gift from CIBA GEIGY).

Reversal Potentials

The reversal potentials for the $IPSC_A$ and $IPSC_B$ were calculated using the Nernst Equation and are as follows: ECl^-, -58mV ($IPSC_A$); EK^+, -92mV ($IPSC_B$). The reversal potentials for the individual currents were measured by taking the difference between the peak of the current and the baseline of the voltage step immediately prior to the stimulus artefact and plotting the values against the voltage.

6.3 Results

Pharmacological and physiological studies of whole cell current recordings from CA1 pyramidal cells after isolation of the individual components of the EPSC/IPSC are presented.

Whole Cell Excitatory and Inhibitory Currents Recorded from CA1 Pyramidal Cells *in vitro*.

Single cells were voltage clamped at -60mV and the input resistance measured by stepping the voltage from the holding potential (-80mV to -40mV) and taking the gradient of the linear regression line after plotting the current against the voltage. The cell usually stabilised, after about 15 minutes. Cells with input resistances of >100 mΩ were then kept for further study (n = 40). All individual results for the peak amplitudes of the synaptic currents are an average of five responses.

Direct orthodromic stimulation to the interneurones in the lacunosum/moleculare (L/M) area, still evoked EPSC's, probably as a result of activation of a small number of nearby excitatory afferents (**Figure 6.1A**). These had a mean peak amplitude of -90.70±8.4pA which was smaller than those evoked by stimulation of the excitatory afferents directly (chapter 5) and a latency to peak of 6.7±.64msec (n=22).

In order to isolate the IPSC, D-APV (50μM) and CNQX (20μM) were added to the ACSF in order to block all the NMDA and non-NMDA receptors (**Figure 6.1B**) This revealed a large, fast inhibitory current which could be blocked by application of bicuculline (6μM) (n=10) and was therefore taken to be the $IPSC_A$. The mean amplitude of the $IPSC_A$ measured at the peak, was 46±5pA with a latency to peak of 35.25±1.7msec (n=12)

Once a pure biphasic IPSC had been obtained, the next step was to isolate the $GABA_B$ component of the response. The $IPSC_A$ was blocked by bicuculline (6μM) and revealed a smaller current of longer duration which could be blocked by both 2-OH-S (200μM) and CGP 36742 (100μM) (n=22). This was taken to be the $IPSC_B$ (**Figure 6.1B,C**). The mean amplitude of the $IPSC_B$ measured at the peak, was 20±3pA with a latency to peak of 175.95±8.45msec (n=22).

Once successful isolation had been achieved we were then able to carry out a number of pharmacological manipulations to investigate the role of the pre and postsynaptic $IPSC_B$.

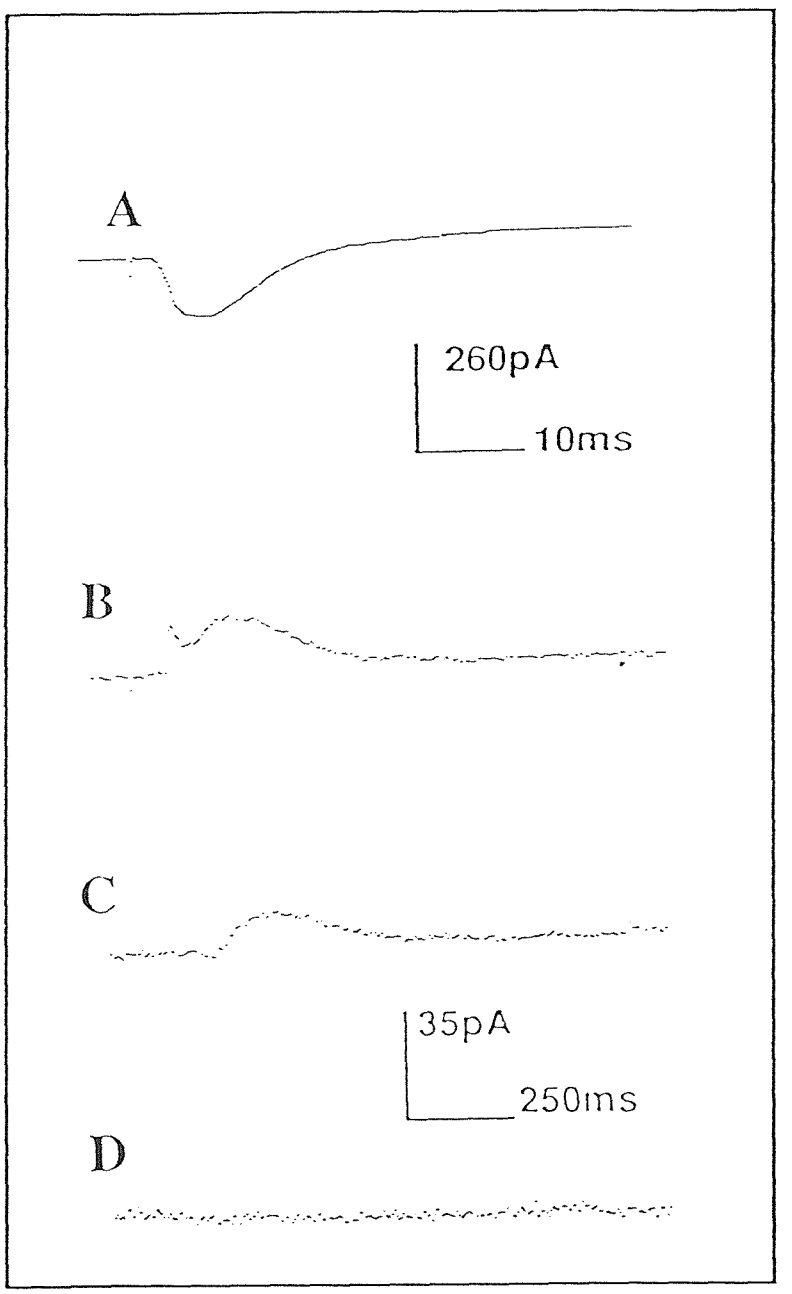


Figure 6.1 The synaptic currents evoked by local stimulation of the lacunosum/moleculare under control conditions are shown. Cells were held at -60mV. A) a single EPSC was evoked using a stimulus set to achieve a maximal response without any breakthrough sodium spikes. Under these conditions it was sometimes possible to see a compound IPSC. B) application of D-APV (50μM) and CNQX (10μM) revealed an IPSC comprising the fast IPSC A and the slow IPSC B (n=12). C) Further application of bicuculline (6μM) revealed the IPSC B (n=10). D) The IPSC B could be blocked by either 2-OH-S (200μM, n=12) or CGP 36742 (100μM, n=10). The top scale bar refers to the EPSC and the bottom scale bar to the three IPSC traces.

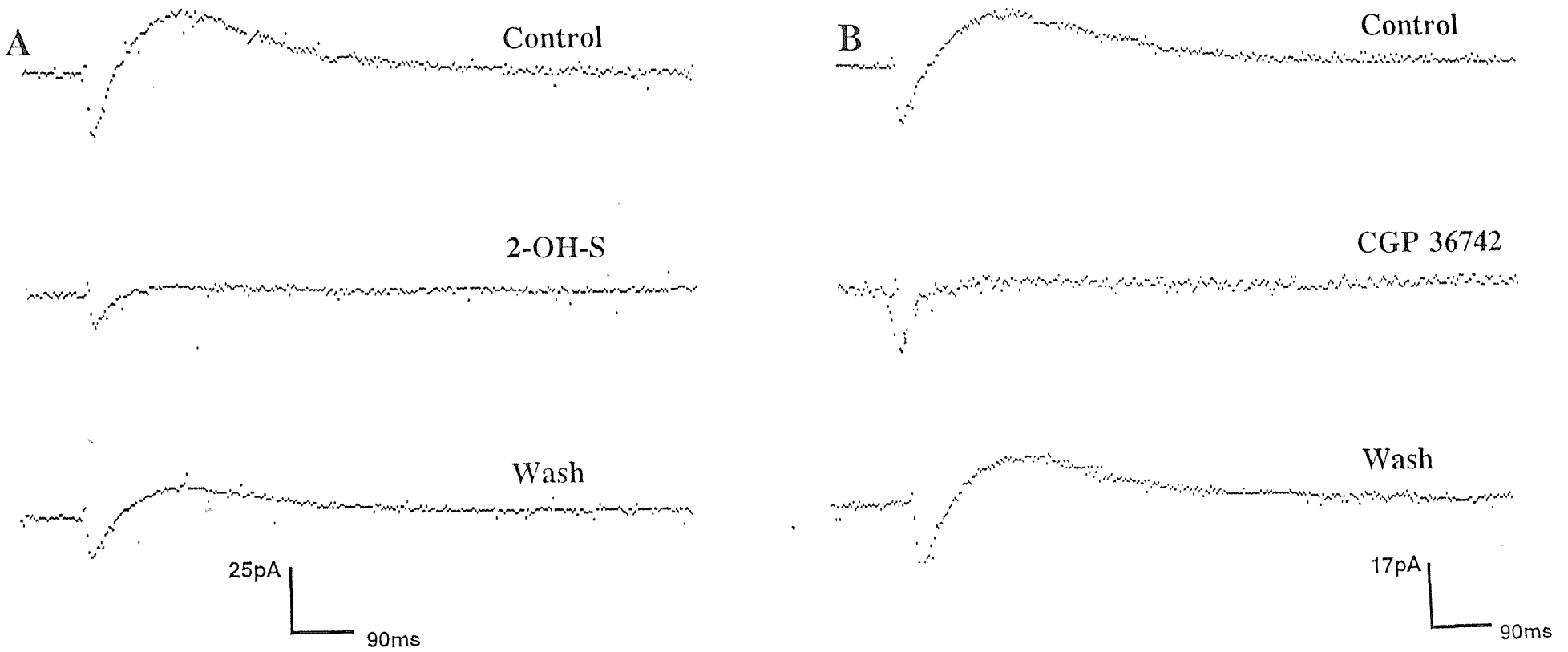


Figure 6.2 Representative traces show the effect of 2-OH-S (200μM, n=5) and CGP 36742 (100μM, n=5) on the peak amplitude of the evoked IPSC B. A) *Top*, control traces obtained after isolation of the IPSC B; *middle*, in the presence of 2-OH-S, which blocked the IPSC B and *bottom*, partial recovery after washing. B) *Top*, control traces; *middle*, in the presence of CGP 36742, which also blocked the IPSC B and *bottom*, after partial recovery on washing.

Effect of 2-OH-S and CGP 36742 on the Isolated $IPSC_B$.

2-OH-S reduced the late $GABA_B$ mediated inhibition seen in extracellular recordings. This was then investigated at the single cell level and compared with the more potent and selective $GABA_B$ antagonist CGP 36742. Following isolation of the $IPSC_B$, as described above, the effects of both compounds were tested. Application of 2-OH-S (200μM, n=5) reversibly and significantly, reduced the $IPSC_B$ by 90.73±1.93%, (control peak amplitudes, 18.29±2.64pA; 2-OH-S, 1.55±.29pA; wash, 13.20±3.8pA, $p<0.01$). Application of CGP 36742 (100μM, n=5) also significantly reduced the $IPSC_B$ by 86.03±1.10% (control peak amplitudes, 13.18±2.25pA; CGP, 2.12±0.45pA; wash, 9.11±.71pA, $p<0.01$). The raw data from one representative cell showing these effects is shown (**Figure 6.2A,B**). The small inward current that can be seen in all the traces immediately before the outward $IPSC_B$ is most likely to be the remains of the EPSC, which may not have been fully blocked. It can be seen from the traces that this was reduced by 2-OH-S but not CGP 36742, and was partially reversible. It was not possible however to observe a significant change on all occasions.

The effects of both of the compounds were quantified as mean±s.e.m. of the peak amplitude of the $IPSC_B$ and are displayed in the histograms in **Figure 6.3A,C.** Both compounds can be seen to significantly reduced the peak amplitude of the $IPSC_B$**.** The ribbon graphs, which were described in chapters 3 and 4, were again used to show the responses of each individual cell to the application of 2-OH-S (6.3B) and CGP 36742 (6.3D). In both cases the trend of the response is reflected in the mean. There is some scatter between the control and the wash responses but all responses to the two drugs are at the same level.

In order to see if the reduction of the peak amplitude of the $IPSC_B$ by 2-OH-S and CGP 36742 was dependant on the stimulus strength, the peak amplitude was measured at increasing stimulus strengths. Responses were evoked for five stimulus intensities (normalised; 1 = minimum to 5 = maximum) and plotted against the peak response (**Figure 6.4A-D**). 2-OH-S reduced the peak of the $IPSC_B$ at all stimulus intensities. This is also reflected in the individual responses shown in the ribbon graph. CGP 36742 also reduced the $IPSC_B$ at all stimulus intensities which is reflected in the ribbon graph. It would appear from these findings that the reduction in the $IPSC_B$ by both compounds is independent of stimulus strength.

Figure 6.3 (next page) The effects of 2-OH-S (200μM, n=5) and CGP 36742 (100μM, n=5) on the $IPSC_B$ are shown quantified as the mean±s.e.m. of the peak amplitude.
A) 2-OH-S significantly reduced the peak amplitude of the $IPSC_B$, which was reversible upon washing.
B) This reduction of the $IPSC_B$ by 2-OH-S is shown for each individual cell to indicate the overall trend of the group of cells and any gross deviation from the mean.
C) CGP 36742 significantly reduced the peak amplitude of the $IPSC_B$, which was also reversible upon washing.
D) This reduction of the $IPSC_B$ by CGP 36742 is shown for each individual cell to indicate the overall trend of the group of cells and any gross deviation from the mean.

Figure 6.4 (...following page) The effects of 2-OH-S (200μM, n=5) and CGP 36742 (100μM, n=5) are shown on the stimulus response curve. The values for the stimulus response curve are normalised and explained in the text. They indicate a consecutive increase in the voltage applied.
A) 2-OH-S significantly affected the stimulus response curve, moving it ~~to the right of~~ downward from the control at all stimulus intensities. The effect was partially recovered on washout.
B) The effects of 2-OH-S on the stimulus response curve of the $IPSC_B$ is shown for each individual cell to indicate any gross deviation from the mean.
C) CGP 36742 also significantly affected the stimulus response curve, moving it to the right of the control at all stimulus intensities. The effect was partially recovered on washout.
D)The effects of CGP 36742 on the stimulus response curve of the $IPSC_B$ is shown for each individual cell to indicate any gross deviation from the mean.

N.B.

The break through current observed in figures 6.2 and 6.6 may be the afferent volley as opposed to any remaining EPSC. This is suggested by the apparent lack of reversal of the event. It can also be seen that this is blocked by 2-OH-S, indicative of a presynaptic action.

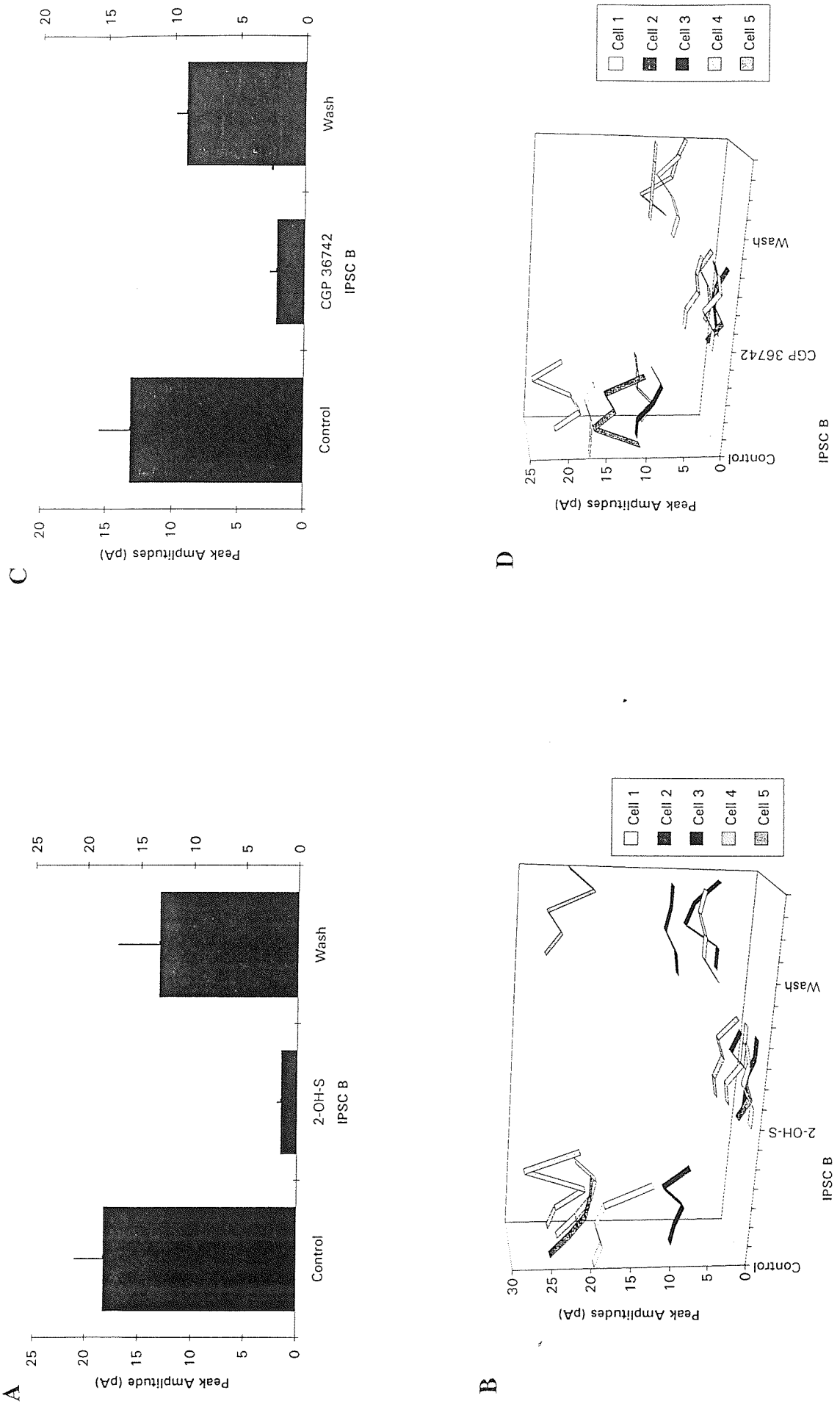


Figure 6.3 2-OH-S & CGP 36742, $IPSC_B$ amplitude

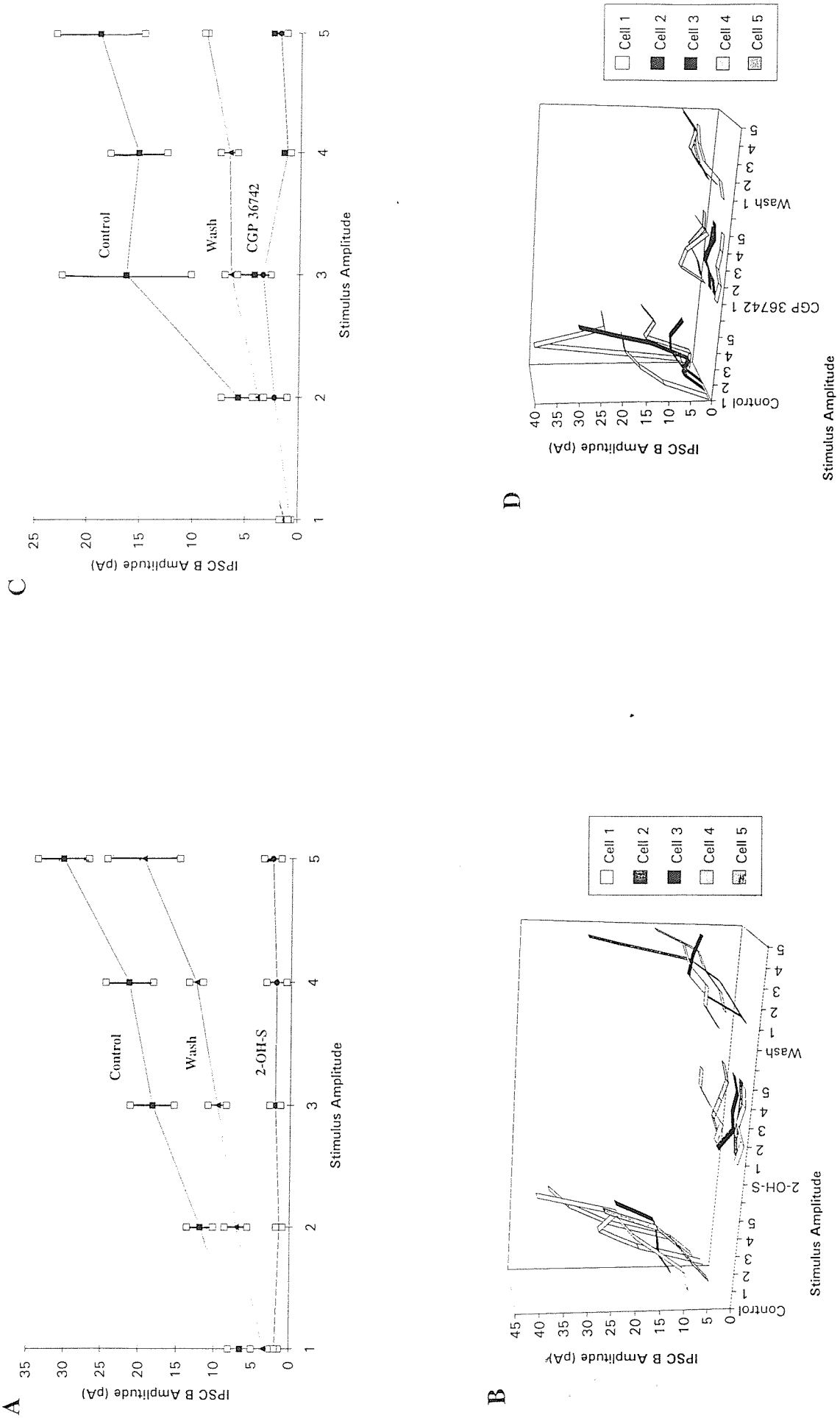


Figure 6.4 2-OH-S & CGP 36742, SR Curves

The effects of 2-OH-S (200μM, n=5) and CGP 36742 (100μM, n=5) were investigated on other parameters of synaptic function to see if a direct postsynaptic action could be responsible for the reduction of the peak amplitude of the $IPSC_B$. The input resistance was calculated as indicated at the beginning of this section. Changes in the input resistance should indicate any increase or decrease in the number of ion channels that are open on the postsynaptic membrane. 2-OH-S did not have any significant effect on the input resistance (mean±s.e.m. Control, 214.81±34.28MΩ; 2-OH-S, 235.10±46.72MΩ; **Figure 6.5A**). Neither did CGP 36742 have a significant effect (mean±s.e.m. Control,204.85±19.74MΩ; CGP 243.02±19.31MΩ; **Figure 6.5C**).

The DC (direct current) offset values which reflect a change in the conductance of the postsynaptic membrane were also measured for 2-OH-S and CGP 36742. An agonist at the postsynaptic receptor would open the ion channels and increase the outward K^+ conductance, which would be reflected as a more **positive** value. An antagonist would close the ion channels if they were tonically active, which is unlikely as tonic $GABA_B$ inhibition has not been shown. This would decrease the outward K^+ conductance and result in a more **negative** value. There was no significant change in the offset values with either 2-OH-S (**Figure 6.5B**) or CGP 36742 (**Figure 6.5D**).

The reversal potential for controls and with 2-OH-S and CGP 36742 were analysed. Unless any of the drugs altered the ionic gradient across the postsynaptic membrane which determines the reversal potential, or the ion selectivity, no change would be expected. Under control conditions the cell was held at -60mV and the reversal potential of the $IPSC_B$ was calculated by stepping the voltage from -100mV to -50mV and plotting it against the peak current to obtain the intercept of the linear regression line. The mean reversal potential of the $IPSC_B$ was -106.48±10.64mV. Neither 2-OH-S or CGP 36742 appeared to cause a significant change in the reversal potential (2-OH-S, -88.02±17.51mV; CGP, -72.35±11.49mV) The raw data traces from representative cells are shown in **Figure 6.6**. The measure of the reversal potential could only be approximate because the size of the event was so small and the reversal of the response was not always clear. Quantification of the results are shown in **Figure 6.7.** Representative current /voltage (IV) plots are shown in control and drug conditions for both 2-OH-S and CGP 36742.

The difficulty in measuring the above three parameters accurately, results from the remote location of the receptors from the cell body. It is not known whether the voltage that the soma is subjected to is the same as that seen by the dendrites and this may result in the large errors and variability of the measurements. However, if there was a consistent and definite change in either of them elicited by the drugs, it may be assumed that it would be observable.

2-OH-S and CGP 36742 both reversibly attenuated the $IPSC_B$. However, there did not appear to be any evidence for a postsynaptic action of 2-OH-S. The presynaptic effects of each compound on the $IPSC_A$ were then investigated in addition to the effects on the $IPSC_B$. 2-OH-S had already been observed to reversibly reduce the conditioning response and the early $GABA_A$ inhibition in the extracellular experiments.

Figure 6.5 (next page) The effect of 2-OH-S (200μM, n=5) and CGP 36742 (100μM, n=5) on the input resistance and the changes in DC offset values are shown.
A) 2-OH-S did not cause any significant change in the input resistance of the cell. **B**) 2-OH-S did not produce a reliable reversible change in the DC offset values of the cells. **C**) CGP 36742 also had no significant effect on the input resistance of the cell. **D**) CGP 36742 did not produce any significant changes in the DC offset values of the cells.

Figure 6.6 (...following page) Representative current traces obtained when stepping the cell from -100mV to -50mV (top trace in each case = -50mV) with an evoked IPSC during the step are shown under each experimental condition. All cells were held at -60mV.
A) Left hand side: *top*, control traces; *middle*, addition of 2-OH-S (200μM, n=5) which depressed the $IPSC_B$ with no significant effect on the reversal potential and *bottom*, recovery. **B**)Right hand side: *top*, control traces; *middle*, addition of CGP 36742 (100μM, n=5) which depressed the $IPSC_B$ with no significant effect on the reversal potential and *bottom*, recovery.

Figure 6.7 (..following page)The average reversal potentials for the $IPSC_B$ are shown along with the effects of 2-OH-S (200μM, n=5) and CGP 36742 (100μM, n=5) and in each case accompanied by a representative graph of an I/V plot for one cell.
A) 2-OH-S had no significant effect on the reversal potential of the $IPSC_B$. This is also reflected in **B**) where the I/V plot is not significantly different from control and 2-OH-S. Inset: The trace of the voltage steps used to calculate the reversal potential with the top line representing a voltage step to -50mV. **C**) CGP 36742 had no significant effect on the reversal potential of the $IPSC_B$. This is also reflected in **D**) where the I/V plot is not significantly different from control.

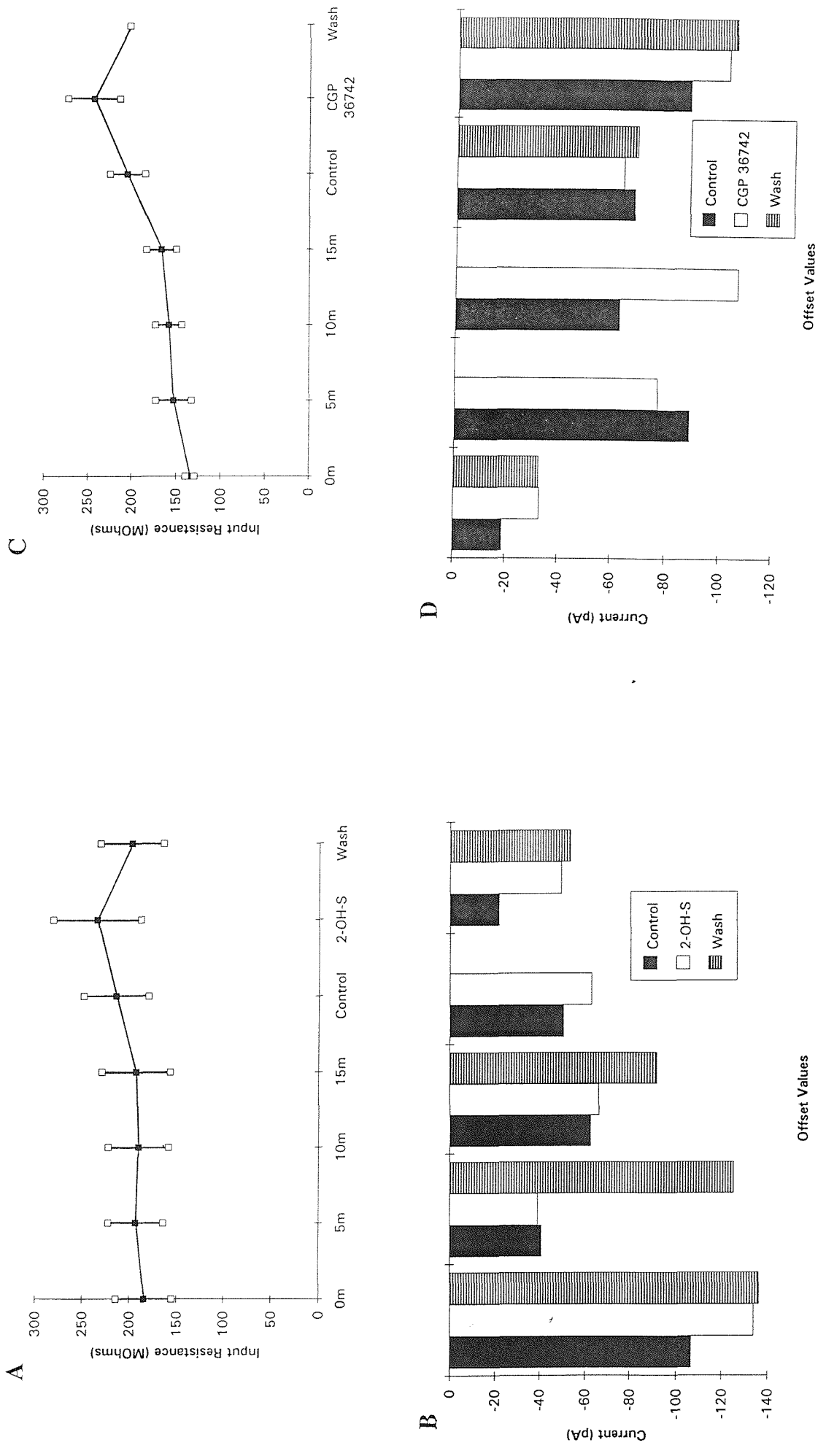


Figure 6.5 2-OH-S & CGP 36742, IR & DC Offsets

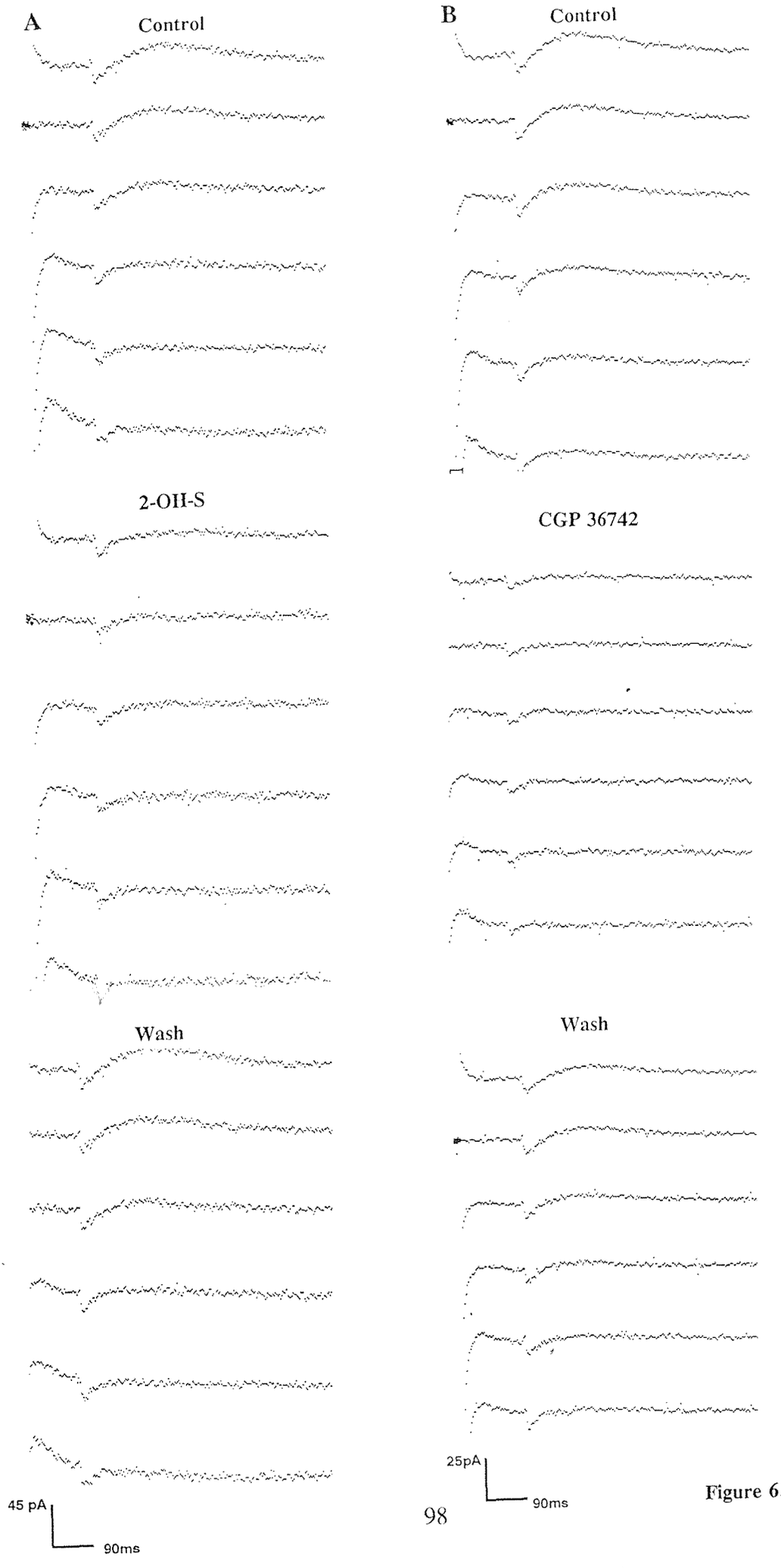


Figure 6.6 2-OH-S & CGP 36742, IV raw data

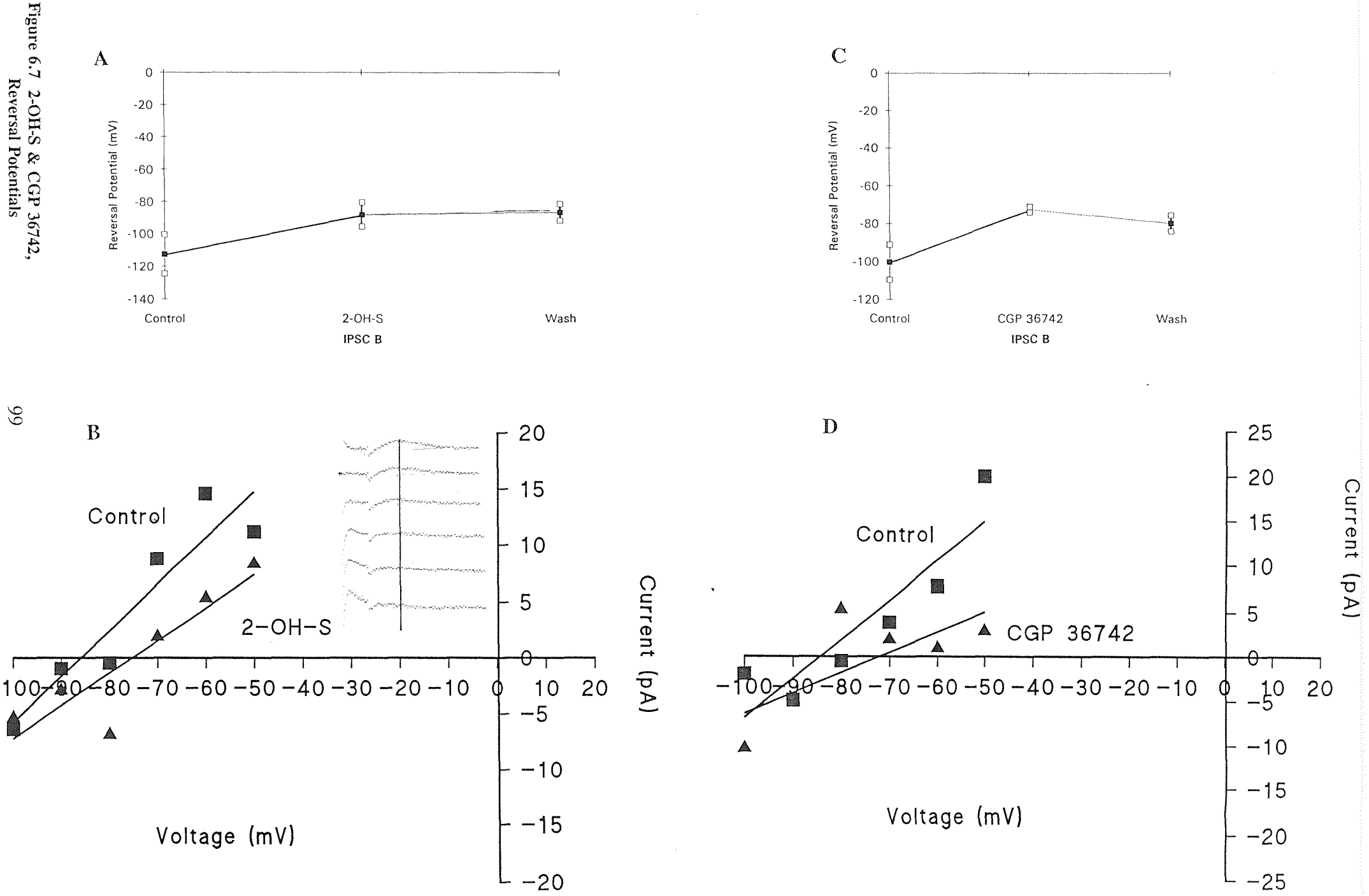


Figure 6.7 2-OH-S & CGP 36742, Reversal Potentials

Effects of 2-OH-S and CGP 36742 on the Isolated $IPSC_A$ and $IPSC_B$.

The effects of 2-OH-S (200μM, n=7) and CGP 36742 (100μM, n=5) were investigated on the isolated $IPSC_A$ and $IPSC_B$. As neither of the drugs are known to have any direct effect at the $GABA_A$ receptor, then any change in the $IPSC_A$ would be presumed to be as a result of an indirect effect at the presynaptic $GABA_B$ receptors on the terminals of the inhibitory basket cells, thereby altering the release of GABA and subsequent activation of the $GABA_A$ receptors.

CGP 36742 reversibly reduced the peak amplitude of the $IPSC_B$ by 44.38±8.24% (control, 22.05±2.3pA; CGP, 12.28±1.97pA, $p<0.01$) with no significant effect on the peak amplitude of the $IPSC_A$ (0.19±6.6%; control, 47.87±5.6pA; CGP, 48.15±6.67pA). It is likely that the decrease in the amount of reduction of the $IPSC_B$ here and in the previous section is due to the residual $IPSC_A$ which remains where the peak of the $IPSC_B$ was measured. Application of 2-OH-S reversibly blocked the peak amplitude of both the $IPSC_A$ (53.8±3.79%, control, 43.84±8.74pA; 2-OH-S, 19.98±3.86pA, $p<0.01$) and the $IPSC_B$ (83.13±3.13%, control, 17.21±5.09pA; 2-OH-S, 2.87±1.27pA, $p<0.01$). Representative traces to show the effects of the two drugs are shown (**Figure 6.8**).

Looking at the traces it would appear that the $IPSC_A$ rides on top of the $IPSC_B$. From this it might be considered that the reduction in the $IPSC_A$ may be due to the reduction in the amplitude of the $IPSC_B$. However, the argument against this being the case, is that CGP 36742 while significantly depressing the $IPSC_B$, does not reduce the $IPSC_A$. Also we could not measure any significant current at the latency of the peak of the $IPSC_A$ when the $IPSC_B$ was studied in isolation.

The effects of both of the compounds were quantified as mean±s.e.m. of the peak amplitude of the $IPSC_A$ and $IPSC_B$ and are displayed in the histograms in **Figure 6.9A,C** ($IPSC_A$) and **Figure 6.10A,C** ($IPSC_B$). Both compounds can be seen to significantly reduced the peak amplitude of the $IPSC_B$ but only 2-OH-S reduced the peak amplitude of the $IPSC_A$. The ribbon graphs show the responses of each individual cell to the application of 2-OH-S (**6.9B, 6.10B**) and CGP 36742 (**6.9D, 6.10D**). In most cases the mean is a reflection of the trend of the responses of the individual cells. There is some scatter between the control and the wash responses but all responses to the two drugs appear similar. A noticeable outlying result is the response to 2-OH-S of the $IPSC_A$ for one cell (**6.9B**) which has a much larger peak amplitude than the rest of the group, but the reduction of the response is still apparent.

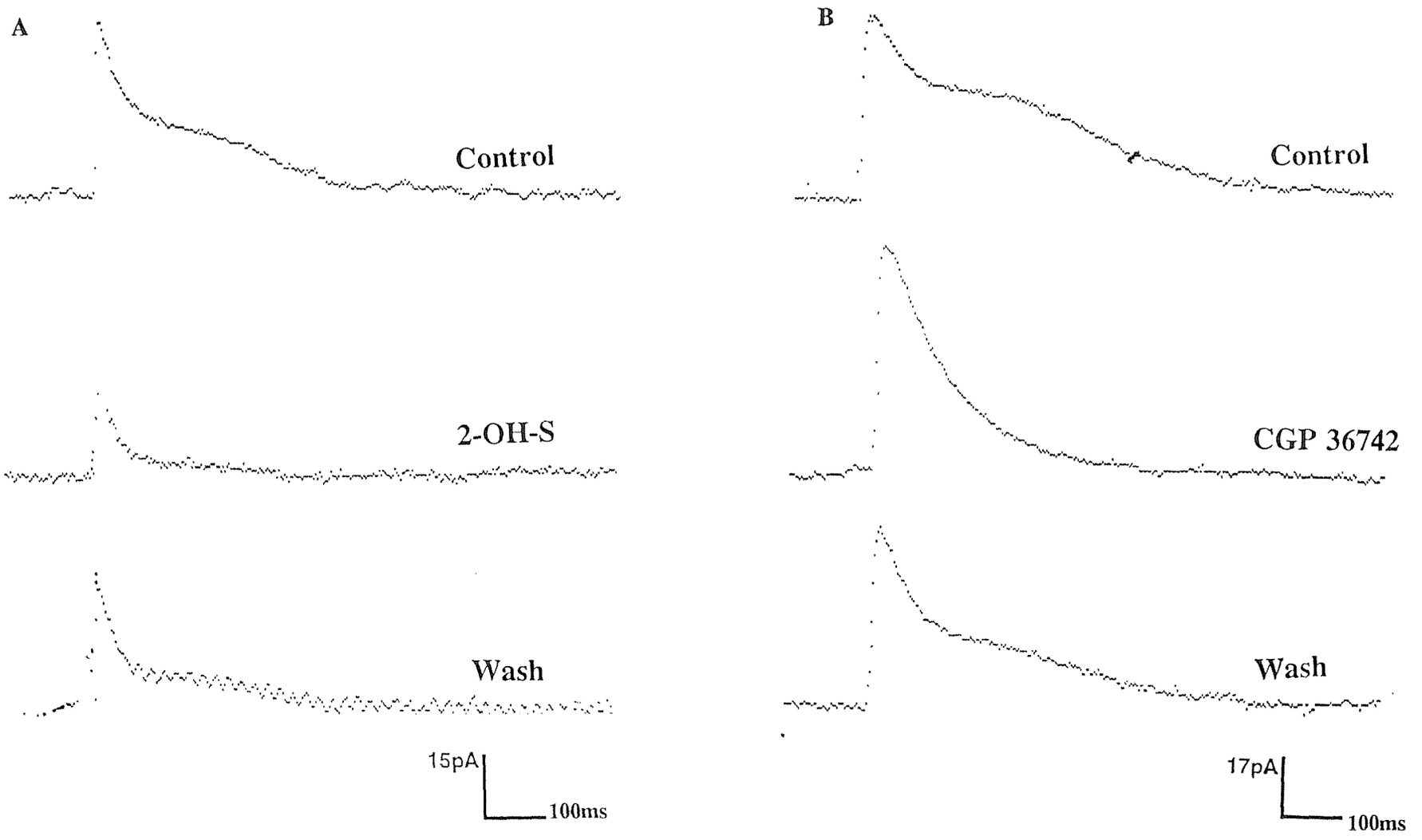


Figure 6.8 Representative traces show the effect of 2-OH-S (200μM, n=7) and CGP 36742 (100μM, n=5) on the peak amplitude of the evoked IPSC A and IPSC B. A) *Top*, control traces obtained after isolation of the IPSC A and IPSC B; *middle*, in the presence of 2-OH-S, which blocked the IPSC B and significantly reduced the IPSC A; *bottom*, partial recovery after washing. B) *Top*, control traces; *middle*, in the presence of CGP 36742, which also blocked the IPSC B, but had no effect on the IPSC A and *bottom*, after partial recovery on washing.

Figure 6.9 (next page) The effects of 2-OH-S (200μM, n=7) and CGP 36742 (100μM, n=5) on the $IPSC_A$ and $IPSC_B$ are shown quantified as the mean±s.e.m. of the peak amplitude.

A) 2-OH-S significantly reduced the peak amplitude of the $IPSC_A$, which reversed on washing.

B) This reduction of the $IPSC_A$ by 2-OH-S is shown for each individual cell to indicate the overall trend of the group of cells and any gross deviation from this trend.

C) CGP 36742 did not effect the peak amplitude of the $IPSC_A$.

D) The effect of CGP36742 is shown for each individual cell to indicate the overall trend of the group of cells and any gross deviation from this trend

Figure 6.10 (...following page) The effects of 2-OH-S (200μM, n=7) and CGP 36742 (100μM, n=5) on the $IPSC_B$ are shown quantified as the mean±s.e.m. of the peak amplitude.

A) 2-OH-S significantly reduced the peak amplitude of the $IPSC_B$, which reversed on washing.

B) This reduction of the $IPSC_B$ by 2-OH-S is shown for each individual cell to indicate the overall trend of the group of cells and any gross deviation from this trend.

C) CGP 36742 also reduced the peak amplitude of the $IPSC_B$ which was reversible on washing.

D) The effect of CGP36742 is shown for each individual cell to indicate the overall trend of the group of cells and any gross deviation from this trend

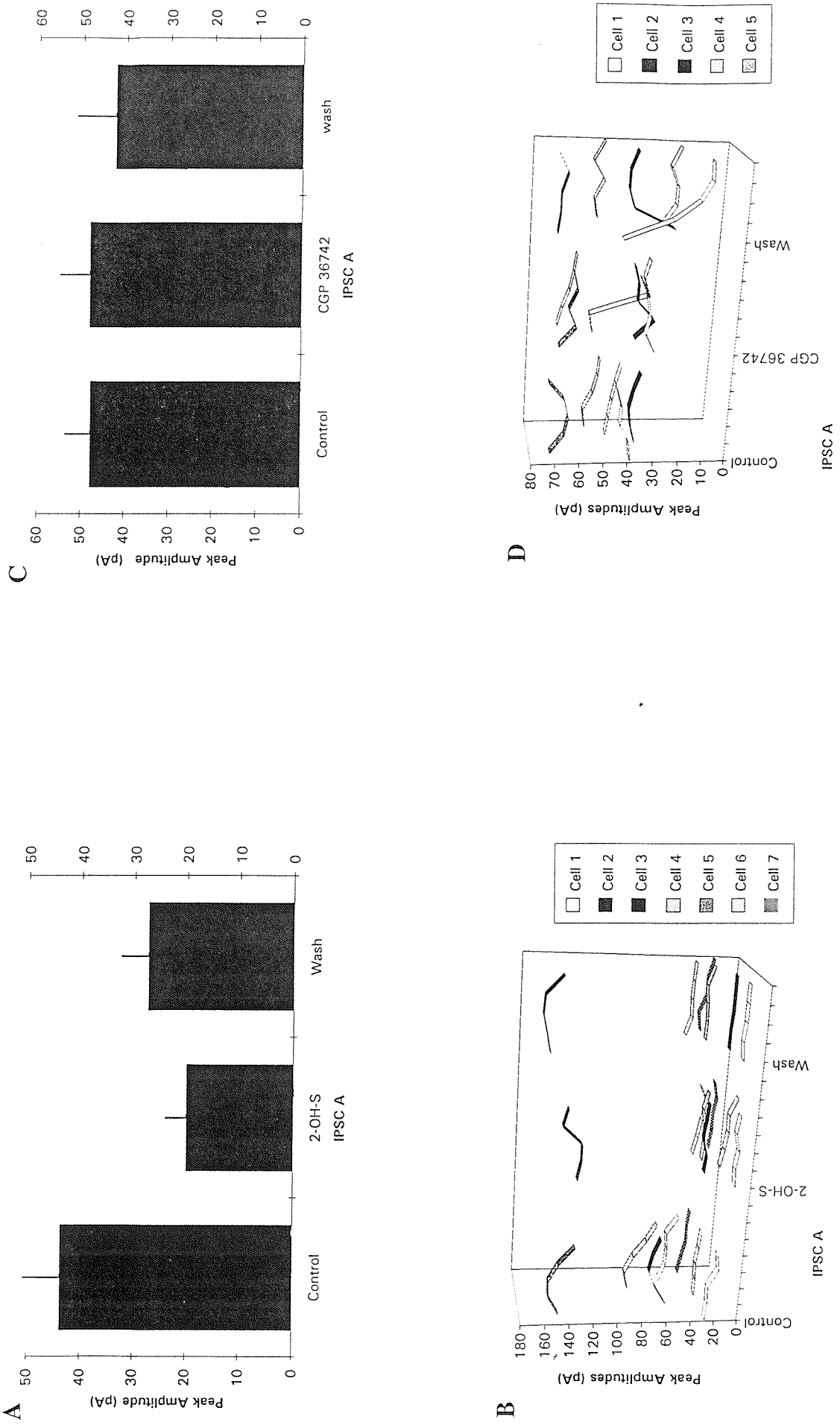


Figure 6.9 2-OH-S & CGP 36742, IPSCA amplitude

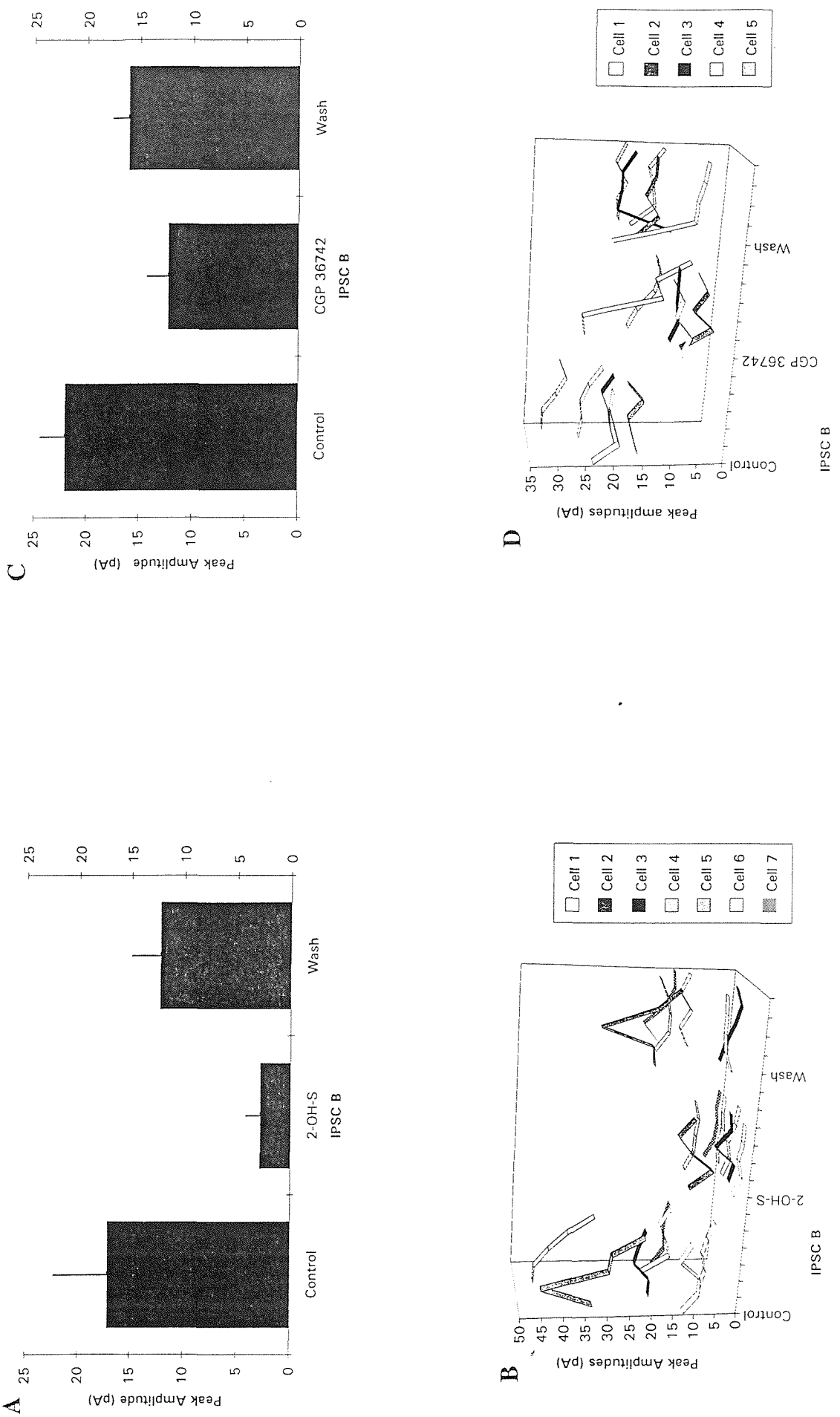


Figure 6.10 2-OH-S & CGP 36742 IPSCB amplitude

Stimulus/response curves were again constructed to see if the reduction in the $IPSC_A$ by 2-OH-S was dependant on the stimulus intensity, following the same protocol as set out earlier. This indicated a reduction in the amplitude of the $IPSC_A$ (**Figure 6.11A**) and $IPSC_B$ (**Figure 6.12A**) at all stimulus intensities for 2-OH-S. A reduction in the amplitude of the $IPSC_B$ (**Figure 6.12C**) was seen at all stimulus intensities but there was no significant change in the $IPSC_A$ for CGP 36742 (**Figure 6.11C**). Again, the spread of the raw data is shown in the ribbon graphs for the reduction of the $IPSC_A$ and $IPSC_B$ (**B,D** in each figure) and shows that the mean responses appear to be an accurate reflection of the individual responses.

In order to assess whethe their effects were mediated by actions at a postsynaptic site, the input resistance was again measured as detailed earlier in the section. There was no significant difference with either 2-OH-S (control, 159.33±26.43MΩ; 2-OH-S, 139.57±22.28MΩ, **Figure 6.13A**) or CGP 36742 (control, 166.84±14.39MΩ; CGP 188.45±22.93MΩ, **Figure 6.13C**).

As a further test the DC (direct current) offset values for 2-OH-S and CGP 36742 were again measured and are shown (**Figure 6.13B,D**).There was no evidence for an activation of an outward conductance by 2-OH-S. in 5 out of 7 cells and there appeared to be no recovery from any changes suggesting that any change was due to physiological changes in the membrane and not pharmacological effects. This was also true for the effects of CGP 36742.

The reversal potential for the $IPSC_B$ was measured as set out before. Under control conditions the mean reversal potential of the $IPSC_B$ was -93.25±3.35mV. 2-OH-S had no significant effect on the reversal potential (2-OH-S, -80.68±5.24mV). This was also the case for CGP 36742 (CGP, -92.39±5.25mV). Traces of a representative cell are given to show the effect of 2-OH-S and CGP 36742 on the reversal potential of the $IPSC_B$ (**Figure 6.14A,B**). Quantification of the results are shown in **Figure 6.15A,C.** Representative current /voltage (IV) plots are shown (**B,C**) in control and drug conditions for both 2-OH-S and CGP 36742.

As mentioned earlier in the results, there is a difficulty in measuring the above three parameters accurately, due to the remote location of the receptors from the cell body. However, once again, if there was a consistent and definite change in either of them elicited by the drugs, it may be assumed that it would be observable in the results.

Figure 6.11 (next page) The effects of 2-OH-S (200μM, n=7) and CGP 36742 (100μM, n=5) are shown on the stimulus response curve of the $IPSC_A$. The values for the stimulus amplitude are normalised and indicate a increase in the stimulus voltage applied.
A) 2-OH-S significantly affected the stimulus response curve of the $IPSC_A$ moving it to the right of the control at all stimulus intensities and this effect was partially reversible on washing.
B) The effects of 2-OH-S on the stimulus response curve of the $IPSC_A$ are shown for each individual cell to indicate any gross deviation from the mean.
C) CGP 36742 did not significantly affect the stimulus response curve of the $IPSC_A$.
D) The effects of CGP 36742 on the stimulus response curve of the $IPSC_A$ are shown for each individual cell to indicate any gross deviation from the mean.

Figure 6.12 (...following page) The effects of 2-OH-S (200μM, n=7) and CGP 36742 (100μM, n=5) are shown on the stimulus response curve of the $IPSC_B$. The values for the stimulus amplitude are normalised and indicate a increase in the stimulus voltage applied.
A) 2-OH-S significantly affected the stimulus response curve of the $IPSC_B$ moving it ~~to the right of~~ the control at all stimulus intensities and this effect was partially reversible on washing.
B) The effects of 2-OH-S on the stimulus response curve of the $IPSC_B$ are shown for each individual cell to indicate any gross deviation from the mean.
C) CGP 36742 also significantly affected the stimulus response curve of the $IPSC_B$ moving it ~~to the right of~~ the control at all stimulus intensities and this effect was partially reversible on washing.
D) The effects of CGP 36742 on the stimulus response curve of the $IPSC_B$ are shown for each individual cell to indicate any gross deviation from the mean.

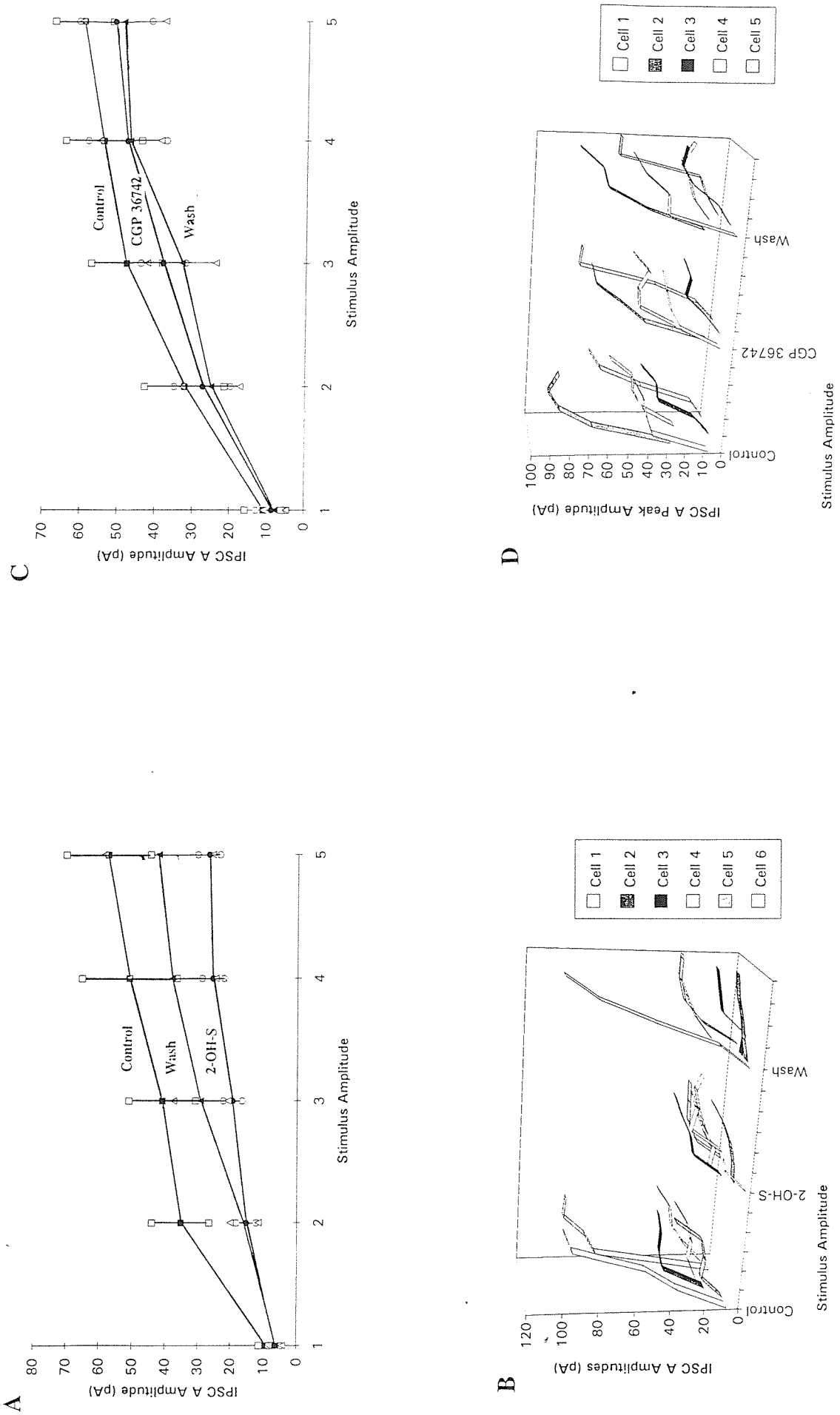


Figure 6.11 2-OH-S & CGP 36742, IPSCA, SR curve

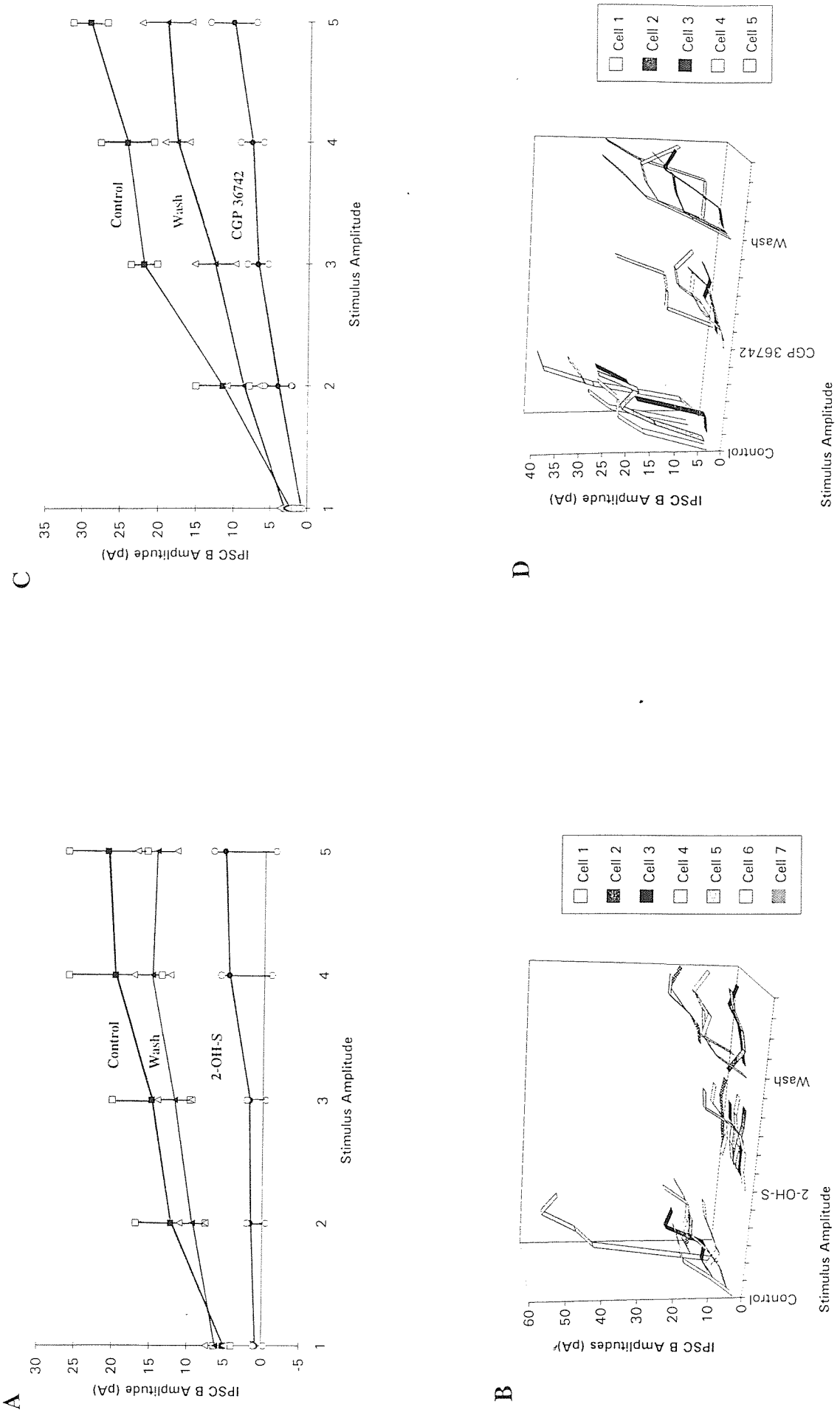


Figure 6.12 2-OH-S & CGP 36742, IPSCB, SR curve

Figure 6.13 (next page) The effect of 2-OH-S (200μM, n=7) and CGP 36742 (100μM, n=5) on the input resistance and the changes in DC offset values are shown.
A) 2-OH-S did not cause any significant change in the input resistance of the cell. **B**) 2-OH-S did not produce a reliable reversible change in the DC offset values of the cells. **C**) CGP 36742 also had no significant effect on the input resistance of the cell. **D**) CGP 36742 did not produce any significant changes in the DC offset values of the cells.

Figure 6.14 (...following page) Representative current traces obtained when stepping the cell from -100mV to -50mV (top trace in each case = -50mV) with an evoked IPSC during the step are shown under each experimental condition. All cells were held at -60mV.
A) Left hand side: *top*, control traces; *middle*, addition of 2-OH-S (200μM, n=7) which depressed the $IPSC_A$ and $IPSC_B$ with no significant effect on the reversal potential and *bottom*, recovery. **B**)Right hand side: *top*, control traces; *middle*, addition of CGP 36742 (100μM, n=5) which depressed the $IPSC_B$ with no significant effect on the $IPSC_A$ or the reversal potential and *bottom*, recovery.

Figure 6.15 (....following page)The average reversal potentials for the $IPSC_B$ are shown along with the effects of 2-OH-S (200μM, n=7) and CGP 36742 (100μM, n=5) and in each case accompanied by a representative graph of an I/V plot for one cell.
A) 2-OH-S had no significant effect on the reversal potential of the $IPSC_B$. This is also reflected in **B**) where the I/V plot is not significantly different from control and 2-OH-S. Inset: The trace of the voltage steps used to calculate the reversal potential with the top line representing a voltage step to -50mV. **C**) CGP 36742 had no significant effect on the reversal potential of the $IPSC_B$. This is also reflected in **D**) where the I/V plot is not significantly different from control.

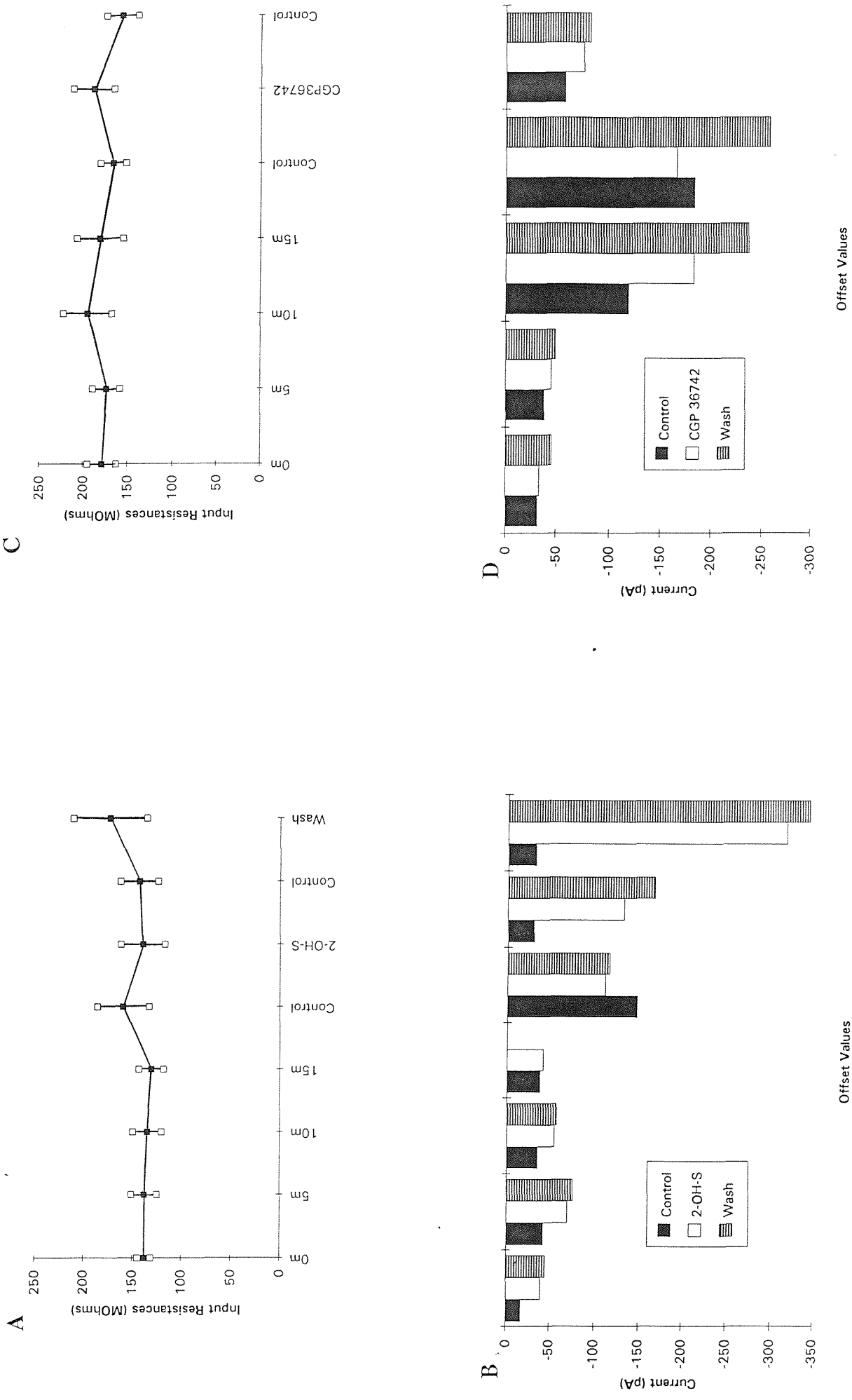


Figure 6.13 2-OH-S & CGP 36742, IR, DC Offsets

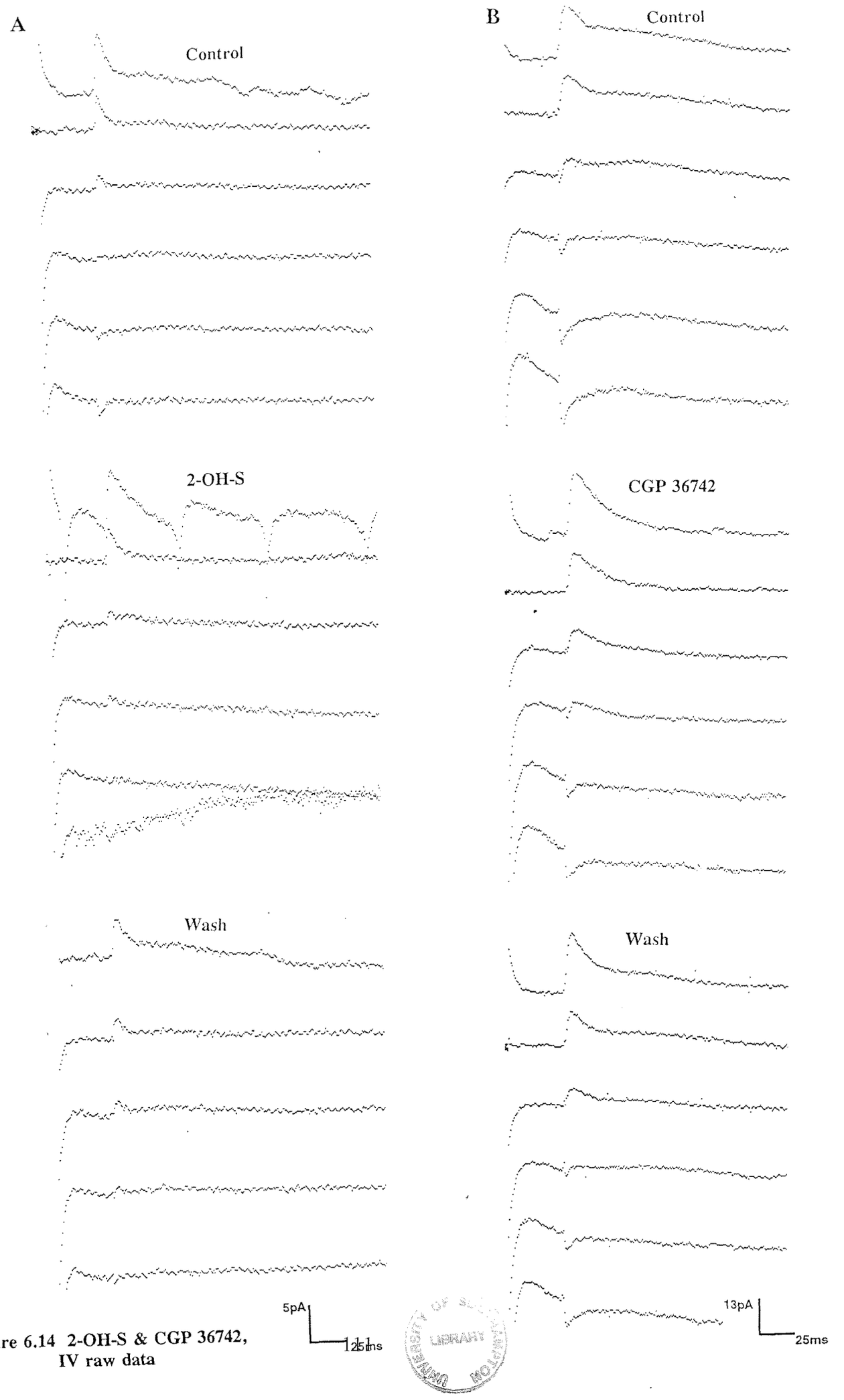
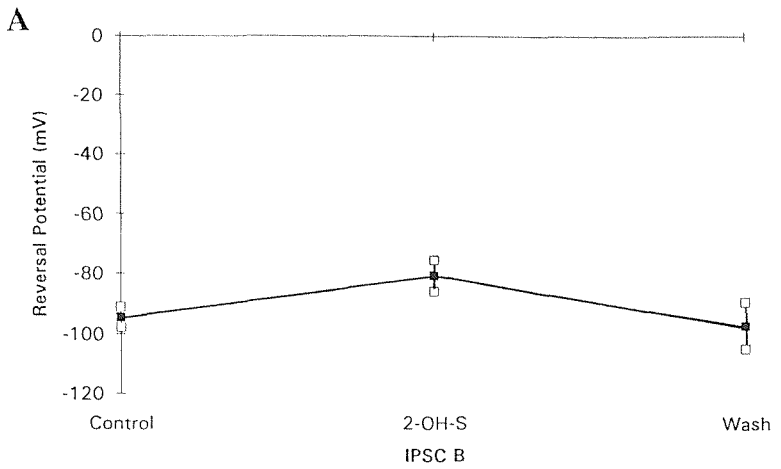
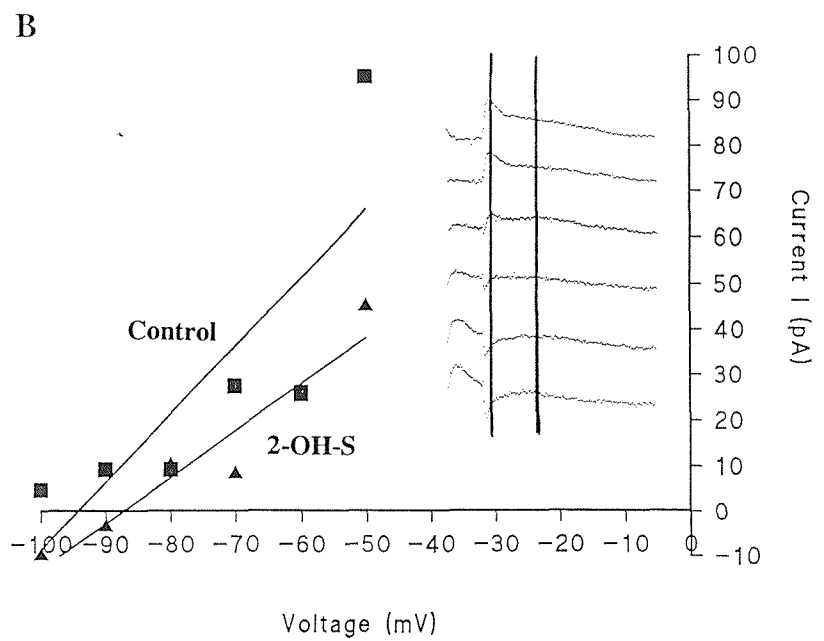
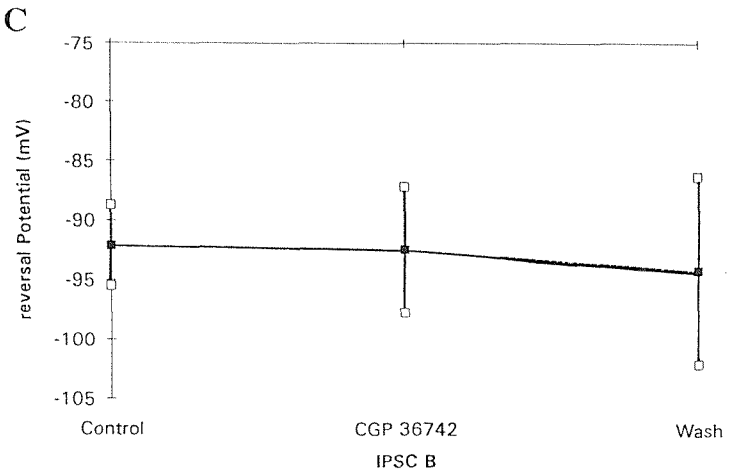


Figure 6.14 2-OH-S & CGP 36742, IV raw data







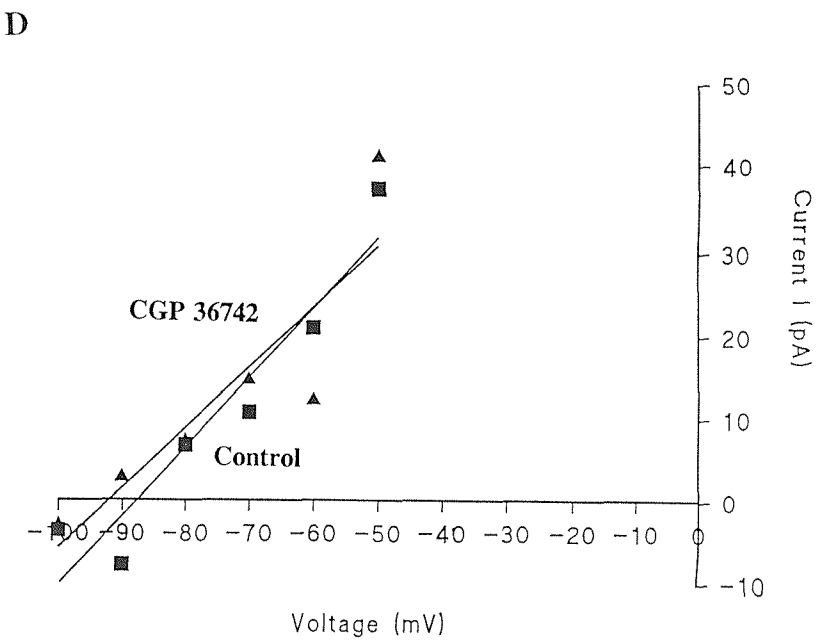


Figure 6.15 2-OH-S & CGP 36742 Reversal Potentials

Having established that 2-OH-S and not CGP 36742 was able to reduce the $IPSC_A$ via what would appear to be a presynaptic action to depress GABA release, we decided to investigate whether 2-OH-S could depress the EPSC via an action on $GABA_B$ receptors on the excitatory terminals

Effects of 2-OH-S and (-) Baclofen on the Isolated EPSC.

In order to investigate the presynaptic actions of 2-OH-S at $GABA_B$ receptors on excitatory terminals the EPSC was isolated by blocking the postsynaptic GABAergic mechanisms. This was done by blocking the $GABA_A$ receptors with bicuculline (6μM) in the ACSF and the postsynaptic K^+ conductance which is activated by GABA B receptors by inclusion of QX-314 (10mM) and CsOH (replacing KOH) in the recording electrode. The effects of 2-OH-S (200μM, n=6) were tested and compared to those of (-) baclofen (10μM, n=3).

The peak amplitude of the EPSC was significantly and reversibly reduced by 2-OH-S (64±14%; n=6, $p<0.01$) and (-)baclofen (66±16%; controls -77.19±17.21pA; (-) baclofen, -21.11±4.12pA, $p<0.01$, n=3;). Representative traces are shown in **Figure 6.16A,B**. Initially the effect of 2-OH-S was tested on EPSC's evoked by a maximal stimulus as before.. However, in some cells this resulted in breakthrough spikes because the $GABA_A$ inhibition had been blocked and so smaller EPSC's were evoked by lower stimulus intensities. For the small EPSC's (n=3) there appeared to be a larger reduction in the peak amplitude of the EPSC (controls, -38.08±2.18pA, 2-OH-S, -11.07±5.2pA) than for the large EPSC's (n=3; controls, -218.09±31.65; 2-OH-S, -118.05±27.02pA) although it is not clear why this should be the case and whether it is significant. These results were quantified and the mean±s.e.m. of the peak amplitudes of the EPSC's are shown as histograms (**Figure 6.17A,B,D**) accompanied by the ribbon graphs showing the individual results (**C,E**).The results for the 6 cells treated with 2-OH-S are shown together for comparison. There is a spread of the responses in the individual cells but the trend between them appears to be reflected in the mean values.

Stimulus/response curves were constructed for the EPSC to see if the effects were intensity dependant (**Figure 6.18A,B,D**). They indicated a reduction in the amplitude of the EPSC at all stimulus intensities for 2-OH-S and (-)baclofen. (n=6, n=3, $p<0.01$). From these results it would appear that there may be a gretaer % block for the lower stimuli/smaller EPSC's. Again, the spread of the raw data is shown as ribbon graphs (**C,E** in each) and shows that the mean responses appear to be an accurate reflection of the individual responses.

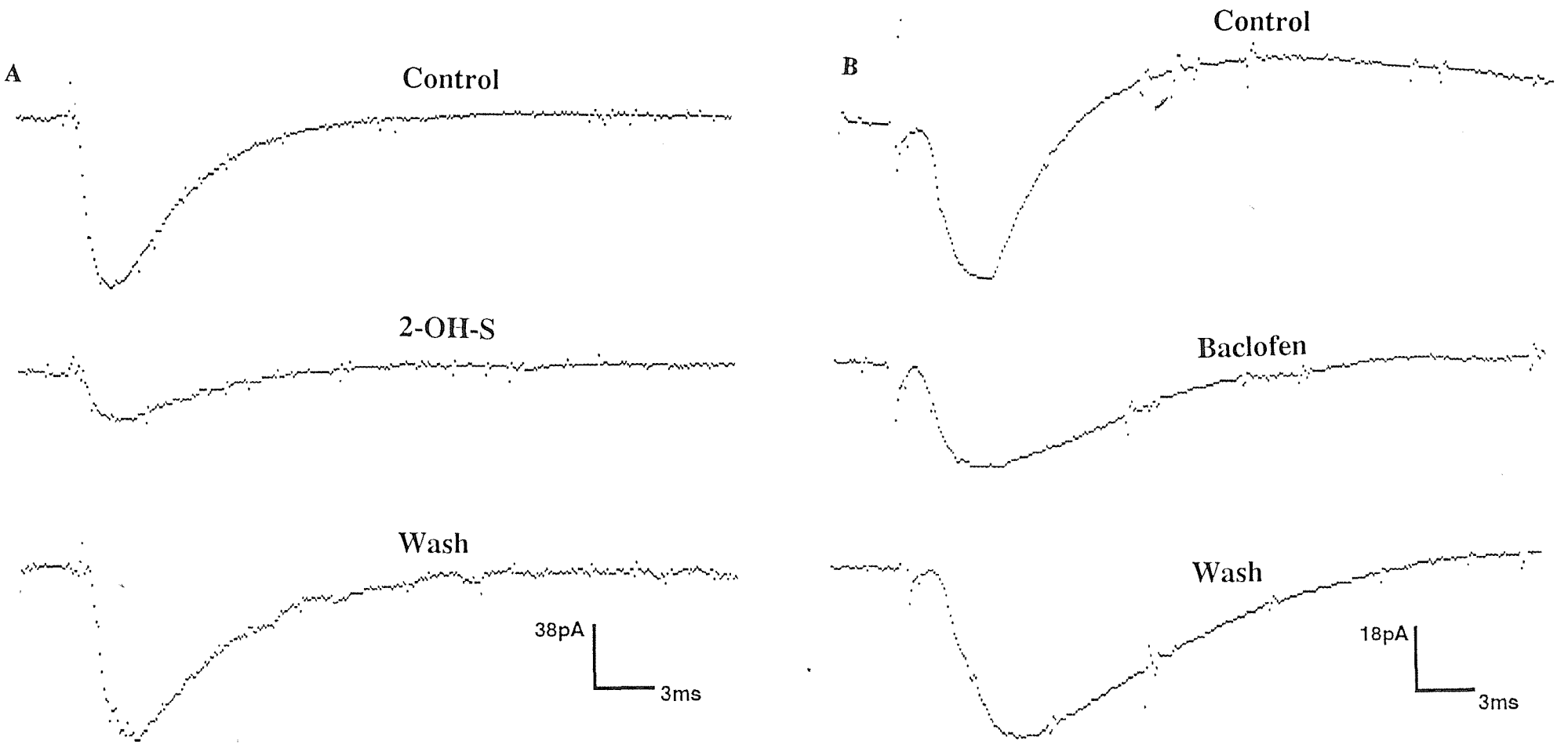


Figure 6.16 Representative traces show the pre synaptic effects of 2-OH-S (200μM, n=6) and(-) baclofen (10μM, n=3) on the peak amplitude of the evoked EPSC. The postsynaptic K+ current was blocked with QX-314. A) *Top*, control traces obtained after isolation of the EPSC; *middle*, in the presence of 2-OH-S, which significantly reduced the EPSC and *bottom*, recovery after washing. B) *Top*, control traces; *middle*, in the presence of (-) baclofen which also reduced the EPSC and *bottom*, recovery on washing.

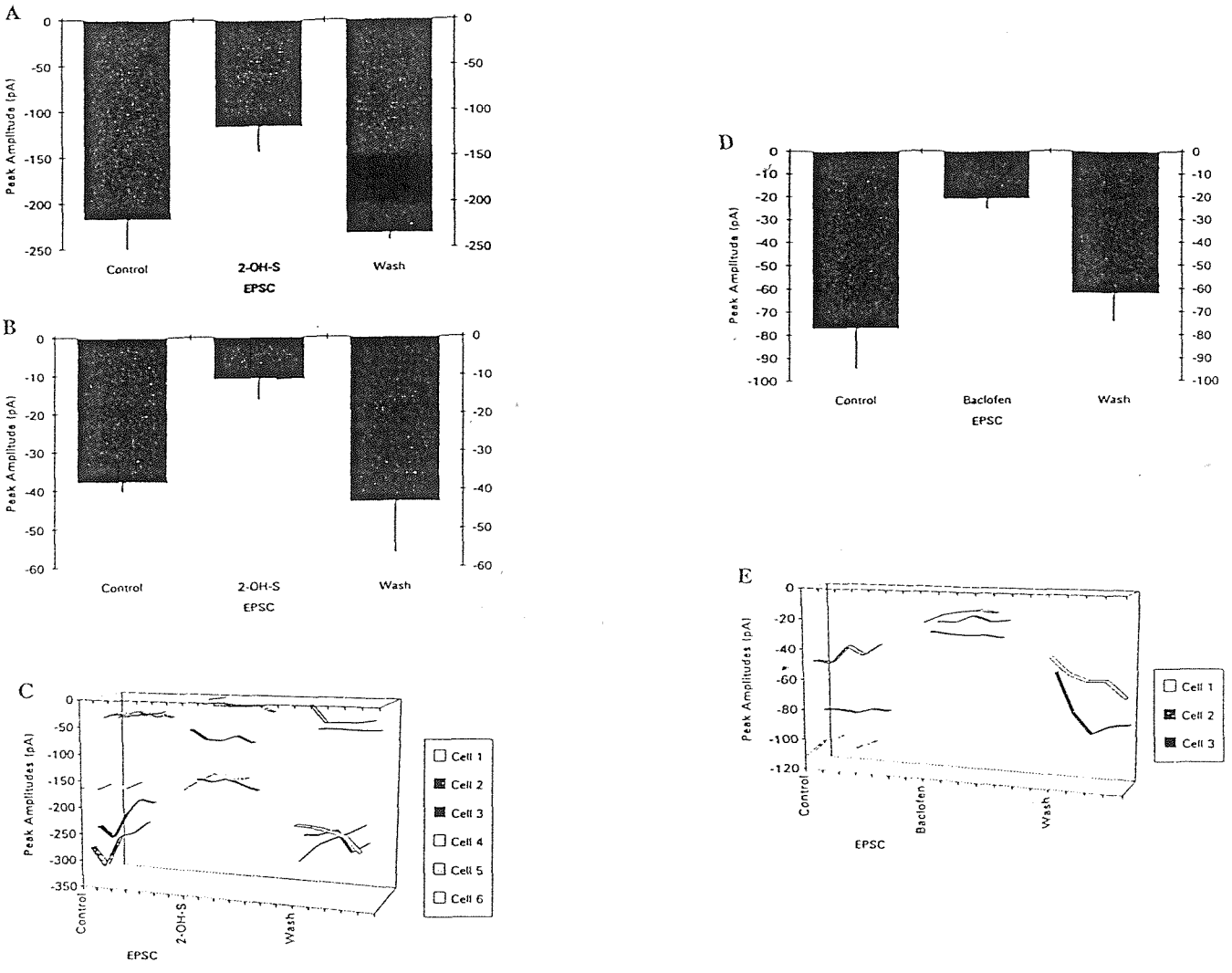


Figure 6.17 The effects of 2-OH-S (200μM, n=6) and (-) baclofen (10μM, n=3) are shown quantified as the mean$\pm$s.e.m. of the peak amplitude. The results for 2-OH-S are split into two groups based on their peak amplitude sizes, small and large, as a result of the experimental protocol. A) 2-OH-S (n=3, large) significantly reduced the peak amplitude of the EPSC, which was reversible upon washing. B) 2-OH-S (n=3, small) also significantly reduced the peak amplitude of the EPSC. C) This reduction of the EPSC by 2-OH-S is shown for each individual cell (n=6) to indicate the overall trend of the group of cells and any gross deviation from this trend. D) (-) baclofen significantly reduced the peak amplitude of the EPSC, which was reversible upon washing. D) This reduction of the EPSC by (-) baclofen is shown for each individual cell to indicate the overall trend of the group of cells and any gross deviation from this trend.

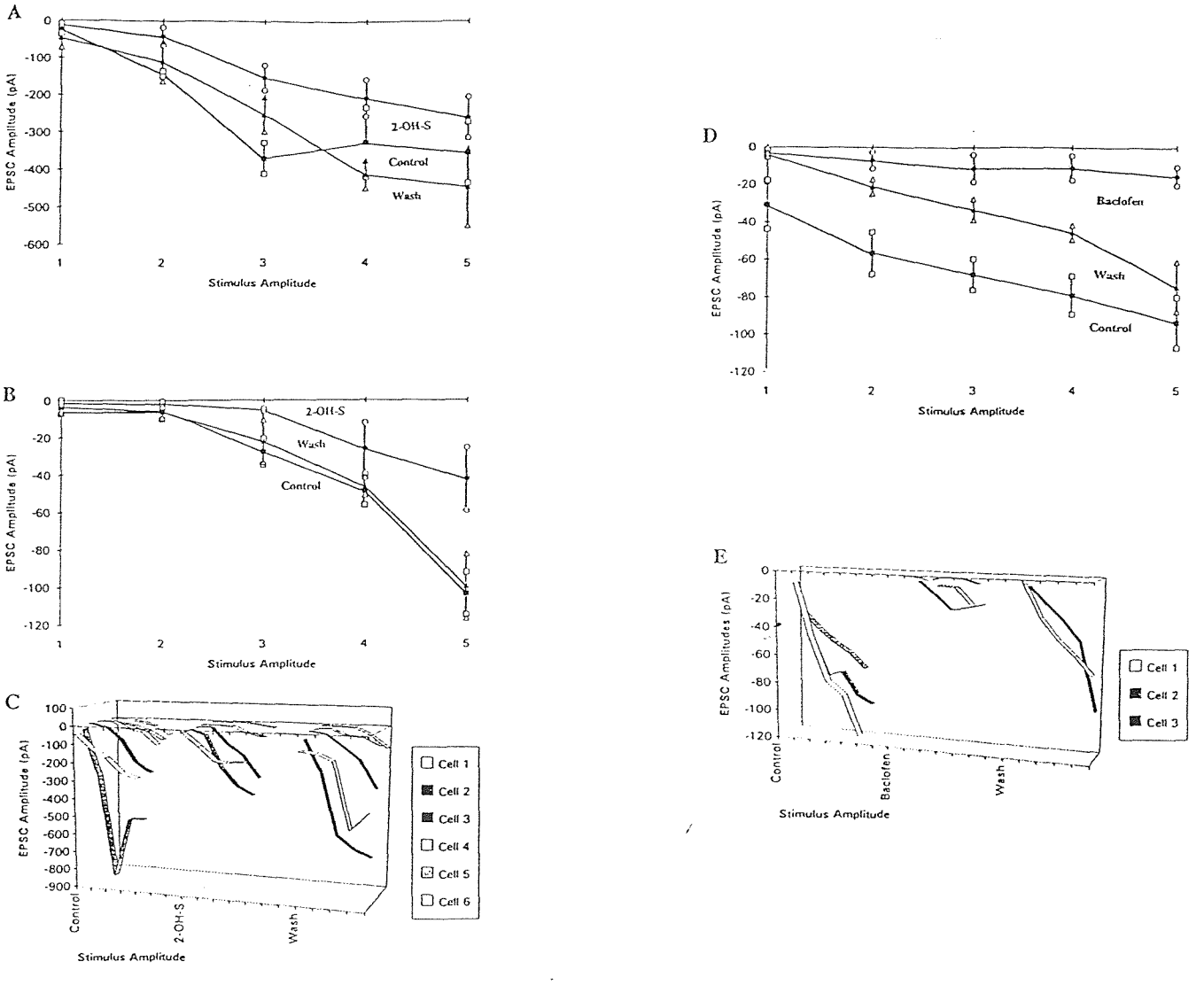


Figure 6.18 The effects of 2-OH-S (200μM, n=6) and (-) baclofen (10μM, n=3) are shown on the stimulus/response curve. The values for the stimulus amplitude indicate a consecutive increase in the stimulus voltage applied. Again, the results for 2-OH-S are split into two groups based on their peak amplitude sizes, small and large, as a result of the experimental protocol. A) 2-OH-S (n=3, large) significantly affected the stimulus response curve, moving it to the right of the control, at all stimulus intensities and this effect was partially reversible upon washing. B) 2-OH-S (n=3, small) also significantly affected the stimulus response curve, moving it to the right of the control at all stimulus intensities with recovery on washing. C) The effects of 2-OH-S on the stimulus response curve of the EPSC is shown for each individual cell (n=6) to indicate any gross deviation from the mean. D) (-) baclofen also significantly affected the stimulus response curve, moving it to the right of the control at all stimulus intensities. This effect was partially reversible upon washing. E) The effects of (-) baclofen on the stimulus response curve of the EPSC is shown for each individual cell to indicate any gross deviation from the mean.

Representative traces of the raw data of the voltage steps used to look at the reversal potential of the EPSC are shown in **Figure 6.19A,D**. No actual reversal of the EPSC was observed under the conditions employed. Examples of plots are shown to illustrate that there was no actual reversal and that computer calculated potentials would be completely innacurate (**Figure 6.20A,D**).

The input resistance was measured and there appeared to be no significant change after application of 2-OH-S (control, 223.9±17MΩ; 2-OH-S, 206.7±26MΩ, **Figure 6.20B**) or (-)baclofen (control, 212.08±44.79MΩ; (-) baclofen, 158.41±25.25MΩ, **Figure 6.20E**). Assuming that the postsynaptic K+ conductance was fully blocked, there should be no decrease in the input resistance by (-) baclofen as would normally be expected from activation of the conductance by an agonist.

The DC offset values for 2-OH-S and (-)baclofen were measured and are shown (**Figure 6.20C,F**).There was no evidence for an activation of an outward current by 2-OH-S (n=6). or (-) baclofen. This would be expected because of the block of the postsynaptic K^+ current.

From this set of experiments it would appear that in addition to being able to reduce inhibitory currents by a presynaptic mechanism, 2-OH-S also depresses excitatory currents by a presynaptic mechanism. Although 2-OH-S appears to exert its effects at the same type of $GABA_B$ receptors, it cannot be determined whether the actions activate the same transduction mechanisms.

Figure 6.19 (next page) Representative current traces obtained when stepping the cell from -100mV to -50mV (top trace in each case = -50mV) with an evoked EPSC during the step are shown under each experimental condition. All cells were held at -60mV.
A) Left hand side: *top*, control traces; *middle*, addition of 2-OH-S (200μM, n=6) which depressed the EPSC with no significant effect on the reversal potential and *bottom*, recovery. **B**)Right hand side: *top*, control traces; *middle*, addition of (-)baclofen (10μM, n=5) which also depressed the EPSC with no significant effect on the reversal potential and *bottom*, recovery.

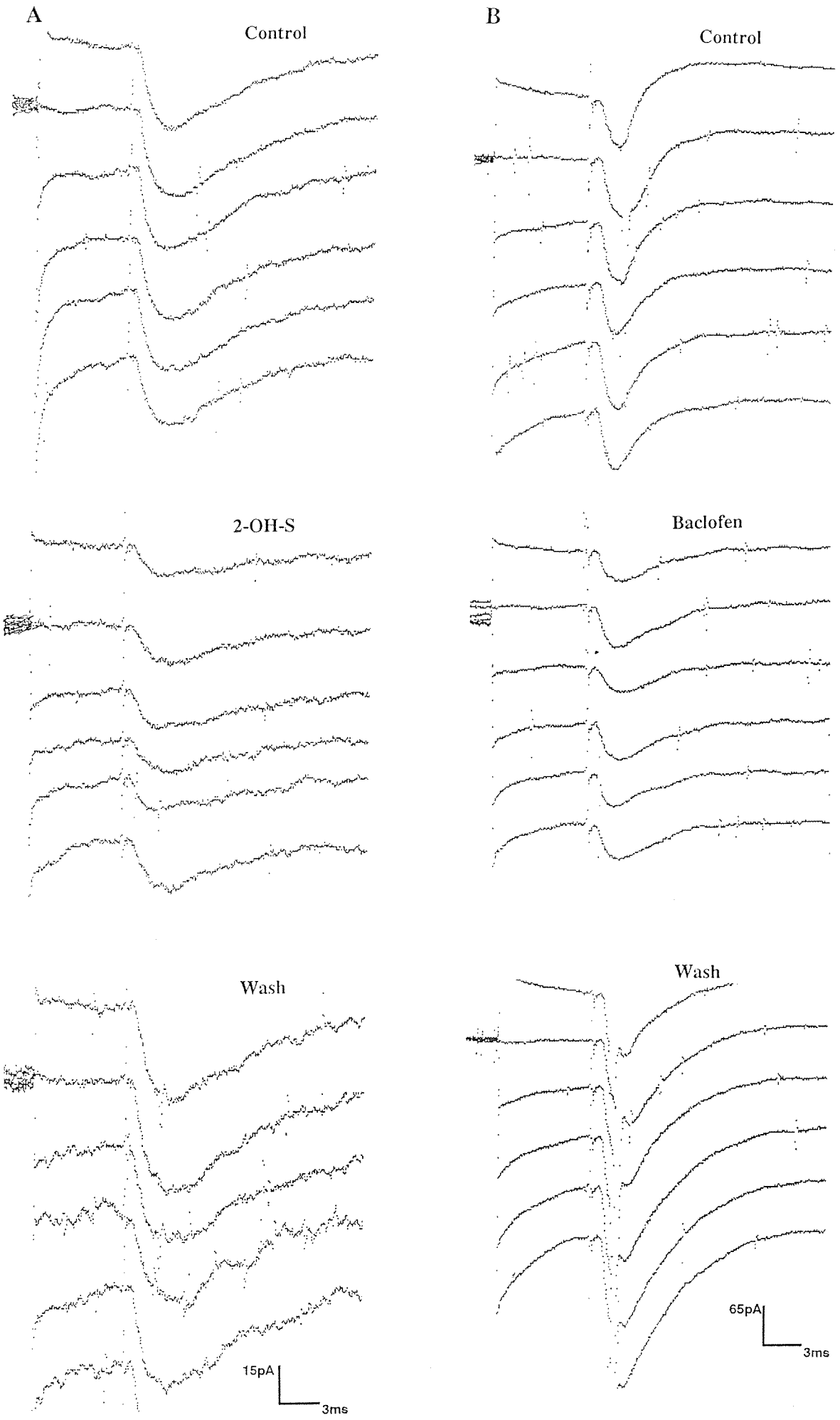


Figure 6.19 2-OH-S & Baclofen IV raw data

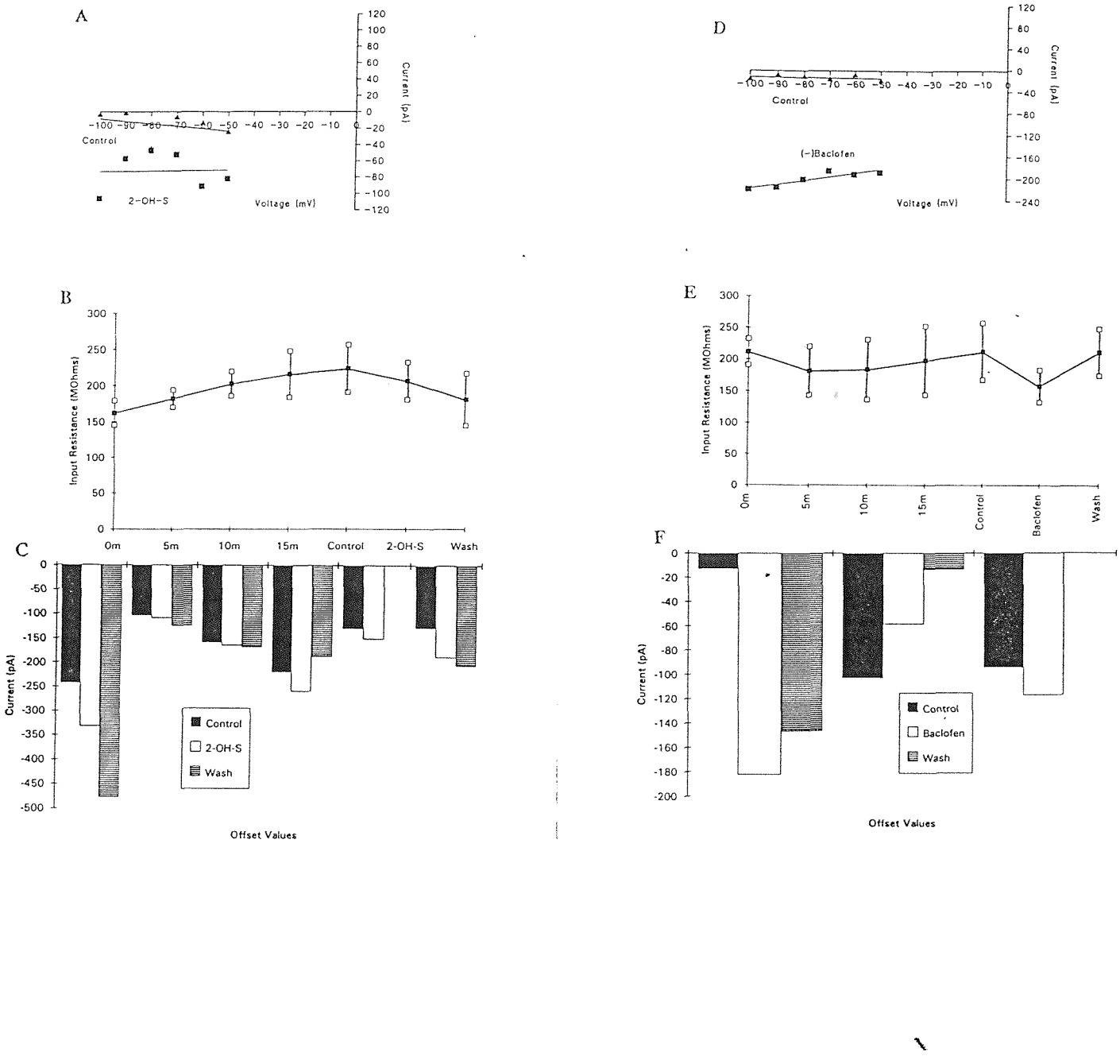


Figure 6.20 The effect of 2-OH-S (200μM, n=6) and (-) baclofen (100μM, n=3) on the reversal potentials, input resistance and the changes in the DC current offset values are shown. Cells were held at -60mV. A&D) Represenative computer calculated plots of the reversal potentials are shown which indicate that ther was no actual reversal of the EPSC. B) 2-OH-S did not cause any significant change in the input resistance of the cell. C) 2-OH-S did not produce any reliable, reversible changes in the DC offset values of the cells. E) (-) baclofen also had no reliable, reversible effect on the input resistance or F) the DC offset values of the cells.

GABAergic Inhibitory Control of the Paired Pulse Depression and Facilitation of the EPSC

The mechanisms and time course of the inhibitory control of the EPSC were further investigated to establish the time course of the presynaptic inhibition of the EPSC. A paired pulse protocol was used to look at the time course of the depression of the EPSC.
In the first set of experiments the recording electrode was placed as for the extracellular experiments to activate the excitatory afferents in CA3 and the solution in the recording electrode was standard with no ion channel or receptor blockers. The external solution was also standard ACSF. Two identical stimuli were given in succession at intervals of 10-500msec and % inhibition was calculated and quantified as for the extracellular responses {(C-T)/C}. Under these conditions there was a small amount of inhibition at 10msec (1.35±14.8%) and a negative inhibition (facilitation) at 20-200msec (e.g. 50msec -73.12±15.14%) which became more positive at 300-500msec (e.g. 300msec -19.75±12.23%). Representative traces from one cell are shown (**Figure 6.21A**) and the effect of paired pulse inhibition is quantified as the mean±s.e.m. of the % inhibition and presented in **Figure 6.22A.** The responses of the individual cells (**B**) are represented on a ribbon graph (n= 5). One cell appears to deviate quite noticeably from the others and has biased the mean value for the inhibition at 200msec.

It is possible that any effect of the pre or postsynaptic $GABA_B$ receptors on the paired pulse facilitation/depression of the EPSC may be masked by the effects of the stronger $GABA_A$ mediated inhibition. Bicuculline (6μM) was therefore added to the external solution to block any $GABA_A$ mediated effects. Addition of bicuculline increased the size of the EPSC and in order to avoid breakthrough spikes the stimulus intensity was lowered. Under these conditions (n=4) negative inhibition (facilitation) was still observed at intervals of 10-200msec and 500msec (10msec, -139.9±32.67%; 100msec, -71.3±18.4%; 500msec, -38.55±1.31%). However, paired pulse inhibition was now seen at 300msec unlike before (24.25±9.17%). Representative traces from one cell are shown in **Figure 6.21B** and the effect of paired stimuli on the EPSC is quantified as % inhibition and presented as mean±s.e.m. The time base of the scale bars are implied from the intervals between stimulation (**Figure 6.22C,D**). The individual cells are represented in the ribbon graph, as before.

These results indicated that the inhibition of the EPSC by presynaptic $GABA_B$ receptor activation had been masked by a component of $GABA_A$ inhibition. The inhibition of the EPSC appeared to occur at the same latency as the paired pulse inhibition of the population spike (300msec) which was quite different from the latency (175msec) of the peak of the postsynaptic $IPSC_B$.

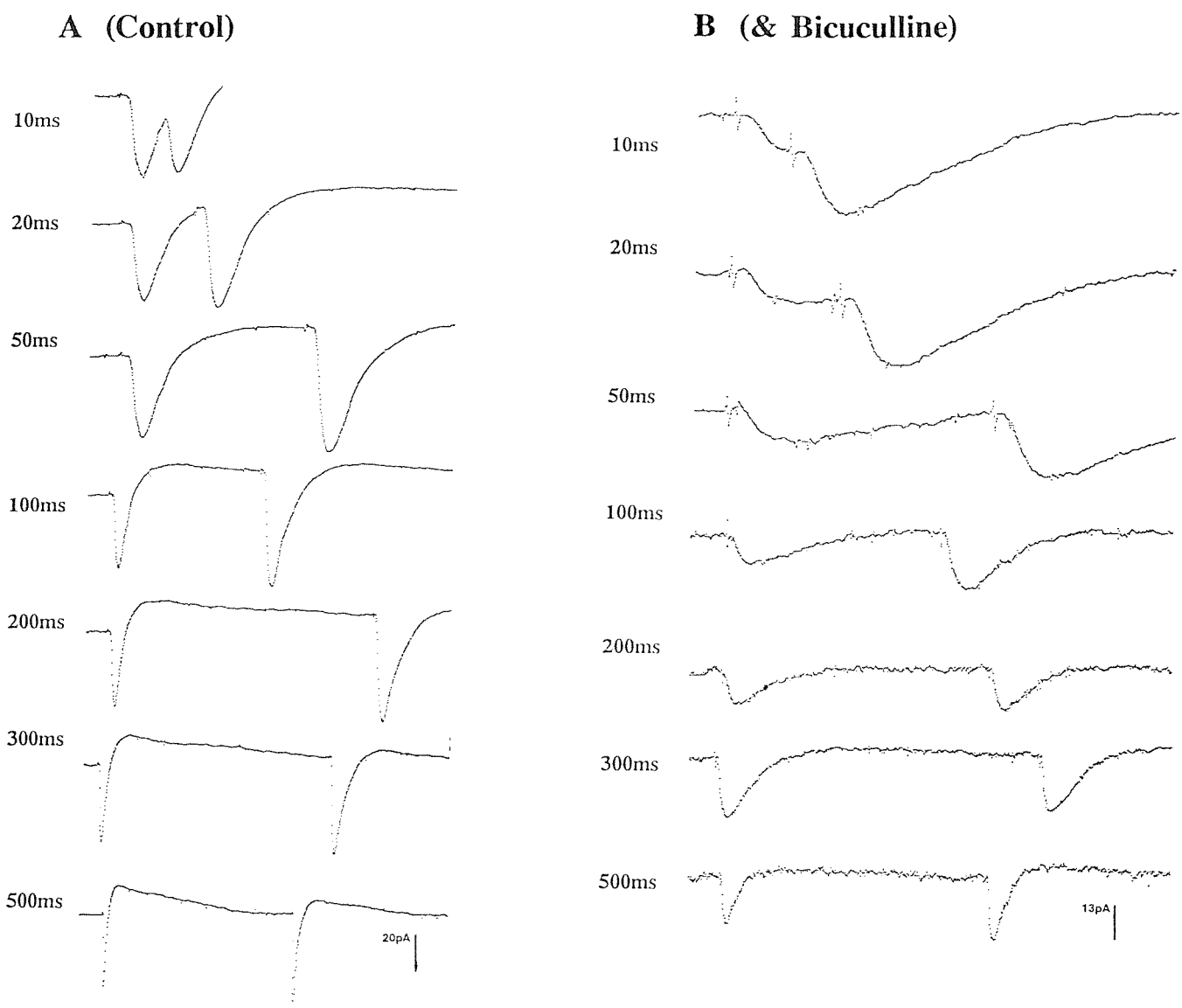


Figure 6.21 Representative currents obtained after delivering two consecutive pulses orthodromically to the excitatory afferents with different interpulse intervals. The left hand column A) shows responses elicited by paired pulses delivered in control conditions (I.E, standard ACSF) (n=5). Facilitation can be seen at all interpulse intervals but is most prominent at 50ms. The right hand column B) shows responses elicited by paired pulses in ACSF containing bicuculline (6μM, n=4). Facilitation can still be observed as before, but at 300ms there is an inhibition of the second pulse.

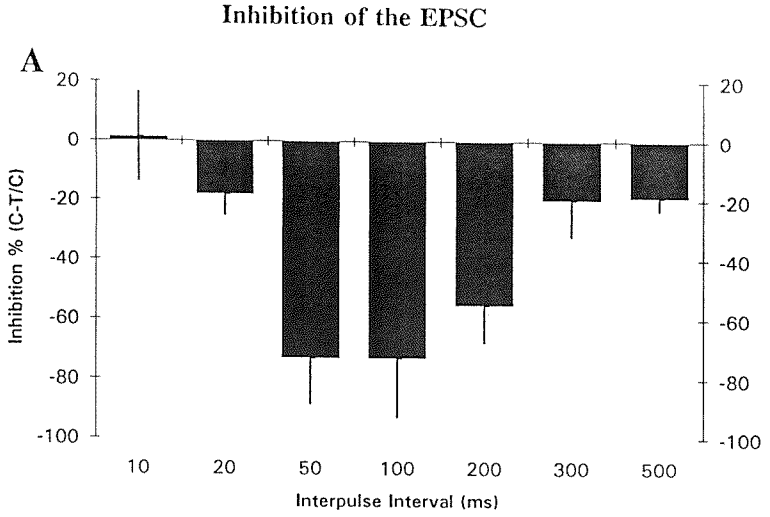
Figure 6.22 (next page) The paired pulse facilitation and inhibition were quantified [(C-T)/C %] and are represented in the histogram as mean±s.e.m..

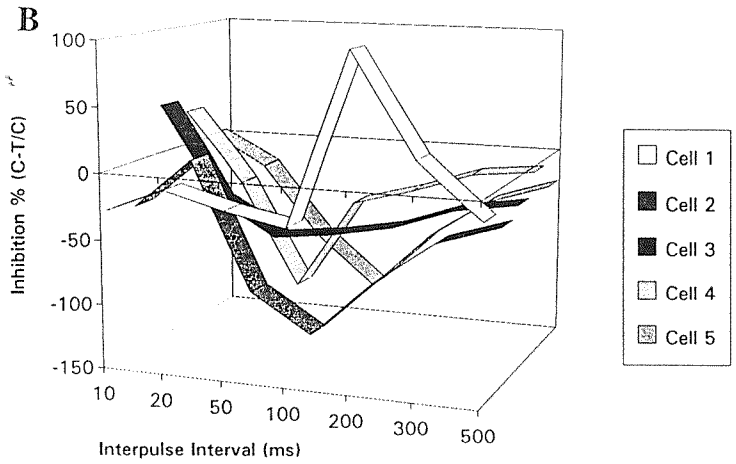
A) Facilitation appears to be prominent at all intervals in control medium (n=5) except for 10msec.

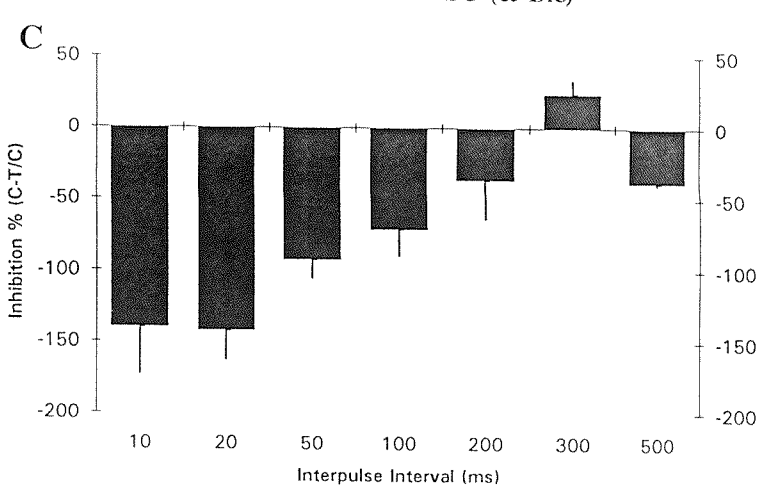
B) The responses of the individual cells are shown and appear to follow a similar trend apart from a large deviation by one cell at 200msec which has distorted the mean.

C) Facilitation is also apparent at most intervals in medium containing bicuculline (6μM, n=4) with a small amount of inhibition being apparent at 300msec.

D) The responses of the individual cells are also shown and again they appear to follow the same trend.







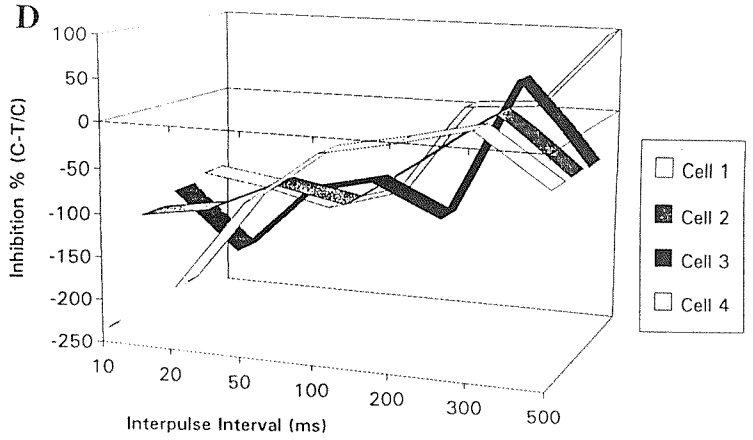


Figure 6.22 Paired Pulse Inhibition of the EPSC ± Bicuculline

6.4 Discussion

Whole cell patch clamp studies were carried out on CA1 pyramidal cells in the hippocampus to investigate GABAergic inhibition mediated by pre- and postsynaptic $GABA_B$ receptors. Successful isolation of the inhibitory currents was achieved by blocking the excitatory NMDA and non-NMDA components with D-APV and CNQX respectively and the $GABA_A$ receptors with bicuculline. Postsynaptic $GABA_B$ effects mediated by K^+ were blocked with QX-314 (Nathan et al., 1990b; Andrade, 1991; Nunez & Buno, 1992) when investigating the presynaptic effects, although we have no direct way of proving that a complete block was achieved. Local stimulation in lacunosum/moleculare provided a direct activation of the interneurones and enhanced the inhibitory currents observed. The results obtained here cast doubt on the validity of the use of 2-OH-S as a selective $GABA_B$ antagonist in area CA1 of the hippocampal slice preparation and attempt to elucidate it's mechanism of action along with investigating the involvement of presynaptic $GABA_B$ receptors in the inhibition of the excitatory and inhibitory currents in this area.

Whole Cell Currents In CA1 Pyramidal cells

Gigaohm seals of individual CA1 pyramidal cells enabled stable recordings to be made for up to ninety minutes and cells that were unstable usually died within the first fifteen minutes before any critical recordings were taken. The mean peak amplitudes and latencies of the $IPSC_A$ and $IPSC_B$ were comparable to those in the literature (Davies et al., 1990; Solis & Nicoll, 1992a; Solis et al., 1992b), without the presence of GABA uptake blockers, which increase the size and duration of the currents (Roepstorff & Lambert, 1992; Solis & Nicoll, 1992a; Solis et al., 1992b; Isaacson et al., 1993).

The size of the EPSC observed with local stimulation of the interneurones in the L/M was, as would be expected, less than that observed with orthodromic stimulation of the excitatory afferents from CA3 (see chapter 5). Although there is the obvious problem of space clamp in these cells due to the fact that they have long diffuse processes, the whole cell currents recorded appeared to be stable. However, measurement of the reversal potentials of the dendritic $GABA_B$ currents and any direct current changes, may have been

hampered by the lack of full space clamp and are therefore not as accurate as we might have hoped for.

The Depression of the $IPSC_A$ and $IPSC_B$ by 2-OH-S

2-OH-S has been used to date as a selective antagonist of the effects of baclofen and GABA at the pre- and postsynaptic $GABA_B$ receptors (Curtis,Gynther,Beattie,Kerr, & Prager, 1988; Kerr et al., 1988; Lambert et al., 1989; Al-Dahan,Jalilian Tehrani, & Thalmann, 1990; Davies et al., 1990; Harrison et al., 1990; Scholz & Miller, 1991b). In the experiments presented here the isolated postsynaptic $IPSC_B$ could be blocked by 2-OH-S as previously shown and also by CGP 36742, a potent $GABA_B$ antagonist at pre and postsynaptic $GABA_B$ receptors (Olpe,Steinmann,Frerat,Pozza,Greiner,Brugger, et al., 1993).

However, in addition to this 2-OH-S also significantly depressed the $IPSC_A$, which was unaffected by CGP 36742. This depression of $GABA_A$ mediated inhibition appears to correlate with the reduction of the early inhibition in response to 2-OH-S and not phaclofen, observed in the extracellular paired pulse recordings. The effects of 2-OH-S were shown not to be mediated by a direct action of 2-OH-S at $GABA_A$ receptors and therefore one strong possibility, is that 2-OH-S could depress the $IPSC_A$ via an agonist, or partial agonist effect, on the pre-synaptic $GABA_B$ receptors located on the terminals of the interneurones (basket cells or axo-axonic cells), which, when activated, depress the release of GABA onto the somatic $GABA_A$ receptors. The depression of GABA release would then lead to a reduction of the postsynaptic $IPSC_A$. It is unlikely that an agonist or antagonist action on postsynaptic $GABA_B$ receptors could produce a change in the ionic conductance of the membrane sufficient to depress the $GABA_A$ response postsynaptically and in these experiments there was no detectable change to the postsynaptic receptor which could result in this. Interestingly, a depressant effect of 2-OH-S upon the $IPSC_A$ has been observed before, but this was not investigated and instead the stimulus was increased to compensate for the depression (Davies et al., 1990).

2-OH-S may exert its actions at presynaptic $GABA_B$ receptors on excitatory terminals to reduce the release of glutamate, decrease the excitation of basket cells and subsequent release of GABA onto the postsynaptic $GABA_A$ receptors and depress the $GABA_A$ response. However, this would not seem to be the case as direct stimulation of the interneurones in L/M was employed and the excitatory amino acid receptors were blocked

In addition to this, we cannot rule out the possibility that the depression of the postsynaptic $IPSC_B$ by 2-OH-S was as a result of agonist/partial agonist effects at the presynaptic $GABA_B$ receptor. This would cause a depression of the $IPSC_B$ as observed, but this would also be the case for any postsynaptic antagonist effects of 2-OH-S. However, we could not see any significant DC shift of the postsynaptic membrane conductance or a change in the input resistance caused by 2-OH-S that would indicate activation of a postsynaptic K^+ current. This would have been expected with application of baclofen.

While these results clearly indicate that 2-OH-S is able to depress the $IPSC_A$ via presynaptic agonist/partial agonist action, the effect on the $IPSC_B$ is less conclusive and from the experiments performed here it is only possible to speculate on the actions, if any, of 2-OH-S on the postsynaptic receptors. It may be possible that the dissimilarity between these results and studies which show that 2-OH-S antagonises the presynaptic effects of baclofen and disinhibition, could arise if 2-OH-S was a partial agonist at the pre and postsynaptic $GABA_B$ receptors, reducing the effects of baclofen and GABA by competing for the receptor sites. This effect could be blocked by an antagonist such as phaclofen, which was seen to be the case in the extracellular studies. Indeed, 2-OH-S has been reported to have only a very weak antagonistic effect on $GABA_B$ hyperpolarisations (Segal, 1990).

The Depression of the EPSC by 2-OH-S and (-)Baclofen

The results obtained in this study were in full agreement with (-)baclofen depressing the evoked EPSC by activation of presynaptic $GABA_B$ receptors (Bowery et al., 1980; Lanthorn & Cotman, 1981; Ault & Nadler, 1983; Dutar & Nicoll, 1988b; Harrison, 1990; Thompson & Gahwiler, 1992a; Thompson,Scanziani,Capogna, & Gawhiler, 1992). This was seen in the reduction of the peak amplitude of the EPSC at all stimulus intensities.

2-OH-S also depressed the EPSC (64±14%) to an extent comparable with the depression caused by baclofen (66±16%) (Figures 6.16 & 6.17). As the postsynaptic K^+ conductance was blocked by QX-314, it would appear that this effect was mediated by an agonist/partial agonist action on the presynaptic receptors of excitatory terminals to depress the release of glutamate. This would appear to agree with the observations in chapters 3 and 4, that 2-OH-S depressed the evoked conditioning response and that this could be reversed by phaclofen and not bicuculline. The possibility that the depression of the EPSC by 2-OH-S and (-)baclofen may be a result of activation of $GABA_B$ receptors on the postsynaptic membrane that do not activate a K^+ conductance cannot be determined from these

experiments but has not been implied from other studies. Experiments showing the depression of the EPSC by (-)baclofen without QX-314 would have been useful in showing the Activation of a postsynaptic conductance.

In previous studies the depression of the EPSC induced by baclofen was antagonised by 2-OH-S (Curtis et al., 1988; Harrison et al., 1990). These results may be as a result of partial agonist actions, with 2-OH-S competing for the presynaptic receptor sites. Again, the potentials measured and the stimulus employed could all play a part in distorting the actions of this compound.

GABAergic Modulation of the Paired Pulse Facilitation/Depression of the EPSC

The time course of paired pulse inhibition of the EPSC was investigated to determine whether it was similar to the inhibition of the population response observed in chapters 3 and 4. Homosynaptic paired stimuli evoked EPSC's which exhibited paired pulse facilitation (negative inhibition values) at all intervals except 10msec. The facilitation was greatest at 50-100msec which corresponds with the facilitation seen in the extracellular paired pulse studies in chapters 3 and 4. The facilitation may result from an increase in the release of glutamate and/or GABAergic disinhibition.

Further investigation was carried out into the possibility that under normal circumstances, the $GABA_A$ component of inhibition masks some of the effects of the $GABA_B$ component (Conners et al., 1988) and by blocking the former it may be possible to identify any contribution of the $GABA_B$ receptors to paired pulse depression. When bicuculline was added to the control medium and the same experiments were repeated, facilitation was still observed, peaking at 50-100msec, but at 300msec a small amount of inhibition was apparent. Bicuculline had caused an increase in the initial size of the conditioning EPSC which meant that no further facilitation could be observed due to a maximum response already being attained. The block of the $GABA_A$ receptors meant that the only GABAergic inhibitory mechanism remaining was via $GABA_B$ receptors. A recent study showed that the facilitation at 50-100msec and the depression of the EPSC at 300msec indicated the involvement of presynaptic $GABA_B$ receptors and their physiological role in the regulation of excitatory synapses (Isaacson et al., 1993). Although we observed facilitation and inhibition at similar intervals, it is likely that the use of a heterosynaptic paired pulse stimulus accounted for any difference in the amount of inhibition and facilitation observed

between our results and those from that particular study. The inhibition that had been observed at 10msec was also completely blocked suggesting that there may be an early feedback inhibition of the EPSC mediated by $GABA_A$ receptors. These results correlate with the paired pulse inhibition of the population response mediated by $GABA_B$ receptors which was observed in the extracellular studies in earlier chapters. Both of these results however do not correlate with the time course of the postsynaptic $IPSC_B$ suggesting further evidence for the role of the presynaptic $GABA_B$ receptor in modulation of excitatory transmission.

In summary, in our hands, 2-OH-S appears to exert agonist/partial agonist actions at presynaptic receptors leading to a reversible decrease in the amplitude of the EPSC, $IPSC_A$ and $IPSC_B$. This may be via activation of a K^+ conductance, as already described for the postsynaptic $IPSC_B$ or via a reduction of a Ca^{2+} current. Paired pulse facilitation of the EPSC may result from GABAergic disinhibition and paired pulse inhibition of the EPSC may be mediated by activation of presynaptic $GABA_B$ receptors on excitatory afferents to depress glutamate release, giving support for a functional role of $GABA_B$ receptors in synaptic transmission. A schematic model of the location of actions of 2-OH-S is shown in **Figure 6.24**.

It should be mentioned that there is always the possibilty that the anomalous results obtained with 2-OH-S were due to contamination of the compound with an agonist such as baclofen. However, the results with 2-OH-S were consistent throughout the length of this study and a number of different batches from the same source were used. If there was contamination, one would expect a variability in the amount of contamination and the effects subsequently seen. Also in support of these results there was no evidence for an action of 2-OH-S at the postsynaptic site, which if contaminated with baclofen would be in evidence. Taking all this into acount it would most likely that contamination was not a determining factor here in this study.

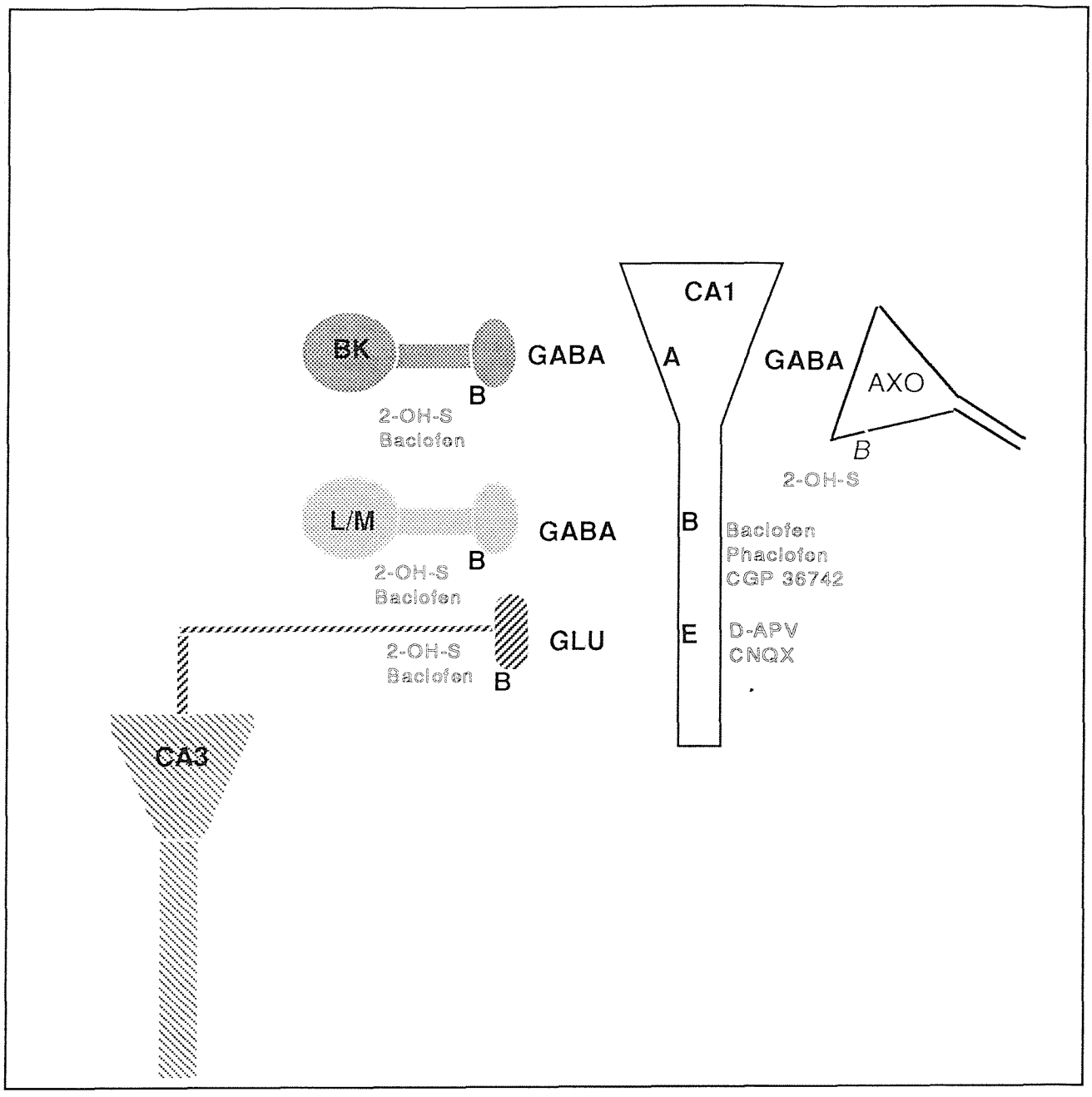


Figure 6.23 The effects of 2-OH-S on GABAergic inhibition in a single cell are shown. 2-OH-S may act as a partial agonist with similar but weaker effects to those of baclofen at $GABA_B$ receptors (B) on the terminals of the basket cells (BK), the lacunosum/moleculare cells (L/M) or/and the excitatory afferents from CA3. There may also be $GABA_B$ receptors on the soma of the chandelier cell (AXO) at which 2-OH-S could act. There is no evidence from these experiments that 2-OH-S acts at the postsynaptic $GABA_B$ receptors like phaclofen, baclofen and CGP 36742.

CHAPTER SEVEN

7

General Discussion

The aim of this work was to study GABAergic inhibition mediated by pre- and postsynaptic $GABA_B$ receptors and to investigate the involvement of the two in the modulation of excitatory transmission in area CA1 of the hippocampal slice. Extracellular recordings of a population of cells, coupled with whole cell patch recordings of single cells, were successful in revealing the inhibition mediated by $GABA_A$ and $GABA_B$ receptors in this area. Investigations were carried out with 2-hydroxy-saclofen on the responses of single cells and a population of cells in order to understand the mechanisms of inhibitory control and to relate the two together The results that were obtained will be discussed below, in the context of physiological relevance of $GABA_B$-mediated inhibitory mechanisms in normal and pathological conditions.

Pre- and Postsynaptic $GABA_B$ Receptors: A Physiological Role in Hippocampal Function ?

The role of the presynaptic $GABA_B$ receptor has been studied more closely over the last few years and it is without doubt that they can have a major influence on synaptic processing. Activation of these receptors by the endogenous agonist GABA, or other agonists, such as baclofen, leads to the depression of both excitatory and inhibitory potentials, as a result of a decrease in transmitter release. Some doubt has been cast as to the importance of the $GABA_B$ autoreceptor on inhibitory terminals under normal physiological conditions (Waldmeier et al., 1988; Waldmeier,Wicki,Bittiger, & Baumann, 1992). However, other evidence indicates that the reduction in recurrent inhibition induced by tetanic stimulation can be reversed by $GABA_B$ antagonists (Mott et al., 1990) and indeed a number of studies including this present one, indicate the reduction of excitatory and inhibitory potentials, by a presynaptic mechanism (Dutar & Nicoll, 1988b; Davies et

al., 1990; Davies et al., 1991; Harrison, 1990; Otis et al., 1993; Otis & Mody, 1992; Thompson et al., 1992).

The reduction of the IPSP by activation of presynaptic $GABA_B$ receptors, has been well documented as providing an important physiological function for the presynaptic receptor in the induction and facilitation of LTP (Mott et al., 1990; Olpe & Karlsson, 1990; Burgard & Sarvey, 1991; Davies et al., 1991; Mott & Lewis, 1991; Davies & Collingridge, 1992; Mott et al., 1992; Mott & Lewis, 1992; Olpe et al., 1992). Activation of the presynaptic receptors results in a decrease in GABA release, a decrease in activation of the postsynaptic hyperpolarisation and subsequently a net increase in the NMDA current which enhances the induction of LTP. This provides an important point of control in the synaptic process of learning and memory and may be a possible target for therapeutic treatment in this area.

$GABA_B$ Mediated Depression of Excitation - Presynaptic Mechanisms

The depression of the EPSP/C is believed to result from activation of presynaptic $GABA_B$ receptors as mentioned above and in the preceding chapters. The results obtained in this study support this view. When the postsynaptic K^+ conductance was blocked with QX - 314, activation of $GABA_B$ receptors by baclofen and 2-OH-S led to a depression of the EPSC (Figure 6.16). This is assumed to be via activation of presynaptic receptors.

Recent work has shown that depression of the EPSC evoked by heterosynaptic paired pulse stimuli is maximal at 300ms (Isaacson et al., 1993). It was shown that underlying this depression is an increase in paired pulse facilitation of GABA release from inhibitory terminals which can be blocked by $GABA_B$ antagonists. The results obtained here with paired pulse stimulation of the population spike also indicated a depression at 300ms that could be blocked by phaclofen (Figure 3.4). In addition paired pulse depression of the EPSC revealed a depression at 300ms (Figure 6.21B). This was only apparent though after addition of bicuculline.

Isaacson et al suggest that a spillover of GABA from the inhibitory synapses onto the nearby excitatory terminals results in the depression of the EPSP/C. This mechanism is suggested to provide a physiological role for presynaptic $GABA_B$ receptors in the depression of the EPSP/C. The uptake mechanisms appear to play an important role in this process by regulating the diffuse inhibitory synaptic action of GABA. Block of the uptake mechanisms was seen to significantly enhance the inhibition of excitatory transmission and to enhance the slow IPSC. Other studies have also shown that block of GABA uptake enhances the size of the slow IPSC (Roepstorff & Lambert, 1992; Solis & Nicoll, 1992a;

Thompson & Gahwiler, 1992b). Under conditions of high frequency activation or excessive excitation these receptors may act to control the strength of excitatory transmission in the hippocampus.

The results in this study, indicate that the latency of the peak of the postsynaptic $GABA_B$ IPSC (175msec) is not the same as the latency of the peak of the late inhibition of the population spike or the paired pulse depression of the EPSC, both of which are maximal at 300msec. The time course of the depression of the initial slope of the field EPSP also peaked at 300msec, further support for a presynaptic effect. This is in agreement with the results of Isaacson et al (above) and further supports the role of presynaptic inhibition in the modulation of excitatory transmission.

The Effects Of 2-OH-S: Evidence For Partial Agonist Actions

2-OH-S depressed the postsynaptic EPSC (Figure 6.16), $IPSC_A$ (Figure 6.8) and $IPSC_B$ (Figure 6.2). This appeared to correlate both qualitatively and quantitatively, with the effects on the extracellular responses. 2-OH-S depressed the conditioning response, the early $GABA_A$ inhibition and the late $GABA_B$ inhibition (Figure 4.1). The time to peak of the early inhibition (approx. 30msec) measured after paired pulse stimulation reflected the time to peak of the $IPSC_A$. However, as mentioned above, the time to peak of the late inhibition (300msec) did not match the time to peak of the $IPSC_B$ (175msec). This indicates a clear difference between pre and postsynaptic inhibition which can be separated on the basis of their time course of action. Activation of the presynaptic receptor would appear to underlie the depression of the EPSC/population spike.

2-OH-S depressed the conditioning response, the slope of the field EPSP and the EPSC by similar amounts. The reduction in the size of the conditioning response and the slope could be reversed by phaclofen (Figure 4.6) and not bicuculline (Figure 4.5) supporting a direct activation of $GABA_B$ but not $GABA_A$ receptors. 2-OH-S depressed the EPSC (Figure 6.16) by a similar amount to that seen with the $GABA_B$ agonist baclofen. This was assumed to be a presynaptic effect as the postsynaptic K^+ conductance was blocked, further evidence to support a role for the presynaptic receptor in the depression of excitation.

The depression of the early inhibition and the $IPSC_A$ by 2-OH-S, probably results from activation of $GABA_B$ receptors on the terminals of interneurones located in the stratum pyramidale, or oriens/alveus. Activation of presynaptic $GABA_B$ receptors would lead to a

decrease in the amount of GABA released and a reduction of the postsynaptic $GABA_A$ response (Nathan et al., 1990a; Nathan & Lambert, 1991). It is unlikely that the same effect could be achieved by an action on postsynaptic $GABA_B$ receptors. The results obtained with bicuculline on the conditioning response show there is no evidence for a direct effect of 2-OH-S on the postsynaptic $GABA_A$ receptor. It would appear that under extracellular conditions, the reduction in the early inhibition was also due in part to the depression of the excitatory drive and subsequent feedback inhibition (Figure 4.4). The size of the test response at early intervals appeared to be enhanced with respect to controls (Figure 4.4), suggesting a block of $GABA_A$ inhibition. In comparison to these actions the selective $GABA_B$ antagonists, phaclofen and CGP 36742, had no effect on the early inhibition or the amplitude of the $IPSC_A$ respectively (Figure 3.4, Figure 6.8).

The depression of the late inhibition and the $IPSC_B$, could also result from activation of presynaptic $GABA_B$ receptors, to reduce GABA release from the terminals of the interneurones in stratum lacunosum/moleculare. It is also possible though, that either of these components could be reduced by an antagonistic action at the postsynaptic $GABA_B$ receptor site. However, we could find no evidence for the activation of an outward conductance by 2-OH-S on the postsynaptic membrane. This is in contrast to baclofen which does activate an outward conductance (Newberry & Nicoll, 1984a; Gahwiler & Brown, 1985; Dutar & Nicoll, 1988b; Gage, 1992). As mentioned in chapter 6 it would have been helpful to show the conductance change with baclofen in the absence of QX-314. The reduction in the conditioning response under extracellular conditions, could contribute to the reduction in the late inhibition. Indeed, the amplitude of the test response at late intervals appeared to be depressed compared to controls (Figure 4.4), although one would expect it to be enhanced if there was a block of the postsynaptic receptors.

2-OH-S, in addition to its effects on paired pulse inhibition, enhanced the paired pulse facilitation of the population spike and the slope of the field EPSP (Figure 4.2). This may reflect an underlying depression of the inhibitory response. The effects of phaclofen were opposite to those of 2-OH-S on the same parameters, while bicuculline appeared to have no direct effect on either of them.

These results, when viewed as a whole, can be quite logically explained by 2-OH-S acting as a partial agonist at presynaptic $GABA_B$ receptors. This would explain why some groups have found that 2-OH-S can antagonise the actions of baclofen and GABA. As a partial agonist, on its own, acting on electrically evoked potentials, it would exert a predominately agonistic effect. In the presence of a true agonist, it would appear to exert weak

antagonistic effects. Contamination of the compound does not seem likely due to the consistency of the results and the lack of effect in activating a postsynaptic conductance.

From this study, it does not appear that 2-OH-S exerts any agonistic effects on postsynaptic receptors (chapter 6). Although it may be the case that 2-OH-S is selective only for the presynaptic receptors, we cannot categorically prove this from these experiments. It may be that 2-OH-S exerts antagonist actions postsynaptically at a different subtype. Further advances will be possible when selective pre- and postsynaptic receptor antagonists become available. What is apparent however, is that it appears to act on presynaptic receptors on both excitatory and inhibitory terminals and as such cannot be used as a tool to separate their effects. The elucidation of the molecular structure of the pre and postsynaptic GABAB receptor should provide us with more information as to whether they are indeed structurally distinct and more than one type of GABAB receptor can be invoked.

$GABA_B$ receptors appear to be capable of exerting both inhibitory and excitatory effects by means of postsynaptic inhibition and presynaptic inhibition and as such provide a number of sites for modulation of hippocampal excitability. This also allows for a number of sites at which to modify this activity and as such may confer a degree of therapeutic potential of these receptors.

Therapeutic Exploitation of $GABA_B$ Receptors

The role of GABAB receptors in a number of disorders are dealt with in two recent reviews (Wojcik & Holpainen, 1992; Bowery, 1993)and some of these are highlighted here.

There are a number of areas in which $GABA_B$ receptors can be exploited pharmacologically, to enhance or depress inhibitory efficacy. Extensive work has been carried out studying the involvement of these receptors in convulsive epilepsies (Prince, Deiz, Thompson, & Chagnac-Amitai, 1992). $GABA_A$ receptors are constantly being activated and any treatment aimed at them may effect not only the desired area, but a number of other normal synaptic processes as well. $GABA_B$ receptors, which may not be subjected to such a major endogenous activation, may produce less side effects for therapeutic use.

In non-convulsive or absence epilepsy models, $GABA_B$ receptor antagonism can reduce the spike-wave discharge that is characteristic of this syndrome (Hosford, Clark, Cao, Wilson, & Lin, 1992; Marescaux, Vergnes, & Bernasconi, 1992; Bernasconi, Lauber,

Marescaux, Vergnes, Martin, Rubio, et al., 1992). Although it has been suggested that the $GABA_B$ receptors are responsible for this syndrome, the underlying mechanisms have still to be elucidated. The increasing availability of new, potent $GABA_B$ receptor antagonists (Olpe et al., 1993) will hopefully be of use in clinical treatment of this condition.

Excessive glutamate can damage neurones and is involved in several neurological disorders (Choi, 1988). Depression of glutamate release by activation of presynaptic $GABA_B$ receptors may provide a promising target for a neuroprotective role by correcting the imbalance between GABA and glutamate

Although activation of presynaptic $GABA_B$ receptors has a role in the induction of LTP, the facilitation of LTP by $GABA_B$ antagonists (Olpe & Karlsson, 1990) leads to the question of a possible target for cognitive improvement. Baclofen has been shown to decrease memory acquisition and retention in rats (Schwarzwelder,Tilson,McLamb, & Wilson, 1987) and it has recently been demonstrated that $GABA_B$ antagonism can improve learning and memory storage in mice, rats and monkeys (Mondadori,Preiswerk, & Jaekel, 1992). Clearly, the nature of the preparation studied and frequency of activation may determine the exact nature of the involvement of $GABA_B$ receptors.

Conclusion

In summary, this work has reinforced the idea of the importance of $GABA_B$ receptors in synaptic processing and shown that the depression in excitatory transmission due to activation of presynaptic receptors on excitatory terminals has a more important role than had previously been thought. The reduced excitability of pyramidal cells due to the postsynaptic $GABA_B$ mediated IPSC precedes the maximal paired pulse depression seen in population responses. The latter correlates well with EPSC depression.

Although there is no solid evidence for a pharmacological difference between the pre and postsynaptic GABAB receptors, there is evidence to suggest that their transduction mechanisms differ (chapter 6). The results here show that there is also a quantitative difference between the pre and postsynaptic inhibition mediated by $GABA_B$ receptors. It would appear that the activation of the postsynaptic $GABA_A$ and $GABA_B$ receptors modulates excitatory transmission by preventing repetitive firing of the pyramidal cells. The delay in presynaptic inhibition is probably a result of the activation by GABA which has diffused across the synapse after being released from inhibitory interneurones. It appears that the presynaptic receptor plays a physiological role in the depression of the EPSC which

underlies the depression of the population response. The actions of 2-OH-S appear to be primarily mediated through partial agonist actions at the presynaptic receptors in these experiments and although no evidence was seen for a postsynaptic effect, this cannot be ruled out. Further advances require specific probes to separate pre and post effects at different sites before a more precise picture can be built up of the role in synaptic function in the CNS.

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