ON THE MECHANISMS OF CEREBELLAR SYNAPTIC PLASTICITY

SONJA JACOBY

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Changes in the strength of signalling between neurones are thought to provide a cellular substrate for learning and memory. In the cerebellar cortex, raising the frequency and the strength of parallel fibre (PF) stimulation leads to a long-term depression (LTD) of the strength of signalling at the synapse between PFs and Purkinje cells (PCs), which spreads to distant synapses to the same cell via a nitric oxide (NO) dependent mechanism (Hartell, 1996). At the same synapse, but under conditions of reduced post-synaptic calcium activity, raised frequency stimulation (RFS) of PFs triggers a long-term potentiation of synaptic transmission (Salin *et al.*, 1996). The aims of the work described in this thesis were to investigate the conditions necessary for LTD and LTP at this synapse following RFS and to identify the origins and second messenger cascades involved in the induction and spread of LTP and LTD.

In thin, parasagittal cerebellar slices whole cell patch clamp recordings were made from PCs and the effects of RFS of one of two, independent PF inputs to the same PC were examined under a range of experimental conditions. Under conditions designed to reduce post-synaptic calcium activity, RFS to a single PF input led to LTP and a decrease in paired pulse facilitation (PPF) in both pathways. This heterosynaptic potentiation was prevented by inhibition of protein kinase A (PKA) or by inhibition of NO synthase with either 7-nitroindazole (7-NI) or N^G Nitro-L-argenine methyl ester. Inhibition of guanylate cyclase (GC) or protein kinase G (PKG) had no effect.

A similar potentiation was observed upon application of the adenylyl cyclase (AC) activator forskolin or the NO donor spermine NONOate. Both of these treatments also resulted in an increase in the frequency of mEPSCs, which provides further evidence for a presynaptic origin of LTP. Forskolin induced potentiation and the increase in mEPSC frequency were blocked by 7-NI. The styryl dye FM1-43, a fluorescent reporter of endo- and exocytosis, was also used to further examine the possible pre-synaptic origins of LTP. RFS or forskolin application enhanced FM1-43 de-staining and NOS inhibitors blocked this effect. Application of NONOate also enhanced FM1-43 de-staining.

When post-synaptic calcium activity was less strictly buffered, RFS to a single PF input led to a transient potentiation that was succeeded by LTD in both pathways. This LTD, which resembled previously described forms, was prevented by inhibition of the NO/cGMP/PKG cascade. Modification of the AC/cAMP/PKA cascade had no effect.

In summary, the direction of synaptic plasticity at the PF-PC synapse in response to RFS depends largely on the level of post-synaptic calcium activity. LTP and LTD were non-input specific and both forms of plasticity were dependent on NOS activity. Induction of LTP was mediated by a presynaptic mechanism and depended on NO and cAMP production. LTD on the other hand was a post-synaptic process and required activity of the NO/cGMP/PKG signalling cascade.

Hartell, N. A. (1996). Strong activation of parallel fibers produces localized calcium transients and a form of LTD that spreads to distant synapses. *Neuron* **16**, 601-610.

Salin, P. A., Malenka, R. C., & Nicoll, R. A. (1996). Cyclic-AMP mediates a presynaptic form of LTP at cerebellar parallel fiber synapses. *Neuron* **16**, 797-803.

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ABBREVIATIONS

AC	Adenylate cyclase
AC1	Adenylyl cyclase, type I
AC8	Adenylyl cyclase, type VIII
ACSF	Artificial cerebrospinal fluid
AMPA	α-Amino-3hydroxyl-5-methyl-4-isoxazole-4-propionic acid
ATP	Adenosine 5'-triphosphate
BAPTA	Bis(2-aminophenoxy)ethane-N,N,N'N'-tetraacetate
BC	Basket cell
cAMP	Cyclic adenosine monophosphate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
cGMP	Cyclic guanosine monophosphate
CF	Climbing fibre
CICR	Calcium induced calcium release
CR	Conditioned response
CREB	cAMP – responsive transcription factor
CRF	Corticotropin releasing factor
CS	Conditioned stimulus
DAG	Diacylglyerol
DMSO	Dimethylsulphoxide
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N'N'-tetraacetic acid
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential

FM1-43	Pyridinium, 4-[2-[4-(dibutylamino)phenyl]ethenyl]-1-[3-(triethylammonio)propyl]-dibromide
GABA	γ-aminobutyric acid
GC	Guanylate cyclase
GFAP	Glial fibrially acidic protein
5'-GMP	Guanosine-5'-monophosphate
G-protein	Guanine-nucleotide binding protein
GTP	Guanosine triphosphate
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
IPSC	Inhibitory postsynaptic current
IP_3	Inositol-1,4,5,-triphosphate
L-NAME	N^G-Nitro-L- arginine methyl ester
L-NARG	N^G-Nitro-L- arginine
LTD	Long-term depression
LTP	Long-term potentiation
L-LTP	Late phase of LTP
mEPSC	Miniature excitatory postsynaptic current
mGluR	Metabotropic glutamate receptor
7-NI	7-nitro-indazole
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
ODQ	H-[1,2,4]oxadiazolo[4,3a] quinoxaline-1-one
PC	Purkinje cell

PDE	Phosphodiesterase
PF	Parallel fibre
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA_2	Phospholopase A2
PLC	Phospholopase C
PPF	Paired pulse facilitation
PPR	Paired pulse ratio
PTK	Protein tyrosine kinase
ROI	Region of interest
RFS	Raised frequency stimulation
SC	Stellate cell
STP	Short-term potentiation
tPA	Tissue plasminogen activator
TTX	Tetrodotoxin
UR	Unconditioned response
US	Unconditioned stimulus
VOR	Vestibular ocular reflex

CHAPTER 1

INTRODUCTION

1.1 THE FUNCTION AND GROSS ANATOMY OF THE CEREBELLUM

The cerebellum is important in the maintenance of balance, the coordination of movement and it influences posture and muscle tone. The cerebellum, which comprises 10% of the brain, is a part of the hindbrain that is not a part of the brainstem (Sutton, 1971). Although the cerebellum contains sensory and motor components it is not necessary for perception or muscle movement. The cerebellum acts at an unconscious level and operates by indirectly adjusting the output of the major descending motor system of the brain by comparing and compensating for mismatches between intention and performance. The function of the cerebellum is changed by experience and is therefore important for learning of motor tasks.

The cerebellum is connected to the underlying pons via a pair of thick cerebellar penduncles. The peduncles consist of thousands of fibres, the axons of neurones that send information into or out of the cerebellum. Figure 1.1 illustrates the location and the shape of the cerebellum in medial (Figure 1.1.A) and lateral (Figure 1.1.B) view. Two longitudinal furrows divide the cerebellum into three regions; the midline zone called the vermis and lateral on either side the left and right cerebellar hemispheres (Figure 1.1.C) (Sutton, 1971). Each hemisphere is composed of an intermediate and lateral part. There are also two deep transverse fissures one of which, the primary fissure, divides the cerebellum into anterior and posterior lobes. The second fissure called the horizontal fissure lies in the posterior lobe (Figure 1.1.C).

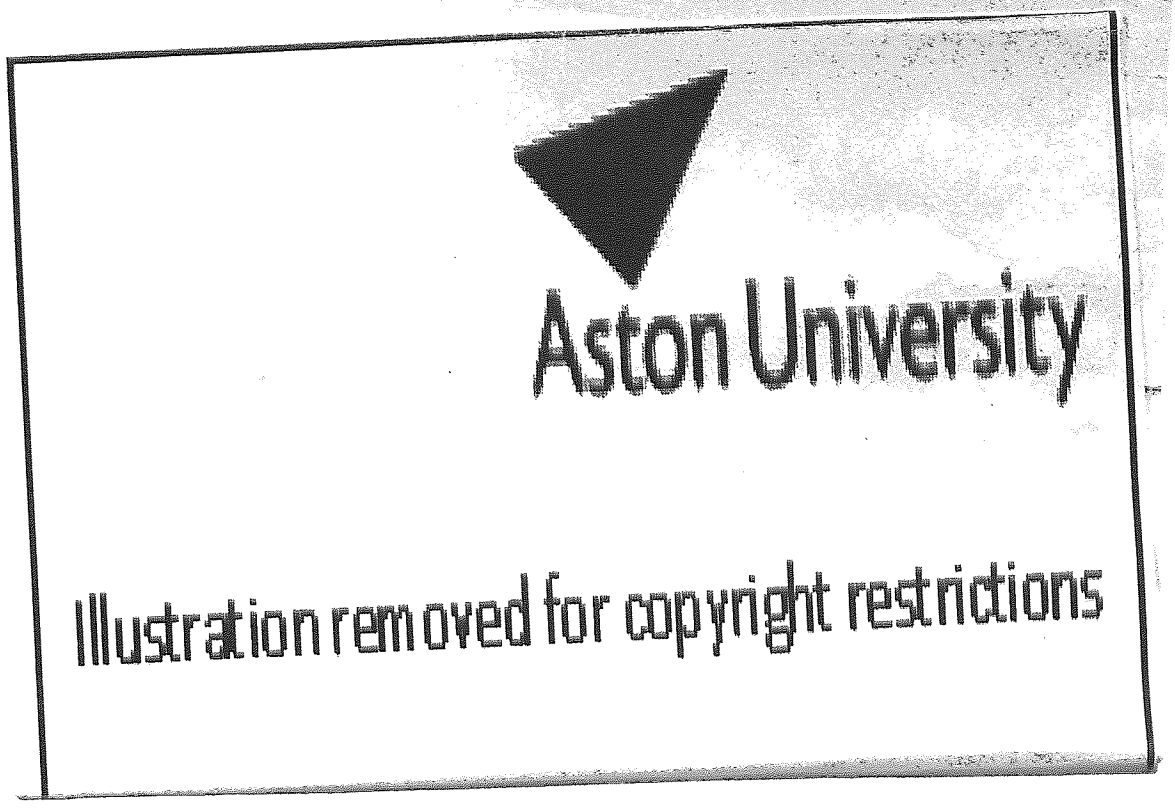


Figure 1.1 The cerebellum is pictured in medial (A) and lateral (B) view. A portion of the occipital cortex that lies above the cerebellum has been removed in order to see the dorsal aspect of the cerebellum (C) (Adapted from "The global cerebellum'97 by John Harting.)

The vermis and each part of the two hemispheres have outputs to different deep cerebellar nuclei and to different components of the descending system. The vermis projects to the fastigial nucleus which in turn projects to cortical and brain stem regions that give rise to the medial descending system which controls proximal muscles. The intermediate zone of both hemispheres projects via the interposed nucleus to the cortical and the brain steam regions that give rise to the lateral descending system through which the distal limb muscles are controlled. The lateral zones project to the dentate nucleus, which connects with the motor cortex (for review see Voogd, 1992; Voogd & Glickstein, 1998).

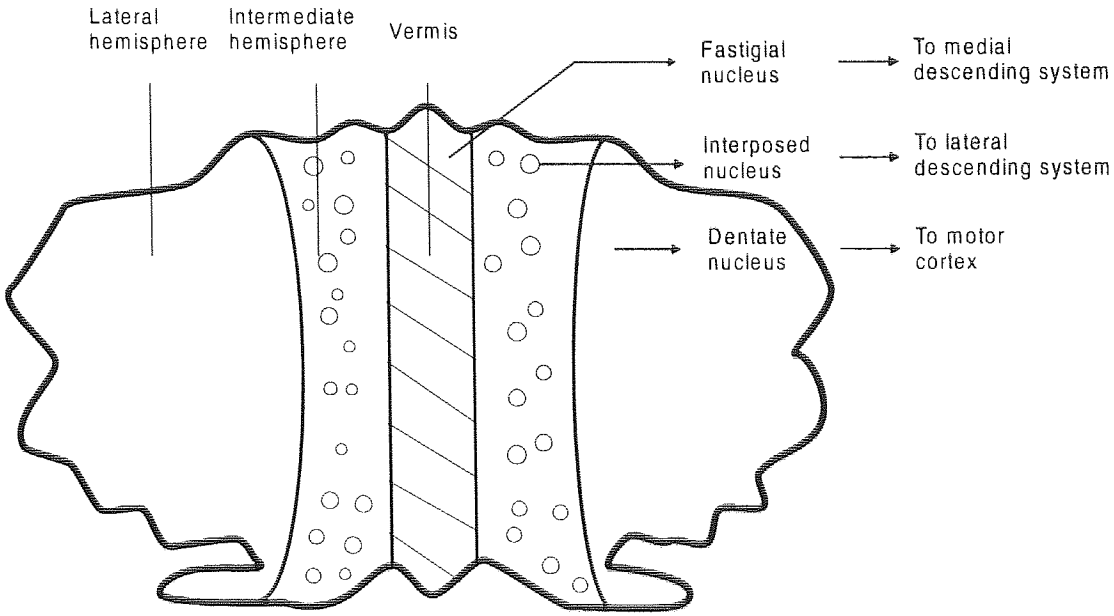


Figure 1.2 The three distinct regions of the cerebellum have different outputs.

1.2 The Cellular Structure Of The Cerebellum

The cerebellar cortex is divided into three layers, which contain five different types of neurones. The granule cell layer, which is the most inner layer, is composed of small, round cells. The granule cells are the most numerous elements in the cerebellar cortex and their axons run outwards to the molecular layer where they bifurcate and give rise to parallel fibres (PFs) (Sutton, 1971). Besides granule cells there are also a few larger Golgi cells at the outer border of the granule cell layer (Voogd & Glickstein, 1998). The second layer consists of Purkinje cells, which are arranged side by side in a row. Purkinje cells are large, inhibitory neurones, which serve as the sole output from the cerebellar cortex. Their extensive dendritic tree extends in the outer molecular layer where they make synaptic contacts with PFs. Each Purkinje cell receives inputs from more than 150,000 PFs (Eccles *et al.*, 1967). The molecular layer also contains stellate and basket cells, which function as inhibitory interneurones (for review see Ross *et al.*, 1990; Sutton, 1971; Voogd & Glickstein, 1998).

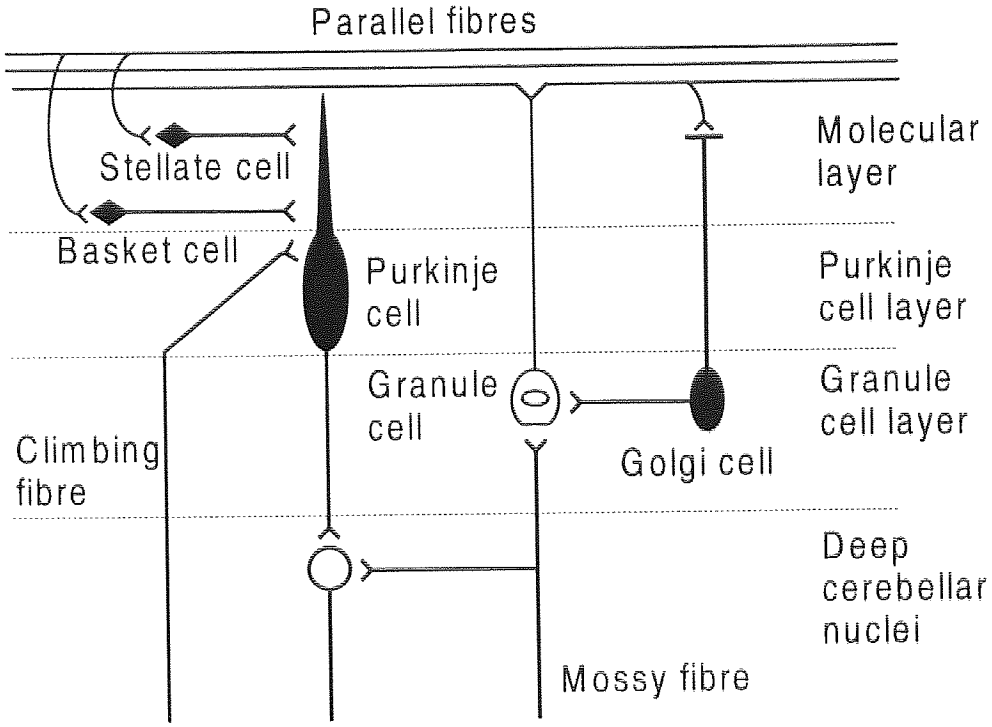


Figure 1.3 The inputs and outputs to and from the cerebellar cortex.

The cerebellar cortex receives excitatory, glutamatergic input from two sources. One of which is from the mossy fibres, which originate in the spinal cord and the brainstem nuclei and form excitatory synaptic connections with granule and Golgi cells (Eccles *et al.*, 1967). The climbing fibres (CFs), the other excitatory input, originate from the inferior olivary nucleus. Their axons enter the cortex where they make contact directly with Purkinje cell dendrites. Each CF contacts only one Purkinje neurone, and each Purkinje cell receives synaptic input from only a single CF (Eccles *et al.*, 1967; and for review see Ross *et al.*, 1990; Voogd & Glickstein, 1998). Both the mossy fibres and the CFs also make excitatory contacts with cells in the deep cerebellar nuclei. The inhibitory Purkinje cells, which serve as the sole output from the cerebellar cortex, can then modulate the ongoing activity.

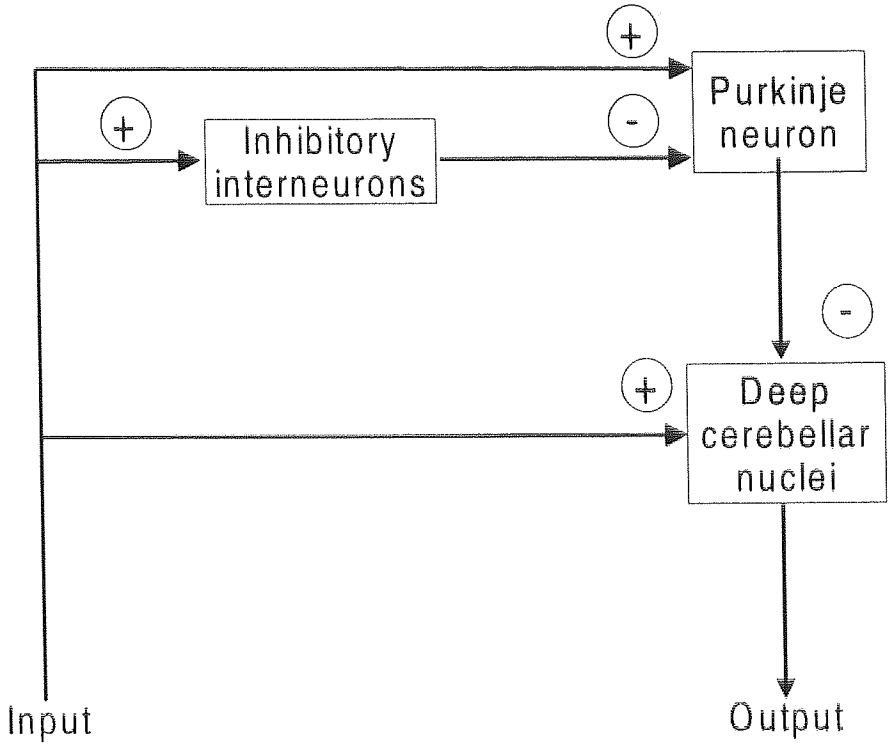


Figure 1.4 The primary cerebellar circuit is formed by excitatory inputs, which directly activate the deep cerebellar nuclei. The inhibitory Purkinje cells then modulate the activity of the primary circuit.

1.3 Cerebellar Long–Term Depression

A role for the cerebellum in learning of motor skills was first suggest in a report by David Marr in which he proposed that the CF input on a specific PC acts to increase synaptic transmission between PFs and the PC for a prolonged period of time (Marr, 1969). James Albus then suggested that the CF input reduces synaptic transmission at the PF–PC synapse, thereby acting as an error signal to correct mismatches between intended and actual movement (Albus, 1971). A study by Ito and co-workers later demonstrated experimentally that repetitive and simultaneous activation of CFs and PFs leads to a long–term depression (LTD) in the strength of transmission from PFs to PCs (Ito *et al.*, 1982). LTD is therefore considered to be associative since it requires activation of PFs and CFs within a similar time frame (Chen & Thompson, 1995;

Karachot *et al.*, 1995; Schreurs *et al.*, 1996) and input specific as only specifically activated PFs undergo depression (Ekerot & Kano, 1985).

1.3.1 THE CELLULAR MECHANISM UNDERLYING LTD

Three basic requirements are thought to be necessary for the induction of conventional LTD. Firstly, post-synaptic calcium influx via voltage-gated calcium channels is required (Sakurai, 1990; Linden & Connor, 1991; Shibuki & Okada, 1992). Physiologically this element is thought to be met through activity of CFs (Konnerth *et al.*, 1992; Ross & Werman, 1987). It has been shown that CF activation can be replaced by depolarisation of the post-synaptic PC (Crepel & Krupa, 1988; Hirano, 1990a; Glaum *et al.*, 1992) or release of caged calcium (Kasono & Hirano, 1994). Introduction of the calcium chelators EGTA or BAPTA in the post-synaptic PC prevents the induction of LTD (Sakurai, 1990; Linden *et al.*, 1991).

The second requirement is activation of AMPA receptors (Linden *et al.*, 1993; Hemart *et al.*, 1995), which mediate fast transmission at the PF-PC synapse (Konnerth *et al.*, 1990). Third is activation of metabotropic glutamate receptors (mGluR)(Linden *et al.*, 1991; Hartell, 1994; Shigemoto *et al.*, 1994; Conquet *et al.*, 1994) both of which are activated via the PF input (Konnerth *et al.*, 1990; Batchelor *et al.*, 1994). In culture, PCs PF stimulation can be replaced by application of glutamate or quisqualate, an agonist of AMPA and mGluRs, whereas the application of AMPA receptor agonists alone is ineffective (Linden *et al.*, 1991). Several lines of evidence suggest that in cerebellar slices as well as in cultured Purkinje cells activation of both AMPA receptors and mGluRs is deemed necessary for the induction of LTD. Application of the AMPA receptor antagonist CNQX prevents LTD induced by pairing PF stimulation and

Purkinje cell depolarisation in cerebellar slices (Hemart *et al.*, 1995) or induced by conjunctive stimulation with quisqualate and PC depolarisation in cultured Purkinje cells (Linden *et al.*, 1991). Application of inactivating antibodies against mGluR1 blocks LTD induced by conjunctive stimulation using glutamate and depolarisation in cultured Purkinje cells (Shigemoto *et al.*, 1994). Moreover, in cerebellar slices of knockout mice lacking functional mGluR1 LTD induced by PF stimulation and cell depolarisation is significantly reduced (Conquet *et al.*, 1994; Aiba *et al.*, 1994a).

1.3.2 Activation of PKC is required for the induction of LTD

Activation of mGluR1s, which link to G-protein and regulate phospholipase C results in the production of 1,2 diacyglycerol (DAG) and inositiol-1,4,5,-trisphosphate (IP3) (Schoepp & Conn, 1993; Nakanishi, 1994). The latter has been suggested to release calcium from internal, IP3 sensitive stores (Aramori & Nakanishi, 1992). This calcium induced calcium release may be regulated by protein tyrosine kinase (Jayaraman *et al.*, 1996). DAG in combination with sufficiently increased intracellular calcium levels activates protein kinase C (PKC) (Nakanishi, 1994). It has recently been suggested that CFs also contribute to PKC activation via the release of the peptide corticotropin releasing factor (CRF) (Miyata *et al.*, 1999). This additional contribution of CFs may explain why depolarisation is not, as first thought, quite as effective in inducing LTD (Reynolds & Hartell, 2000). Inhibition of PKC blocks LTD induced by pairing PF stimulation with PC depolarisation (Crepel & Jaillard, 1990; Hemart *et al.*, 1995) or CF activation (Hartell, 1994) in cerebellar slices as well as LTD induced by conjunctive application of glutamate and depolarisation of cultured PCs (Linden & Connor, 1991). Moreover, application of PKC-activating phorbol esters depresses PC responses to exogenous glutamate or quisqualate in slices and in cultured preparations (Crepel &

Krupa, 1988; Linden & Connor, 1991). These findings demonstrate PKC involvement in the induction of LTD. PKC may reduce the post-synaptic sensitivity of AMPA receptors to glutamate either via phosphorylating the receptors (Nakazawa *et al.*, 1995) or, as recently suggested, by receptor internalisation (Wang & Linden, 2000).

1.3.3 The involvement of NO in cerebellar LTD

A role for the nitric oxide (NO)/guanylate cyclase (GC)/cyclic 3,5-guanosine monophosphate (cGMP)/ protein kinase G (PKG) pathway in the induction of cerebellar LTD is suggested by numerous studies. NO is produced by calcium/calmodulin dependent nitric oxide synthase (NOS) during the conversion from L-arginine to citrulline. NOS has been found in granule cells, basket cells and Bergman glial cells (Bredt *et al.*, 1990; Southam *et al.*, 1992) but it has not been identified in PCs (Crepel *et al.*, 1994). This suggests that the diffusible messenger NO must then diffuse into the post-synaptic PC and activate GC thereby producing cGMP (Daniel *et al.*, 1993; Boxall & Garthwaite, 1996), which in turn activates PKG (Ito & Karachot, 1992; Hartell, 1994), which is particularly abundant in PCs (Lohmann *et al.*, 1981). In cerebellar slices induction of LTD is prevented by application of NOS inhibitors (Crepel & Jaillard, 1990; Shibuki & Okada, 1991; Daniel *et al.*, 1993). Moreover, application of NO donors or membrane permeable cGMP analogues induce LTD (Daniel *et al.*, 1993; Ito & Karachot, 1990; Blond *et al.*, 1997). LTD is also induced by direct dialysis of cGMP in the PC (Daniel *et al.*, 1993; Hartell, 1994) or by the release of caged NO from the PC (Lev Ram *et al.*, 1995). While these findings are consistent with a role for NO in the induction of LTD, studies carried out in cultures do not support the involvement of NO or PKG in the induction of LTD (Linden & Connor, 1992; Linden *et al.*, 1995). This controversy may be explained by the low number of potential NO sources such as PFs

or basket cells in cultured preparations. However, application of NO donor fails to increase cGMP levels in PCs in slices (DeVente *et al.*, 1990; Southam *et al.*, 1992). Whilst the contribution of NO in the induction of cerebellar LTD is now widely accepted, the way in which activation of PKG results in a decrease of PF induced glutamate currents is not entirely clear. Ito and co-workers suggested that activation of PKG leads to phosphorylation of it's specific endogenous substrate (G-substrate) thereby inhibiting protein phosphatase activity and hence reduced sensitivity of AMPA receptors (Ito & Karachot, 1992), a process that is also thought to be regulated by the actions of PKC. This hypothesis is supported by findings that application of the protein phosphatase inhibitor calyculin A induces LTD (Ajima & Ito, 1995). Alternatively, cGMP may stimulate the production of cyclic ADP-ribose (cADP-ribose) and thereby trigger the release of calcium from ryanodine receptor sensitive calcium stores (Galione *et al.*, 1993). This increased level of calcium could then induce or facilitate LTD induction (Berridge, 1993).

1.3.4 NON-INPUT SPECIFIC LTD

Recent studies demonstrate that LTD induced by either PF stimulation in combination with PC depolarisation (Reynolds & Hartell, 2000; Wang *et al.*, 2000) or by pairing PF and CF activation (Reynolds & Hartell, 2000) is not restricted to the site of activation but spreads to distant synapses. NO, which is produced by molecular layer stimulation at sufficient intensities, is, due to its ability to diffuse over large distances, able to involve distant synapses and reduce the sensitivity of AMPA receptors to glutamate. Although these findings seem to contradict earlier observations, which demonstrate that cerebellar LTD is input specific at the cellular level (Linden, 1994), these earlier studies

were carried out in cultured preparations where the induction of LTD is independent of the NO/cGMP/PKG cascade.

PF stimulation at raised frequency and intensity can, on its own, induce LTD (Hartell, 1996a). Stimulation of enough PFs at sufficient rates leads to a localised calcium influx within spiny dendritic branchlets via activation of voltage-gated calcium channels (Hartell, 1996a; Eilers *et al.*, 1995). Recent studies suggest that IP3 mediated calcium release from internal calcium stores may also contribute to the localised calcium increase that follows raised frequency PF stimulation (Takechi *et al.*, 1998; Finch & Augustine, 1998). These observations indicate that raised frequency and intensity PF stimulation can fulfil all three requirements for induction of LTD namely elevation of calcium, activation of AMPA receptors and activation of mGluRs. PF induced LTD (LTD_{PF}) is not restricted to the site of induction but spreads to distant synapses outside the region of calcium elevation (Hartell, 1996a). The underlying mechanism of LTD_{PF} at the heterosynaptic site varies from depression at the homosynaptic site since it's induction is less sensitive to post-synaptic calcium and unlike LTD at the homosynaptic site, requires activation of NOS and PKG. Whilst the actions of PKC seem to lead to a reduction in the sensitivity of AMPA receptors to glutamate at the stimulation site, activation of PKG seems to be responsible at the distant site (Hartell, 1996b; Hartell, 2000).

1.3.5 THE SOURCE OF NO RELEASE

High frequency stimulation of the molecular layer not only leads to NO release (Shibuki & Kimura, 1997) but it can also potentiate further NO release (Kimura *et al.*, 1998). Together with findings that PF stimulation on it's own is able to induce NO dependent

LTD (Hartell, 1996b) and that PF stimulation can be replaced by application of NO (Lev Ram *et al.*, 1995) PFs, whose cell bodies contain NOS (Bredt *et al.*, 1990) seem to be the most likely source of NO. Other reports, however, assign NO release to the CF (Shibuki & Okada, 1991) or to inhibitory interneurones with Bergman glia, stellate cells and basket cells being the most likely sources (Bredt *et al.*, 1991; Southam *et al.*, 1992).

1.4 Role Of Cerebellar LTD In Motor Learning

It has been suggested that certain forms of motor learning such as adaptation of the vestibulo–ocular reflex and associative eye-blink conditioning require plasticity at the PF-PC synapse (Albus, 1971; Ito, 1982; for review see Thach, 1998).

1.4.1 The Eye-blink Reflex

The principles of classical conditioning of the eyelid response derived from Pavlov's observations with his dogs. If a stimulus (unconditioned stimulus; US), like a puff of air, which triggers an eye blink, is repeatedly paired with a neutral stimulus (conditioned stimulus; CS) such as a tone, then the neutral stimulus applied on its own triggers an eye blink. Stimulation of PFs represent the conditioned stimulus while CF activation can substitute for the unconditioned stimulus (for a review see Yeo & Hesslow, 1998). After repeated pairing of conditioned and unconditioned stimuli, the conditioned stimuli decreases the firing rate of PCs and thereby, via dis-inhibition, increases the firing rate of the deep cerebellar nuclei (Thompson, 1986). Thus, LTD was suggested as a mechanism for this sort of motor learning (Thompson & Krupa, 1994; Chen & Thompson, 1995). Several studies have shown a correlation between LTD and motor learning. Knockout mice lacking mGluR1 do not express cerebellar LTD and show an impaired ability to acquire the eye-blink reflex (Aiba *et al.*, 1994b).

Furthermore, in mutant mice lacking glial fibrillary acidic protein (GFAP) LTD induced by conjunctive activation of PFs and CFs is clearly deficient and the eyeblink reflex is weakened (Shibuki *et al.*, 1996). However, the interpretation of these studies is controversial since knockout mice sometimes show a developmental phenotype (Chen *et al.*, 1995; Kashiwabuchi *et al.*, 1995) and since the gene is deleted in all cells of the body behavioural changes are difficult to assign to dysfunction in a particular structure or cell type (for a detailed discussion see DeZeeuw *et al.*, 1998).

1.4.2 The Vestibulo–Ocular Reflex

The vestibulo–ocular reflex (VOR) maintains the orientation of the eyes on a fixed target when the head is rotated. Reflex motion of the head in one direction is sensed by the vestibular labyrinth, which initiates eye movements in the opposite direction to maintain the image in the same position on the retina. If a visual stimulus moves exactly with the rotation of the head, therefore not requiring the VOR, then subsequent experiments carried out in darkness show a reduction in the amplitude of the VOR. Whereas, moving the visual stimulus in the opposite direction to the head movements causes the VOR to increase in subsequent trials. Therefore, the VOR has been proposed as a good model to examine the process of motor learning (Ito, 1982; Ito, 1989). Several studies suggest that one site of motor learning for the VOR is the PF–PC synapse and the mechanism is LTD-driven by a teaching signal from the CF (Mauk & Donegan, 1997; Raymond, 1998). Various approaches have been taken to establish the role of LTD in VOR learning. The strongest evidence that LTD is indeed required for VOR learning has been provided by a recent study in which transgenic mice, which selectively express an inhibitor of PKCγ in PCs, were used. These transgenic animals

lack both cerebellar LTD and adaptation to VOR (DeZeeuw *et al.*, 1998) suggesting a role for LTD in adaptation to VOR.

1.5 Long–Term Potentiation

Long-term potentiation (LTP), which is one of the most widely studied forms of synaptic plasticity, was first discovered in the hippocampus. The hippocampus is important for certain forms of spatial learning and is the brain region where short–term memory is transferred into long–term memory. Two distinct forms of LTP have been identified in the hippocampus (Nicoll & Malenka, 1995) the N-methyl-D-aspartate receptor (NMDAR) dependent form has been found at CA3-CA1 synapse (for review see Bliss & Collingridge, 1993; Malenka & Nicoll, 1993) as well as at various excitatory synapses throughout the brain. The second form occurs at synapses formed by mossy fibres to CA3 neurones and is entirely independent of NMDA receptors (Harris & Cotman, 1986; Zalutsky & Nicoll, 1990; Weisskopf *et al.*, 1994). Furthermore, a form of LTP has also been found at cerebellar PF-PC synapses that resembles hippocampal LTP at the mossy fibre- CA3 synapse (Shibuki & Okada, 1992; Salin *et al.*, 1996).

1.5.1 LTP In The Hippocampal CA1 Region

In the CA1 region LTP is composed of two distinct temporal phases, a short induction phase lasting seconds and a long expression phase lasting for hours. High frequency stimulation to presynaptic axons causes an increase in calcium entry into post-synaptic neurones through NMDA receptors (Lynch *et al.*, 1983; Malenka *et al.*, 1988). This produces strong depolarisation in the post-synaptic neurone (Malinow & Miller, 1986) and relieves the voltage dependent magnesium block of NMDA receptor-ion channels

thereby increasing intracellular calcium, which is critical for the induction of LTP (Arancio *et al.*, 1995). Alternatively LTP can be produced by weak presynaptic stimulation in combination with post-synaptic cell depolarisation (for review see Schuman & Madison, 1994b). Much evidence indicates that the induction phase of LTP at the CA1 region is mediated via a post-synaptic mechanism. The expression phase however, seems to partially involve a presynaptic increase in transmitter release (Hawkins *et al.*, 1993; Bliss & Collingridge, 1993), suggesting the involvement of a retrograde messenger that is released post-synaptically and targets presynaptic terminals (Bliss & Collingridge, 1993). NO has been suggested as a possible candidate for such a retrograde messenger in various reports (Bohme *et al.*, 1991; Odell *et al.*, 1991; Schuman & Madison, 1991; Haley *et al.*, 1992) but its role is controversial (Hawkins, 1996; Bliss & Collingridge, 1993). Application of caged NO in combination with weak stimulation induces LTP in some (Arancio *et al.*, 1996) but not all cases (Boulton *et al.*, 1994). Inhibition of NOS prevented tetanus induced LTP and this inhibition could be reversed by application of L-arginine (Schuman & Madison, 1991; Bohme *et al.*, 1991; Odell *et al.*, 1991; Haley *et al.*, 1992). However, in other studies application of NOS inhibitors failed to block LTP induced by either weak (Gribkoff & Bauman, 1992) or strong stimulation (Musleh *et al.*, 1993). Williams and co-workers reported that inhibitors of NOS are only capable of blocking LTP if experiments are carried out at room temperature (Williams *et al.*, 1993). Several lines of evidence suggest that NO is produced at the post-synaptic neurone but acts at presynaptic terminals. Firstly, the neuronal and endothelial isoform of NOS have been found in CA1 pyramidal neurones (Dinerman *et al.*, 1994). LTP is reduced in knock-out mice that lack both endothelial and neuronal NOS (Son *et al.*, 1996). Secondly, in cultured cells injection of NOS inhibitors into the post-synaptic neurone blocks LTP whereas presynaptic injection of

NOS inhibitors has no effect (Arancio *et al.*, 1996). Thirdly, extracellular application of haemoglobin, which absorbs NO prevents LTP (Bohme *et al.*, 1991; Odell *et al.*, 1991; Schuman & Madison, 1991; Haley *et al.*, 1992). Since haemoglobin is not taken up by cells (Boulton *et al.*, 1995) NO has to travel between cells to be effective (Bohme *et al.*, 1991; Odell *et al.*, 1991; Schuman & Madison, 1991; Haley *et al.*, 1992). Finally, brief application of NO to cultures produces a long-lasting increase in the frequency of spontaneous miniature synaptic currents, suggesting a presynaptic site of action (O'Dell *et al.*, 1991).

The possible involvement of the diffusible messenger NO in LTP at the CA1 region may also explain the apparent non-input specificity first found by Schuman and co-workers. LTP induced by pairing post-synaptic cell depolarisation with low-frequency stimulation spreads to nearby synapses within a radius of 100μm (Schuman & Madison, 1994a).

The late phase of LTP (L-LTP), which lasts for more than 24 hours is thought to be responsible for converting short-term memory into long-term memory. Several studies suggested that L-LTP requires protein synthesis and PKA. L-LTP is induced by application of membrane permeable cAMP analogues (Frey *et al.*, 1993) and blocked by PKA inhibitors (Matthies & Reymann, 1993; Impey *et al.*, 1996). In transgenic mice, in which a PKA inhibitor is expressed in the neurones of the forebrain L-LTP is significantly attenuated (Abel *et al.*, 1997). In behavioural studies these animals were impaired in long – term memory (Abel *et al.*, 1997). Thus, the PKA- signalling pathway seems to contribute to the maintenance of LTP and memory for prolonged periods of time. Furthermore the cAMP – responsive transcription factor, CREB, seems to be

critically involved in L-LTP and long-term memory. Knockout mice in which the alpha and delta CREB isoforms are deleted show an impairment of L-LTP and long-term memory (Bourtchuladze *et al.*, 1994). These results indicate that the cAMP signalling cascade and an increase in transcription mediated by CREB are required for the induction of hippocampal L-LTP and for long-term memory.

1.5.2 LTP At The Mossy Fibre – CA3 Pyramidal Cell Synapse

Mossy fibre LTP varies from the potentiation that occurs at the CA1 region of the hippocampus in several aspects. Firstly, mossy fibre LTP is completely independent of NMDA receptors (Harris & Cotman, 1986; Zalutsky & Nicoll, 1990). Furthermore, much evidence points towards an entirely presynaptic mechanism for the induction (Ito & Sugiyama, 1991; Castillo *et al.*, 1994; Weisskopf *et al.*, 1994) as well as the expression (Xiang *et al.*, 1994; Weisskopf & Nicoll, 1995) of mossy fibre LTP. Several studies suggest that activation of adenylyl cyclase (AC) and protein kinase A (PKA) is necessary for the induction and maintenance of LTP (Huang *et al.*, 1994; Weisskopf *et al.*, 1994). For example, application of the AC – activator forskolin induces mossy fibre LTP whereas the inactive forskolin homologue, 1,9-dideoxy-forskolin, has no effect (Weisskopf *et al.*, 1994). Furthermore, mutant mice lacking PKA C1β or Riβ subunits do not exhibit mossy fibre LTP (Huang *et al.*, 1995). Since mossy fibre LTP is not mimicked by agonists that stimulate AC via receptor activation, Weisskopf and co-workers suggested that induction of LTP at the mossy fibre synapse it attributed to activation of AC by presynaptic calcium increase (Weisskopf *et al.*, 1994). It has recently been shown that mossy fibre LTP is significantly reduced in mutant mice lacking calcium/calmodulin dependent AC1 (Villacres *et al.*, 1998) suggesting that increases in presynaptic calcium leads to activation of AC1, which in turn via

production of cAMP activates PKA (Villacres *et al.*, 1998). The mechanism by which activation of PKA results in enhanced glutamate release is not yet known although it has been suggested that PKA may directly phosphorylate one or more proteins that control synaptic strength (Trudeau *et al.*, 1996). Synapsins (Greengard *et al.*, 1993) and raphillin-3A (Fykse *et al.*, 1995) are known to be phosphorylated by PKA. Synapsins do not appear to contribute to mossy fibre LTP since mice lacking synapsins express normal LTP (Spillane *et al.*, 1995). However, raphillin-3A which binds to the synaptic vesicle proteins Rab3A and Rab3C (Fykse *et al.*, 1995) seems to be required for LTP since Rab3A mutant mice lack mossy fibre LTP (Castillo *et al.*, 1997).

1.5.3 Cerebellar LTP At The PF–PC Synapse

The LTP that occurs at the synapse formed by PFs and PCs following raised frequency stimulation of PFs under conditions of reduced post-synaptic calcium activity has several elements common to mossy fibre LTP (Sakurai, 1990; Hirano, 1990b; Shibuki & Okada, 1992; Salin *et al.*, 1996). Both, the induction and expression of cerebellar LTP are considered to be presynaptic (Hirano, 1990b; Salin *et al.*, 1996). Several studies show that the induction requires presynaptic calcium influx but is independent of post-synaptic calcium levels. Application of glutamate receptor antagonists (Salin *et al.*, 1996; Linden, 1997) or loading of PCs with calcium chelators (Sakurai, 1990; Shibuki & Okada, 1992; Salin *et al.*, 1996; Linden, 1997) has no effect on the induction of LTP. LTP is, however, blocked by the removal of external calcium during raised frequency stimulation (Salin *et al.*, 1996; Linden, 1997; Linden, 1998). Similarly to mossy fibre LTP a role for cAMP was suggest in cerebellar LTP. Application of the AC activator forskolin mimics LTP whereas inhibitors of PKA block the induction of raised frequency stimulation or forskolin induced LTP (Salin *et al.*, 1996). Moreover, mutant

mice that lack AC1 which is activated by calcium/calmodulin and accounts for approximately 80% of the AC activity in the cerebellum lack LTP (Storm *et al.*, 1998). It has been demonstrated that forskolin induced LTP is not associated with a basal increase in pre-synaptic calcium levels and no changes in mEPSC amplitude were observed indicating that the post-synaptic receptor sensitivity was unaltered (Chen & Regehr, 1997). However the precise mechanism by which cAMP/PKA results in facilitation of presynaptic transmitter release remains to be investigated.

1.6 Aims And Objectives

The work described in the following chapters was prompted by a study undertaken by Salin and co-workers who demonstrated that 8Hz PF stimulation for a period of 15 seconds under conditions of reduced post-synaptic calcium activity was capable of inducing a LTP of synaptic transmission between PFs and PCs (Salin *et al.*, 1996). Induction of LTP required activation of AC and PKA and was proposed to arise through an entirely presynaptic mechanism.

By using several different approaches and techniques the properties and origins of LTP induced by 8Hz PF stimulation have been investigated. In particular, by the use of an additional stimulating electrode, which activated a synaptically distinct PF input to the same PC, the degree of input specificity of LTP associated with 8Hz PF stimulation was examined. Since raised frequency stimulation of the molecular layer is a powerful stimulus for NO production, the possible role of the NO, cGMP cascade has been investigated in the induction mechanism of cerebellar LTP and the interaction of this pathway with the AC/cAMP/PKA cascade. This work forms the substance of chapter 3.

Raised frequency and intensity stimulation of PFs can induce heterosynaptic LTD (Hartell, 1996a; Hartell, 2000). Therefore the possibility that synaptic transmission at the PF-PC synapse induced by 8Hz PF stimulation could also undergo LTD when post-synaptic calcium was not strongly buffered was examined. Finally, the underlying mechanism and the degree of input specificity of LTD that emerged after 8Hz PF stimulations when post-synaptic calcium levels were not buffered were examined. These experiments are described in Chapter 4.

CHAPTER 2

METHODS

2.1 DISSECTION AND BRAIN SLICING

14 to 21 day old male Wistar rats that were bred in house were placed in an anaesthetic box attached to a Boyles apparatus (Fluotec-3, Cyptane limited) and deeply anaesthetized with 5% fluothane in a 3:2 NO_2:O_2 gas mixture. Corneal and pedal reflexes were tested and, when absent, rats were decapitated and the cerebellum was rapidly dissected. Upon removal the cerebellum was submerged in cold (<4ºC), oxygenated artificial cerebro-spinal fluid (ACSF) of the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2.2H_20$, 2.5; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4.7H_2O$, 1.2; Glucose 11, equilibrated with 95% O_2 + 5% CO_2 gas (pH 7.4). To minimise cell damage from anoxia care was taken to ensure that this initial procedure did not take longer than 90 seconds and that the cerebellum was kept cold.

Cerebellar slices were cut according to established techniques (see for example Edwards *et al.*, 1989). The cerebellum was placed on an agar block made with 3% agar in a 0.9% NaCl solution. Two parasagittal cuts were made. The first was made on the right hand side close to the vermis. The second was made on the left side midway across the paravermis. This cut edge was then placed downwards onto a Teflon block lightly coated with cyanoacrylate (RS Components) with the dorsal surface of the cortex facing forwards. The Teflon block was placed into the chamber of a vibroslicer (Campden Instruments, Sileby, England) that was pre-cooled with a peltier device and chilled ACSF was added. To obtain slices with healthy cells close to the surface of the tissue 200μm thick, parasagital slices were prepared with slow forward speed and maximum lateral vibration. By using sagittal, cerebellar slices the Purkinje cell and its dendritic tree can be

preserved whole within the slice. The PFs traverse the cerebellum perpendicular to the PC dendritic tree, which lies in the plane of the slice. This orientation, together with the high number of PFs that make synaptic contact with PCs enables two synaptically independent PF inputs to the same PC to be activated. Slices were placed in a holding chamber containing ACSF and were bubbled gently from below to obtain efficient oxygenation. Slices were incubated for at least 60 minutes at room temperature before being transferred to the recording chamber (Edwards *et al.*, 1989).

2.2 EXPERIMENTAL SET-UP

Slices were placed between two nylon nets on an upright microscope, fully submerged and perfused with oxygenated ACSF at a flow rate of 1.5 – 2 $mlmin^{-1}$. Experiments were carried out initially on a Leitz based microscope adapted by Micro Instruments (Oxford). Later experiments were carried out on an Olympus BX50WI microscope. Electrophysiological responses were amplified with either an Axopatch 1C or an Axopatch 200B amplifier (Axon Instruments). Analogue signals were then digitised with either a Labmaster TL1 or a Digidata 1200A AD converter (Axon Instruments) . Data were filtered at 5 kHz and sampled at 10 kHz. All data were displayed and processed online using the LTP program (Anderson & Collingridge, 1999). Further offline reanalysis was done using custom procedures written with Igor Pro software (Wavemetrics). Purkinje cells were visualised using either a 20x (0.5 NA) or 40x (0.75 or 0.8 NA) water immersion objectives. A schematic diagram representing the basic wiring diagram of the recording system is shown in Figure 2.1.

2.3 WHOLE-CELL PATCH CLAMP RECORDING

Thin walled borosilicate glass with an outer diameter of 1.5 mm and an inner diameter of 1.17 mm was used to pull patch pipettes with resistances of between 3 and 5 MΩ using a Flaming Brown micropipette puller (Sutter Instruments, Model D-97). Pipettes were filled with solutions of the following composition (mM): KGluconate, 132, NaCl, 8, $MgCl_2$, 2, HEPES 30, Na_2ATP, 4, EGTA 0.5, GTP 0.3, adjusted to pH 7.3. In some experiments 0.5 mM EGTA was replaced by 5 or 10 mM BAPTA. Whole cell path clamp recordings were made from the soma of healthy looking Purkinje cells close to the surface, which were identified by their round, smooth appearance. Cells were held in voltage clamp mode at a holding potential of –70 mV (for a description of the patch-clamp technique see Hamill *et al.*, 1981; Edwards *et al.*, 1989; Neher, 1992; Neher & Sakmann, 1992; Hamill *et al.*, 1981; Sakmann & Neher, 1984; Sakmann, 1992).

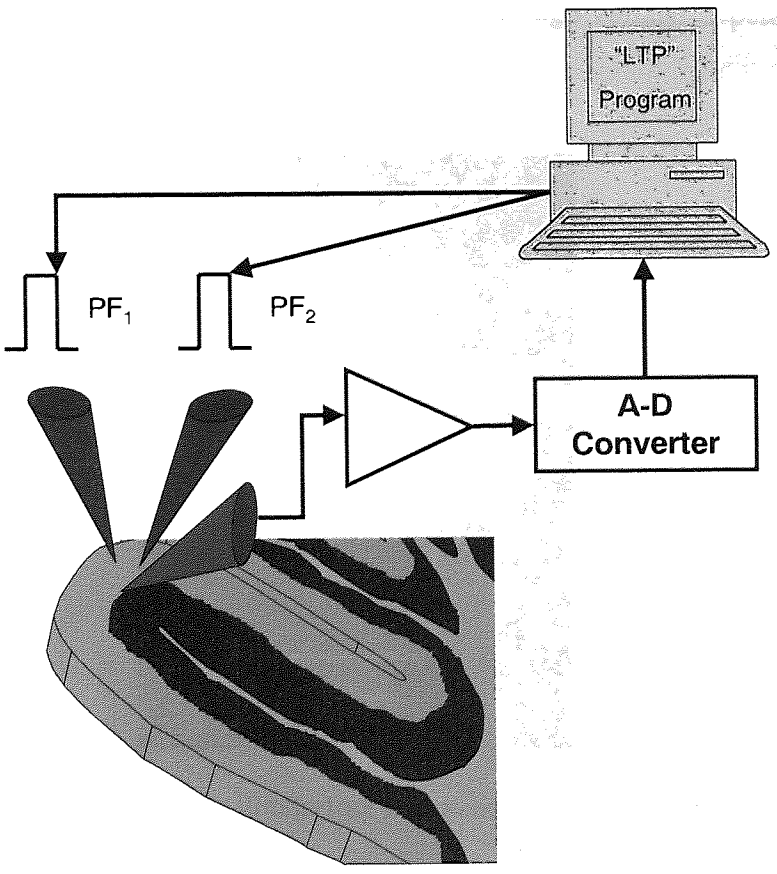


Figure 2.1 Schematic diagram of the experimental equipment used for patch clamp recordings.

2.4 STIMULATION OF SLICES

Pairs of ACSF filled patch electrodes with resistances between 1.5 and 2 MΩ were placed equidistant from the soma in the middle of the molecular layer at an approximate separation of 60μm to stimulate PFs (Figure 2.2). The position midway through the molecular layer was chosen to fulfil both the desire to maintain reasonably good voltage clamp conditions and to allow an average separation between the electrodes and soma of approximately 60μm in order to maximise the likelihood of activating two, synaptically independent PF inputs to the same Purkinje cell. In experiments where the extent of spread of synaptic plasticity was specifically investigated electrodes were placed over a range of 40 to 170μm. The two PF inputs to the cell were activated alternately at a rate of 0.2 Hz with pulses of 100 μs width and with intensities between 1-10 Volts. The amplitude of evoked PF-excitatory postsynaptic currents (EPSCs) was limited to less than 300 pA to minimise the likelihood of calcium influx through voltage dependent calcium channels (Hartell, 1996; Eilers *et al.*, 1995).

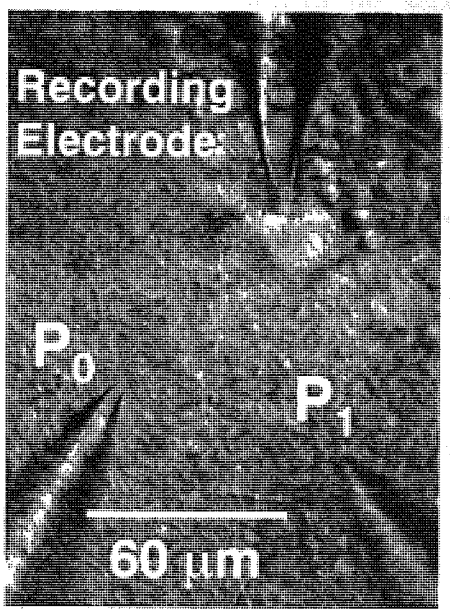


Figure 2.2 A bright field image illustrating the positions of two stimulating electrodes (P_0 and P_1) and the recording electrode (top). The image was taken using a 40 times water immersion lens.

2.5 EXPERIMENTAL PROTOCOL

Once EPSCs elicited by alternate, 0.2 Hz activation were stable in each pathway for at least 10 minutes, the frequency of one input, designated P_0, was raised to 8Hz for 15 seconds. These stimulation parameters were based upon those used in an earlier report by Salin and co-workers who found that 8 Hz PF stimulation effectively induced a LTP of synaptic transmission at the PF-PC synapse (Salin *et al.*, 1996). However, a second stimulation electrode was used in addition in order to examine the degree of input specificity of any induced synaptic plasticity. During the period of raised frequency stimulation (RFS) to P_0 the second input, termed P_1, was not stimulated. After this phase of RFS, alternate stimulation to both pathways was resumed at 0.2 Hz. Figure 2 illustrates the timing pattern of a standard experiment.

Throughout the experiments pairs of pulses were delivered at a separation of 50 ms. Paired pulse stimulation causes the second pulse to increase in amplitude due to a presynaptic accumulation of calcium. The ratio of the second pulse to the first was calculated and plotted over time as an indicator for presynaptic changes. In later experiments the extent of pathway independence was checked before, and 5 and approximately 20 minutes after RFS was applied. Pathways P_0 and P_1 were activated at a 50 msec interval and *vice versa* 150 msec later. The amplitude of P_1, when activated 50 msec after P_0, was compared to a naïve P_1 response and to an EPSC resulting from paired pulse activation at the same interval. The same comparison was carried out for P_0 when stimulated 50 msec after P_1. If P_0 and P_1 shared a significant number of PFs activation of P_0 should cause some facilitation in P_1 and *vice versa*. In some experiments RFS was replaced by the bath application of various drugs.

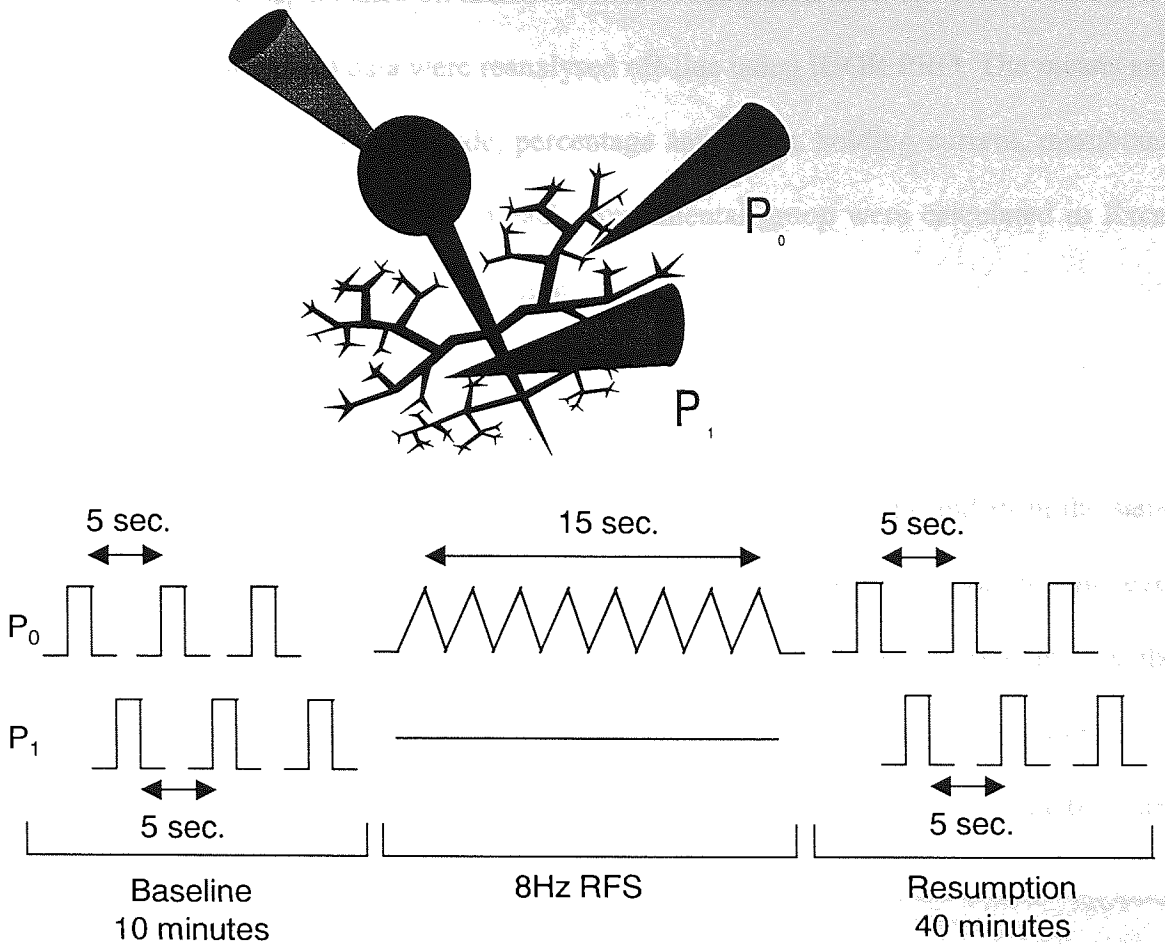


Figure 2.3. Schematic diagram illustrating the relative positions of P_0 and P_1 stimulating electrodes and the standard experimental protocol used for whole cell patch clamp recordings.

2.6 DATA ANALYSIS

Data were initially analysed on-line using the LTP program (Anderson & Collingridge, 1999). Changes in synaptic strength of the average of 6 PF responses in each pathway collected over 30 second periods were monitored by measuring the EPSC amplitudes and these changes were expressed as a percentage of mean baseline levels measured over 10 minutes prior to RFS. Membrane resistance and holding current were measured throughout and data were discarded from the analysis if either of these measurements changed significantly over time. The series resistance was compensated in order to prevent errors in the true membrane potential compared to the command potential due to the flow of ionic currents in the cell membrane and the filtering effect of series resistance

and cell membrane capacitance on measured membrane currents. In order to calculate the paired – pulse ratio (PPR) data were reanalysed off-line using IGOR PRO. The means and standard errors of absolute amplitude, percentage amplitude, holding current, membrane resistance and PPR of 5 to 6 cells in each experimental group were calculated in Excel and then plotted over time using Sigma plot.

2.7 STATISTICS

To determine the level of statistical significance between pathways P_0 and P_1 in the same experiment the Wilcoxon- Matched pair test was performed. In order to examine the level of statistical significance between pathways in two different sets of experiments the Mann-Whitney U test was carried out. P values less than 0.05 were considered significant and are illustrated throughout with a single asterisks (*), P values less than 0.01 are illustrated as double asterisks (**).

2.8 MATERIALS

H89, forskolin and tetrodotoxin (TTX) were supplied by Sigma. H-[1,2,4]oxadiazolo[4,3a] quinoxaline-1-one (ODQ), 7-nitro-indazole (7-NI), N^G-Nitro-L-arginine methyl ester (L-NAME) and spermine NONO-ate were obtained from Tocris Cookson. KT5823 was supplied by Calbiochem. With the exception of spermine NONO-ate and L-NAME, which are water-soluble, compounds were dissolved in DMSO to final concentrations of less than 0.1% DMSO. KT5823 was included in the internal patch solution at a concentration of 0.5 μM. ODQ was applied both intracellularly and extracellularly. H89, rolipram, forskolin, TTX L-NAME, 7-NI and spermine NONOate were all applied extracellularly. The pH of all internal solutions was adjusted to 7.3 and all external solutions to 7.4.

2.9 RECORDING OF MEPSCS

Spontaneous miniature EPSC activity was measured in the whole – cell configuration using 3 – 5 MΩ patch pipettes filled with solution of the following composition (mM): KGluconate 132, NaCl 8, $MgCl_2$ 2, HEPES 30, Na_2ATP 4, BAPTA 10, GTP 0.3, adjusted to pH 7.3. The calcium concentration of the standard ACSF was raised to 5mM and 200 nM TTX was added to block action potential – evoked synaptic currents. Throughout all experiments the holding potential was maintained at –70 mV. Postsynaptic recordings were made at high gain to measure mEPSC amplitudes and frequency. Data were collected over 4 minute epochs using Clampex 7 (Axon Instruments), filtered at 2KHz and sampled at 5 kHz. Events were analysed offline using Mini Analysis (Synaptosoft Inc.). mEPSCs were distinguished from background noise according to their amplitude and area. Events with amplitudes exceeding –10 pA and areas greater then 40 femto coulombs were included for analysis. Forskolin and spermine NONOate were applied extracellularly after a 10 minutes baseline period. In some cases slices were pre-incubated with 7-NI and throughout the experiment. Data collected before and after drug application were compared using the Kolmogorov – Smirnov test for significance. Statistical significance was taken to be at the 5% level. Control experiments during which no drug was applied were carried out to ensure that mEPSC amplitudes and frequencies were stable over time. Input resistance was monitored in all experiments and series resistance was compensated by more than 80%. Data were excluded from the analysis if either of these values changed significantly over time as described above in experiments in which evoked EPSCs were measured.

2.10 Imaging FM1-43 Fluorescence in Cerebellar Slices

2.11 Experimental Set-Up

The styryl dye pyridinium, 4-[2-[4-(dibutylamino)phenyl]ethenyl]- 1-[3-(triethylammonio)propyl]- , dibromide (FM1-43, Molecular probes, Figure 2.4), has been used as a real time marker of endo- and exocytosis (Betz & Bewick, 1992; Betz *et al.*, 1992b; Betz *et al.*, 1992a). Experiments using FM1-43 to assess presynaptic release were performed on an upright microscope (Olympus BX50 WI) equipped with a monochromator (Till Photonics), a dichroic mirror (505), an emission filter (530 ± 20nm) and a cooled, digital CCD camera (Hamamatsu C4880 series). Water immersion objectives (60 x 0.9 NA or 40 x 0.8 NA) were used. FM1-43 was excited with 488 nm light from the monochromator and images were captured with an exposure time of 800 msec (Figure 2.5). The camera offset was chosen at 10% and the camera gain was set to 25%. These values were kept constant throughout to allow a quantitative analysis between experiments. 12 bit images with 2 x 2 pixel binning were acquired at the rate of one frame every 6 seconds on a Macintosh computer using a custom written automations (Openlab, Improvision). A detailed description of the automation used can be found in appendix 1. Once captured, images were analysed offline using custom written procedures in IGOR PRO software.

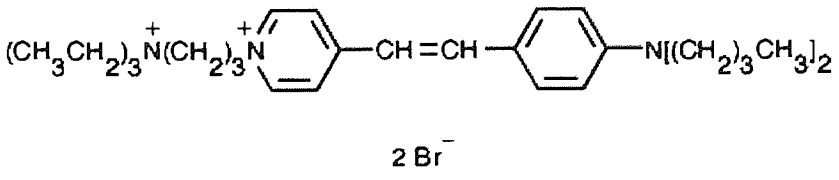


Figure 2.4 Chemical Structure of FM1-43.

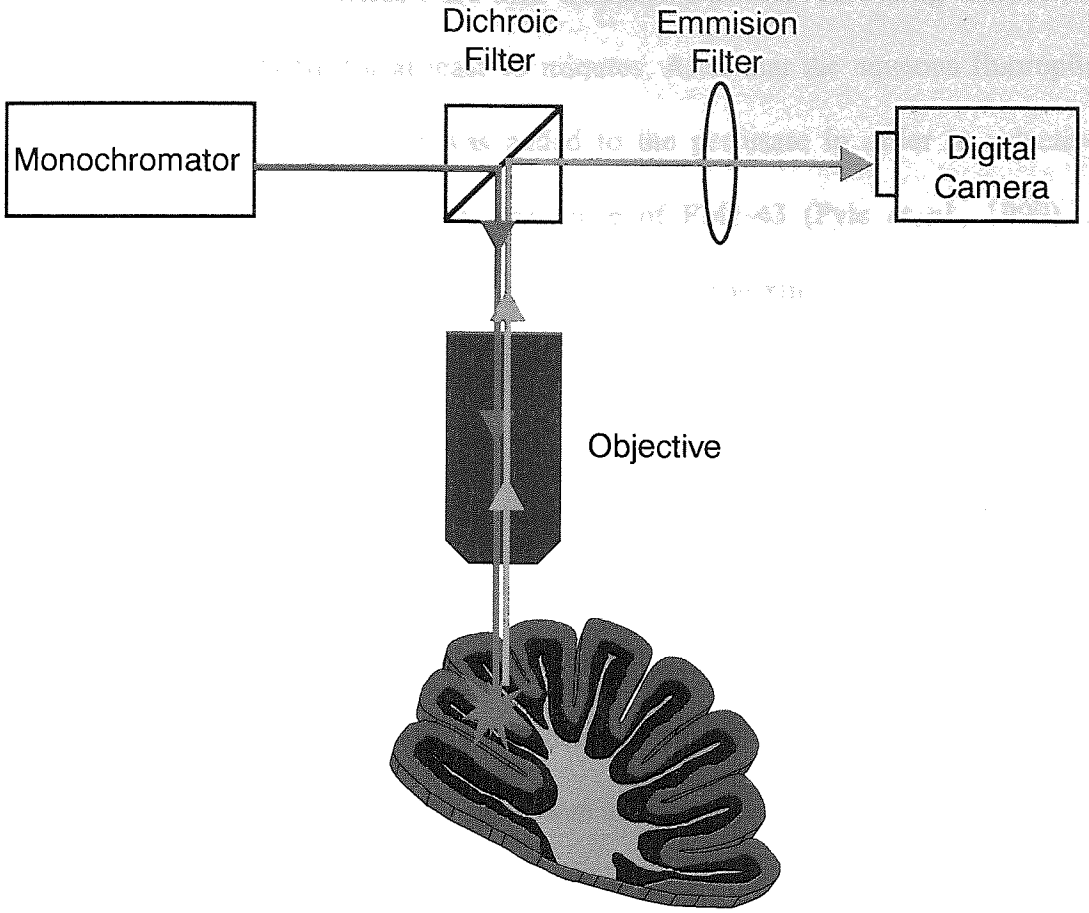


Figure 2.5 Schematic diagram of the experimental equipment used for imaging experiments. FM1-43 was excited at 488nm light produced by a monochromator. Emitted fluorescence was collected through a dichroic mirror (>505 nm) and was detected by a cooled, digital CCD camera after emission filtering at 530± 20nm.

2.12 Loading of Slices with FM1-43

In initial experiments transverse or parasagittal slices were loaded by incubation in ACSF modified to contain 20 mM KCl, 5 mM $CaCl_2$ and 10 μM FM1-43 for 15 minutes. To reduce background fluorescence slice were transferred back into the holding chamber and washed for at least two hours. After that slices were transferred into the recording chamber and perfused with standard ACSF. Since incubation of slices in raised potassium and calcium solutions for so long appeared to elevate the overall level of dead cells in slices, the composition of ACSF was modified in later experiments. The concentration of KCl was raised to 50 mM and the $CaCl_2$ concentration was lowered to standard level of 2.5 mM. Under these conditions, sagittal slices were incubated for 90 seconds in the

presence of 10μM FM1-43. Slices were then transferred into the recording chamber and washed with standard ACSF for at least 15 minutes. After that the aqueous fluorophore sulforhodamine (Molecular probes) was added to the perfusate in order to selectively quench surface bound, non-synaptic fluorescence of FM1-43 (Pyle *et al.*, 1999). All experiments were carried out in the presence of 20 μM picrotoxin.

2.13 Experimental Protocol

In some experiments one or more ACSF filled stimulating electrodes were placed on the surface of the molecular layer to activate PFs, as outlined in section 2.4. Several different stimulation protocols were used to first determine suitable stimulus conditions and to establish the optimum camera settings to detect changes in FM1-43 fluorescence at optimum resolution. First, a single PF input was activated at 8 Hz at 40 V for 5 minutes. Second a single electrode was activated at 8 Hz at increasing stimulus intensities (5, 10, 20, 40 and 80 V) for one minute respectively. Third a single PF input was activated twice at 8 Hz at 20 V for 15 seconds with a 5 minute separation. Finally, two stimulating electrodes were placed in the molecular layer as described in section 2.4 and P_0 was stimulated at 8 Hz at 5V for a 15 second period. In this final set of experiments whole cell patch clamp experiments were also made from Purkinje cells to simultaneously measure changes in fluorescence and the electrical response. In another set of experiments electrical stimulation was replaced by application of various drugs.

2.14 Data Handling

Due to a lack of spatial resolution it was difficult to identify the single puncta thought to represent synaptic boutons (Pyle *et al.*, 1999) therefore several, identically sized regions of interest (ROIs) with diameters between 5 – 15 μm were defined within images taken

of the molecular layer. The use of ROIs instead of single puncta compensated for slight movements of slices during prolonged recording periods and furthermore, the overall signal to noise ratio was improved by using ROIs. The mean change in fluorescence (δF) in response to either drug application or to electrical stimulation was calculated for each ROI.

Since ROIs did not contain identical numbers of puncta the changes in fluorescence were in some cases normalised to the initial mean fluorescence intensity (δF/F) to exclude the possibility that the degree of fluorescence changes was dependent on the initial level of fluorescence. The relative distance of ROIs from the stimulating electrode was measured and the extent of changes in fluorescence was compared to the distance from the point of stimulation.

2.15 MATERIALS

Forskolin (10μM) and spermine NONOate (100μM) were applied for 10 minutes as described above. In some experiments 7-NI (5μM) or L-NAME (100μM) were additionally present throughout the experiments. In some cases the voltage gated Ca^{2+} channel blocker Cadmium (100μM) was present throughout the experiments.

CHAPTER 3

THE NATURE AND THE UNDERLYING CELLULAR MECHANISMS OF CEREBELLAR LONG-TERM POTENTIATION.

3.1 INTRODUCTION

The phenomenon of LTP of synaptic transmission can be found at numerous excitatory synapses throughout the brain. This form of synaptic plasticity has been most intensely studied in the hippocampus in which two main forms of LTP have been described (for reviews see Bliss & Collingridge, 1993; Nicoll & Malenka, 1995). The best understood of these occurs in the CA1 region of the hippocampus and requires the activation of the NMDA subtype of glutamate receptor located at the post-synaptic pyramidal cell. The second form of potentiation is NMDA receptor independent and occurs at synapses between mossy fibres and the CA3 pyramidal cells.

It was recently shown that the synapse between cerebellar PFs and Purkinje cells can also undergo a form of LTP in response to brief raised frequency PF stimulation that is remarkably similar to that observed in hippocampal region CA3. LTP at both sets of synapses share a number of common elements including their dependency on the activation of calcium/calmodulin dependent adenylyl cyclase and PKA (Huang & Kandel, 1994; Weisskopf *et al.*, 1994; Salin *et al.*, 1996; Storm *et al.*, 1998). Furthermore, CA3-LTP in the hippocampus (Tong *et al.*, 1996; Weisskopf & Nicoll, 1995) as well as that in the cerebellum (Salin *et al.*, 1996), are thought to be expressed via a presynaptic increase in transmitter release.

Stimulation of the molecular layer, through which the PFs run, particularly at the higher rates necessary for LTP induction, not only causes the release of the diffusible

messenger NO (Shibuki & Kimura, 1997), it can also trigger a subsequent long-term potentiation of NO production (LTP_{NO}) for a given train of stimuli (Kimura *et al.*, 1998). Intriguingly, both the induction of LTP_{NO} and RFS induced cerebellar LTP can be mimicked by pharmacological activation of cAMP with forskolin and prevented by inhibition of PKA (Kimura *et al.*, 1998; Salin *et al.*, 1996).

There is now considerable evidence to suggest that NO plays a role in the induction or modulation of central synaptic plasticity. As outlined in the general introduction the induction of LTP in the hippocampal area CA1 (Arancio *et al.*, 1996) as well as in layer 5 of the medial frontal cortex (Nowicky & Bindman, 1993) for example, appear to involve NO. In the cerebellar cortex, a number of studies have shown that NO plays a role in the induction of LTD in brain slices (Daniel *et al.*, 1993; Lev Ram *et al.*, 1995; Daniel *et al.*, 1998) but not in Purkinje cell culture models of glutamate desensitisation (Linden & Connor, 1992; Linden *et al.*, 1995). PF activation at raised frequencies and intensities capable of producing a localised post–synaptic increase in calcium (Eilers *et al.*, 1995) results in a non–input specific form of LTD that spreads over tens of microns through a process that requires NOS activity (Hartell, 1996). Although the origin of cerebellar LTD is considered to be post-synaptic the anatomical distribution of nNOS within the cerebellar cortex, when compared to potential targets in PCs, suggests that NO, if released from PFs (Southam *et al.*, 1992), could act as a trans cellular messenger.

Since it has been shown that RFS induced LTP and NO release in the cerebellum have similar characteristics and since cAMP and NO can modulate each others production (Inada *et al.*, 1998; Polte & Schroder, 1998; Dubey *et al.*, 1998) the possibility that NO may also be involved in the induction of cerebellar LTP was examined. In the light of

observations that cerebellar LTD may not be input specific, the degree of input specificity associated with potentiation induced by PF stimulation at raised frequencies was also investigated by applying the induction protocol to one of two synaptically independent PF inputs to the same cell. These experiments were performed under conditions of reduced post–synaptic calcium activity to avoid the concurrent induction of LTD.

3.2 METHODS

The methods used for the experiments detailed in this chapter are described in full in chapter 2. Briefly, EPSCs were elicited by alternate stimulation of the two PF inputs at a rate of 0.2 Hz. Once EPSCs were stable for at least 10 minutes the frequency to one input, designated P_0, was raised to 8Hz for 15 seconds. Stimulation to the other pathway, termed P_1, was stopped during this period. After this phase of RFS alternate stimulation at 0.2 Hz was resumed (see Figure 2.3, Chapter 2). The amplitudes of evoked EPSCs, the PPRs and the decay time constants were measured for each pathway, together with the membrane resistance and holding current.

Figure 3.1 shows the averaged response of 6 successive sweeps and serves to illustrate the standard experimental protocol used for each of the two pathways activated. The membrane resistances of cells were measured using 100 msec, 5 mV hyperpolarizing voltage steps from the usual holding potential of –70mV. This was followed by activation of PF- EPSCs evoked by paired pulse stimulation at a separation of 50msec. The PPR was calculated by dividing the amplitude of the second pulse by the first. The decay time was calculated by fitting a single exponential curve from a point just after the peak of the second EPSC to a time point when the current had completely recovered

to the baseline level. The second EPSC was used because the true baseline of the first may have been truncated by the arrival of the second pulse. For pooling of experimental results, data were subsequently expressed as a percentage of baseline levels and plotted over time for each experiment.

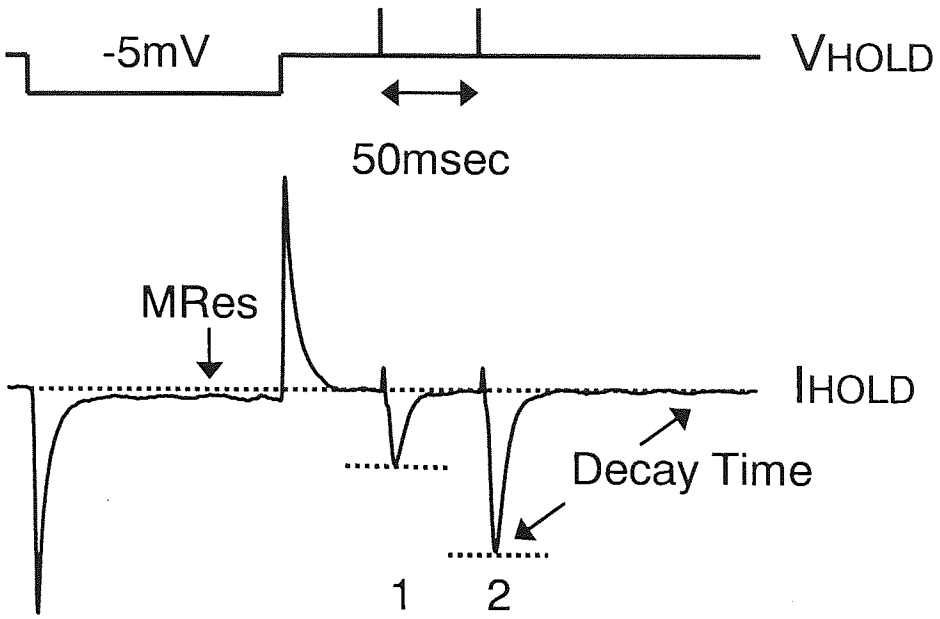


Figure 3.1 The current response (IHOLD) to a 5 mV hyperpolarizing voltage step (VHOLD) from a holding potential of –70 mV is shown, followed by paired pulse activation of PFs. The bottom trace represents the average of 6 successive sweeps. The approximate points on the trace where the membrane resistance (MRes), the first and second EPSC amplitudes and the decay time constant were measured are shown.

Figure 3.2 provides a representative example of a single experiment in which the effects that RFS had on each of these parameters is shown. To ensure consistency with regard to the quality of recordings, cells were not used if their membrane resistances fell below 60 MΩ and if the holding current exceeded -800 pA or if either of these parameters changed significantly or abruptly over the course of experiments.

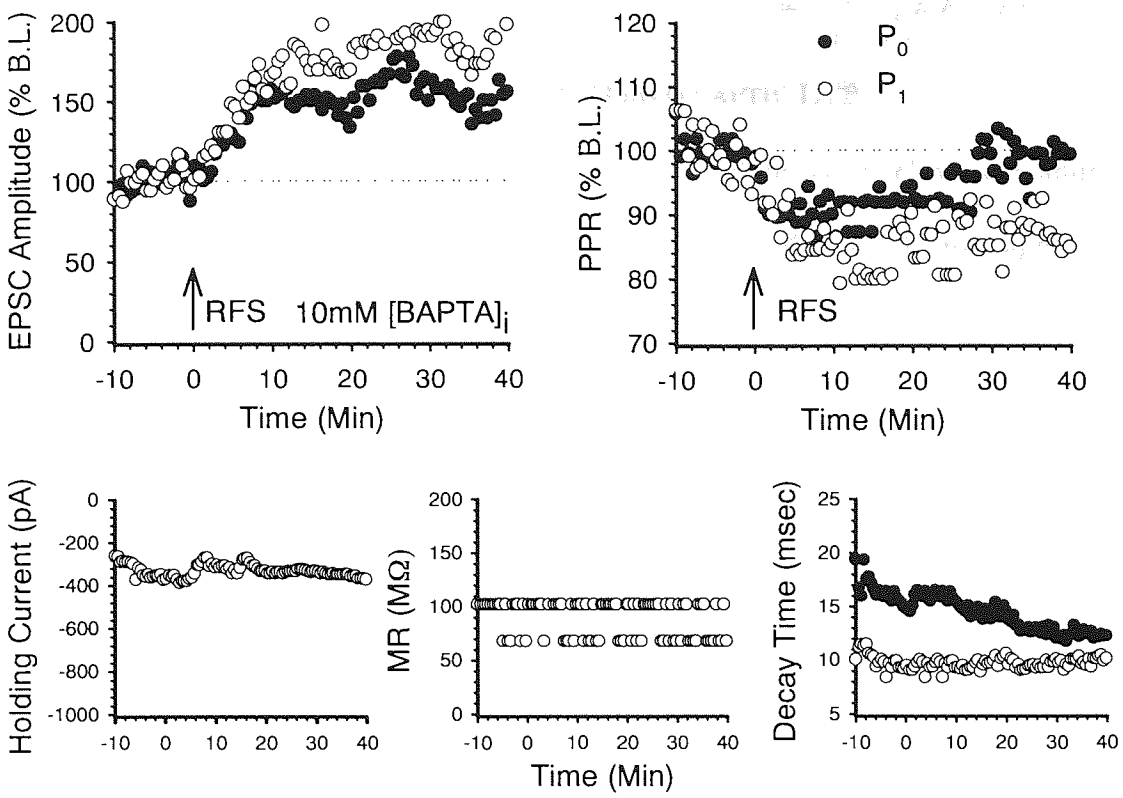


Figure 3.2 A representative example in which the effects of RFS in the presence of 10mM BAPTA on PF-EPSC amplitude and PPR are shown. Holding current, membrane resistance and decay time were also measured throughout the experiment.

The decay time constants of the EPSCs were measured routinely in the hope of gaining some additional insight into the mechanism or origin of any observed change in synaptic strength. Despite the obvious hazards of interpretation of voltage clamp data obtained from cells with extensive dendritic trees such as Purkinje cells, it has been reported that a decrease in EPSC decay time constant can reflect a desensitisation of post-synaptic AMPA receptors (Hemart *et al.*, 1994). Since no consistent or significant changes in decay time were detected in any sets of experiments included in this chapter, this data has not been included. Unless otherwise stated Purkinje cells were voltage clamped at a membrane potential of -70 mV and filled with 10 mM BAPTA to reduce post-synaptic calcium activity in order to prevent the induction of LTD which depends on a rise in post-synaptic calcium.

3.3 RESULTS

3.3.1 PATHWAY SPECIFIC RFS INDUCES HETEROSYNAPTIC LTP

The effects of RFS to one of two, independent PF inputs to the same cell were examined first. With 10 mM BAPTA in the patch pipette RFS induced a statistically significant potentiation of PF responses in both pathways compared to baseline levels 20 minutes after induction (P_0: 146.8 ± 9.7% and P_1: 152.4 ± 9.5%). The level of potentiation between pathways was statistically indistinguishable ($P>0.05$, Wilcoxon signed-rank test, n=6; Figures 3.3 and 3.6).

To help establish the origin of the potentiation, pairs of pulses at a 50 msec interval were applied to P_0 and P_1 throughout the experiments. Under this paired-pulse-paradigm, the second response is facilitated compared to the first due to an accumulation of pre-synaptic calcium. During the first pulse, the calcium that enters the pre-synaptic terminal may not trigger the release of transmitter from all available release sites. If a second action potential invades the terminal before calcium activity has returned to resting levels, the additional calcium will release transmitter from the remaining vesicles. In terminals where the transmitter release probability is high, the second pulse will tend to be less effective than the first and paired pulse depression is seen. When the release probability is low, the elevated calcium accompanying the second pulse will cause transmitter release from quiescent sites and the response will be facilitated. Thus the paired pulse ratio (PPR) of the second pulse to the first depends upon the probability of transmitter release and PPR changes are generally taken to reflect changes in the probability of transmitter release. However, because release probability is sensitive to extracellular calcium concentration, modulation of the PPR infers rather than proves a change in presynaptic release probability.

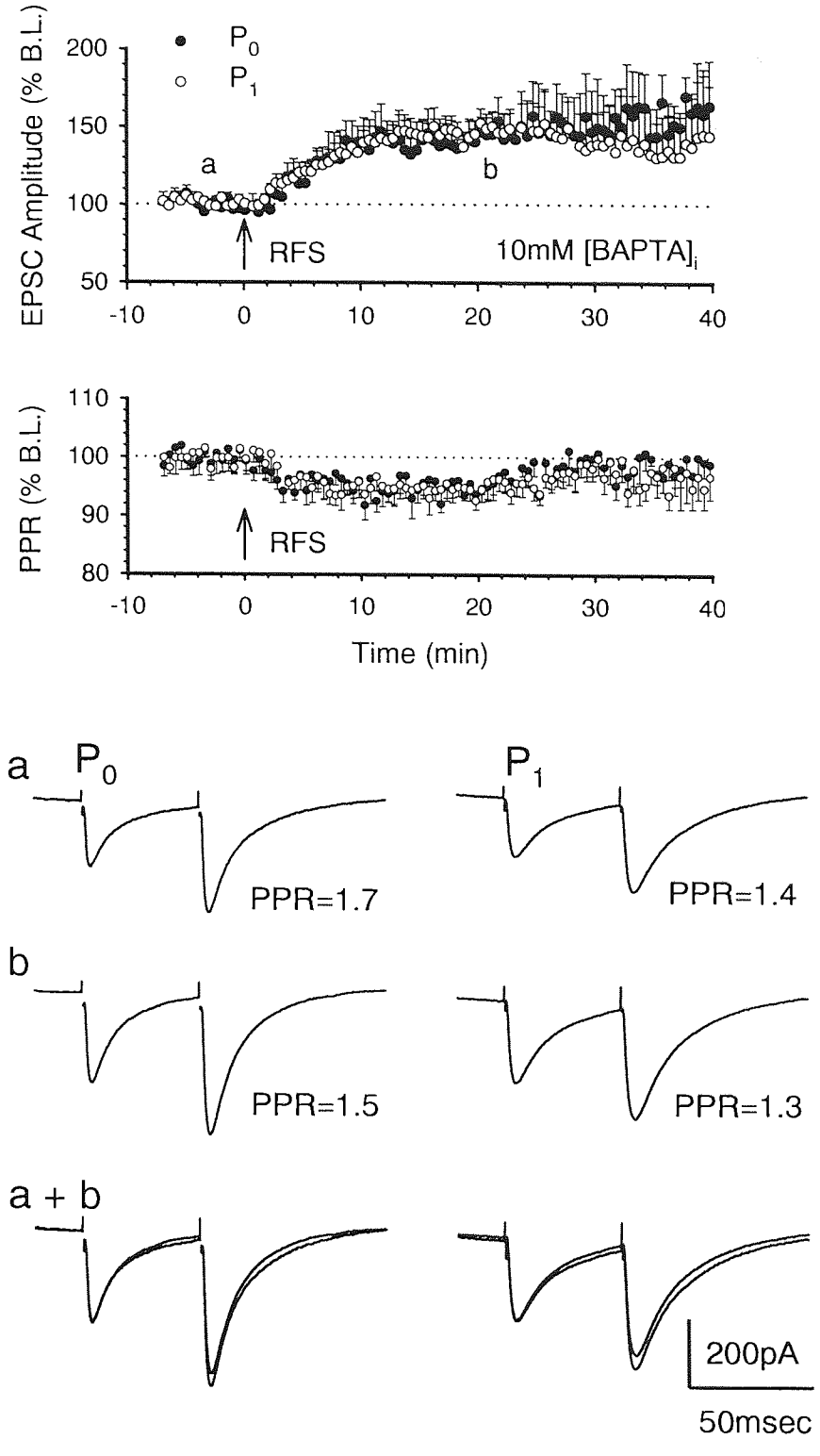


Figure 3.3 With 10 mM BAPTA included in the patch pipette, RFS led to a long-lasting increase in synaptic response and a decrease in PPR in both P_0 (filled circles) and P_1 (open circles) pathways. Data are expressed as percentages of the mean baseline level measured over 10 minutes. The bottom panel provides representative examples of EPSCs elicited by stimulation of P_0 (left) and P_1 (right) pathways at times a and b indicated in the top panel. Each trace represents the average of 6 successive sweeps recorded over 30 seconds. The amplitudes of the first pulse in P_0 and P_1 were normalized to illustrate the accompanying reduction in PPR (a + b).

RFS to P_0 led not only to potentiation in both pathways but also to a decrease in the PPR at both pathways (Figures 3.3 and 3.6). The normalized PPRs of P_0 and P_1 responses 20 minutes after RFS were significantly reduced compared to baseline levels to 94.6 ± 0.9% and 94.5 ± 2.9% respectively ($P<0.05$, n=6, Wilcoxon signed-rank test; Figure 3.6). In contrast to the potentiation, which was sustained for the duration of the recordings (up to 60 minutes), the decrease in paired pulse facilitation consistently recovered towards baseline levels within approximately 25 minutes of RFS (Figure 3.3). This might suggest that sustained potentiation at the PF-PC synapse could consist of an early and a late phase mediated by different mechanisms.

To confirm that the potentiation was mechanistically similar to that previously described (Salin *et al.*, 1996), experiments were repeated with the PKA inhibitor H-89 (Kawasaki *et al.*, 1998) in the extracellular bathing media at a final concentration of 0.2μM. Inhibition of PKA prevented potentiation in P_0 and P_1 pathways (99.4 ± 4.8% and 103.4 ± 4.3% respectively, $P<0.05$, n=6, Mann-Whitney U test, Figures 3.4 and 3.6). No associated reduction in the PPR was seen (100.2 ± 2.6 and 99.9 ± 3.0%, $P<0.05$, n=6, Figures 3.4 and 3.6) after 20 minutes.

Potentiation was also observed in cells containing only 0.5 mM EGTA provided the cells were hyperpolarized to -90mV during the period of RFS (Figures 3.5 and 3.6). After 20 minutes, P_0 and P_1 responses were potentiated to 130 ± 9.3% and 129.3 ± 14.9% and the PPR decreased to 96.1 ± 4.0% and 96.4 ± 1.2%, respectively ($P<0.05$, n=6, Figure 3.5).

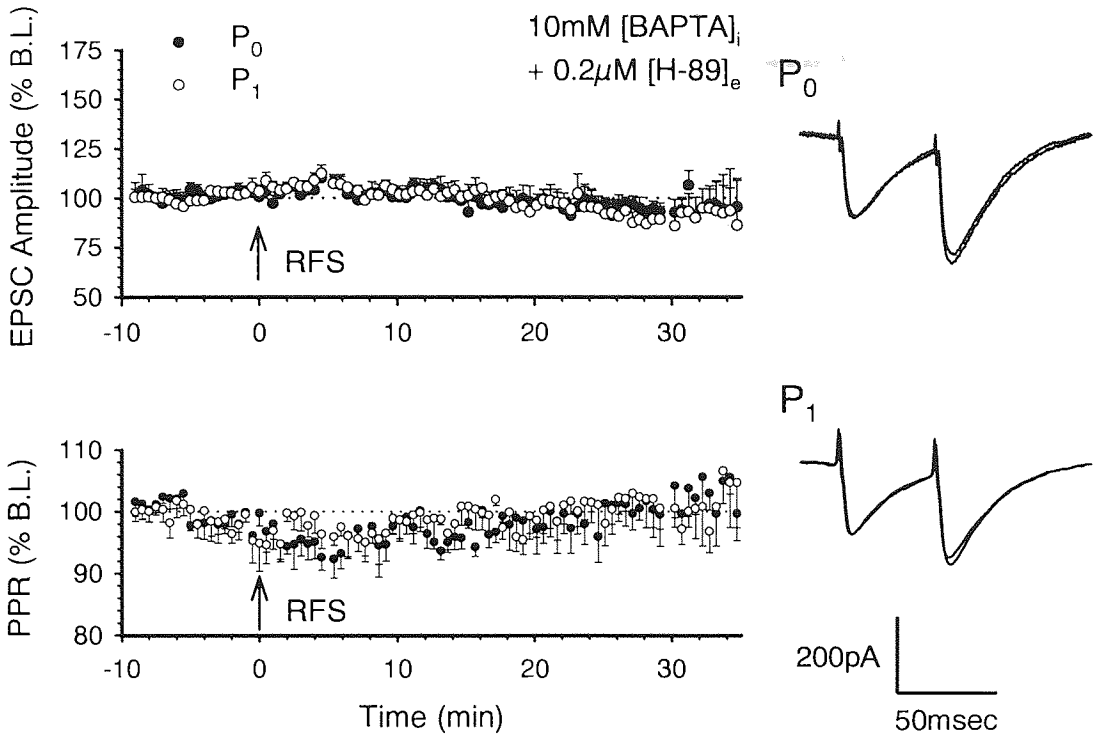


Figure 3.4 RFS induced potentiation and the associated decreases in PPR were completely blocked in the presence of the PKA inhibitor H-89 (n=6). Data are presented as in Figure 3.3.

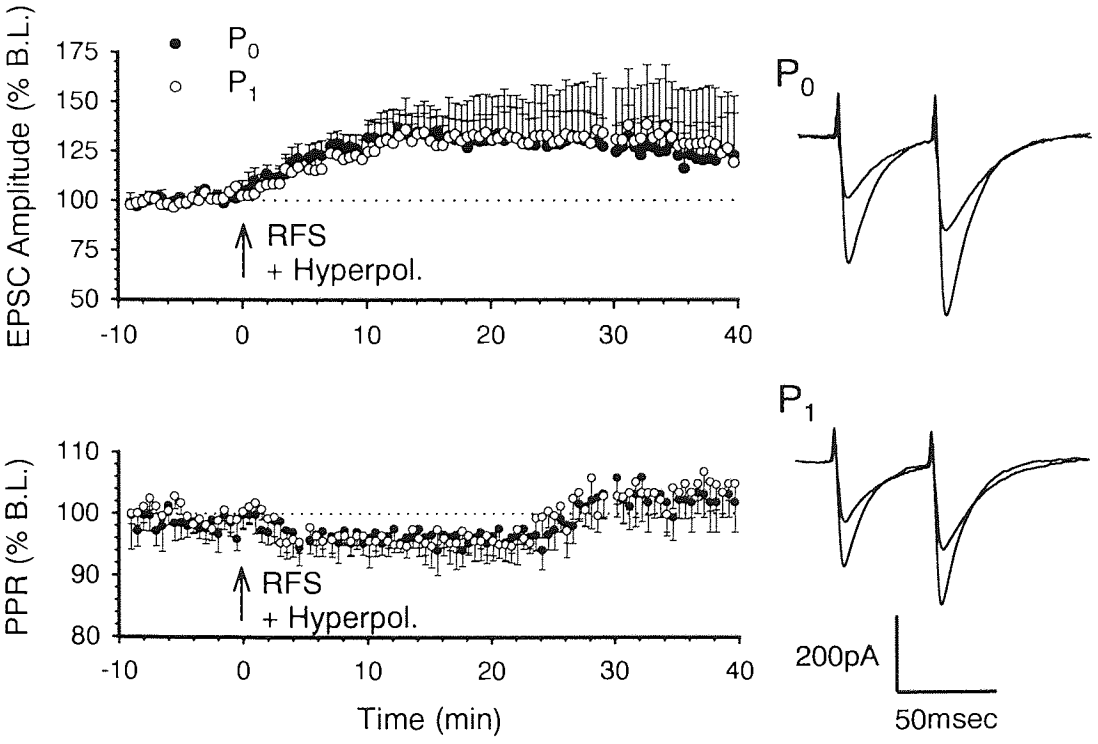


Figure 3.5 In the presence of 0.5mM intracellular BAPTA, hyperpolarization to –90mV during RFS to P_0 led to an increase in synaptic transmission and a decrease in PPR in both pathways. Data are presented as in Figure 3.3.

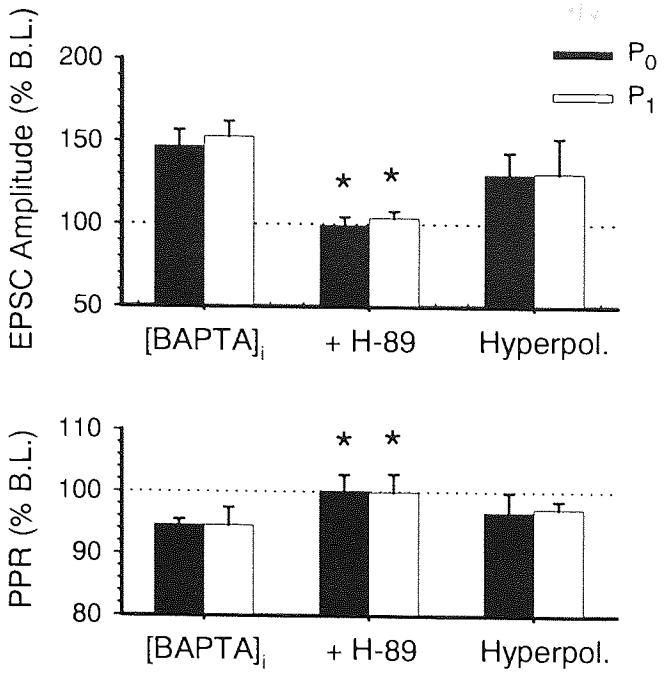


Figure 3.6 The means and standard errors of P_0 (filled bars) and P_1 normalized EPSCs (open bars) measured 20 minutes after RFS are shown under conditions of 10 mM intracellular BAPTA, BAPTA plus 0.2 μM extracellular H-89 and following cell hyperpolarization to –90mV during RFS with 0.5 mM EGTA inside the recording pipette. The bottom graph illustrates the associated changes in PPR measured at the same points in time. Asterisks indicate a statistical difference between test conditions and that in the presence of 10mM intracellular BAPTA (Mann-Whitney U-test, $P<0.05$, $n=6$).

There are several possible explanations for our observation that the potentiation of P_0 induced by RFS under conditions of reduced post-synaptic calcium activity does not remain pathway specific but spreads to the distant P_1 site. The first is that RFS leads to a generalized increase in the post-synaptic sensitivity of AMPA receptors on the Purkinje cell. In view of the accompanying reduction in the PPR and hence the likely presynaptic origin of cerebellar LTP, a more feasible explanation is that RFS leads to a generalized increase in transmitter release. This could arise either through an increase in the probability of transmitter release, an increase in the number of transmitter release sites per fibre and/or an increase in the number of contributing fibres. Although the PF stimulating electrodes were routinely placed at separations of around 60 μm (range 30-

100 μm), the possibility that the pathways were not completely independent was tested in case the loss of input specificity resulted from a significant number of synapses being activated by both P_0 and P_1, either prior to or following the induction of LTP.

To this end, a modified paired pulse protocol was used that allowed an estimation of the degree of overlap between the two pathways before and after LTP induction (Hartell, 1996). The principle is described in the experimental procedures section and illustrated in Figure 3.7. The degree of overlap between P_0 and P_1 was estimated 5 minutes prior to RFS induced potentiation and again 5-10, 15-20 and 30 minutes after using the equation described in experimental procedures.

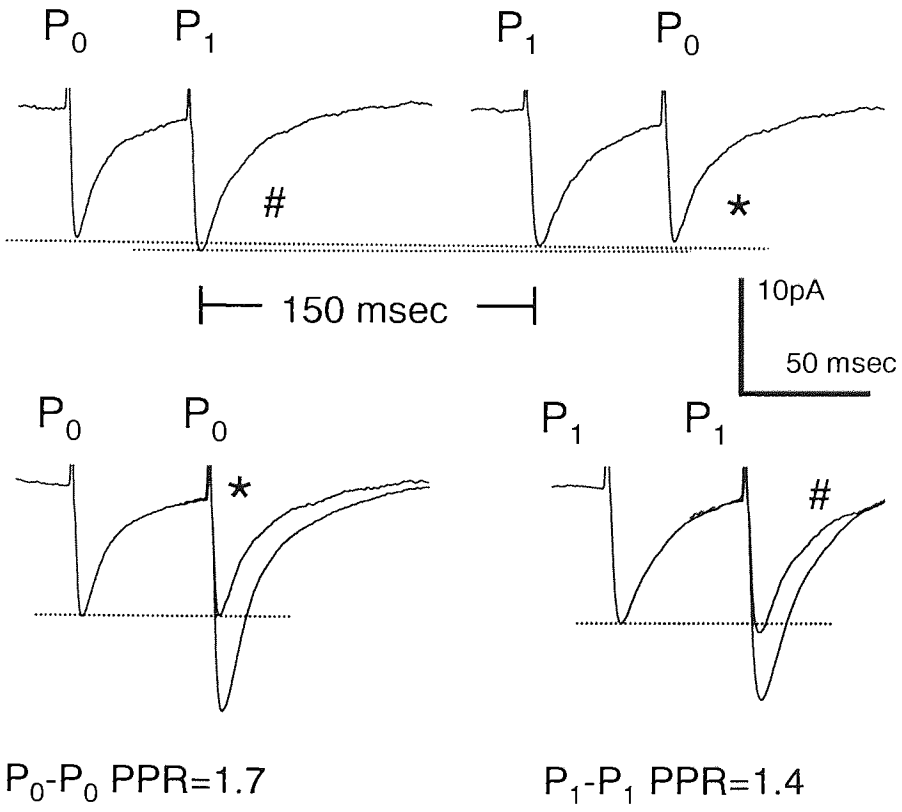


Figure 3.7 Upper graph: Parallel fibre pathways P_0 and P_1 to a single Purkinje cell were activated at 50 msec intervals and again in reverse order 150 msec later. When preceded by activation of the alternate pathway, P_0 and P_1 EPSC amplitudes remained similar to naïve responses, in this an in four other examples. Lower graph: The second response to P_1-P_0 stimulation (left) and P_0-P_1 stimulation (right) are superimposed on P_0-P_0 and P_1-P_1 responses respectively.

Figure 3.7 provides a representative example in which this test for pathway independence was carried out 5 minutes prior to RFS. Measurements taken during the baseline period revealed that P_0 had no influence on P_1 (0 % of the P_1-P_1 PPR; n=5) and *vice versa* (0 % of the P_0-P_0 PPR; n=5). Therefore, it is reasonable to assume that P_0 and P_1 did not share a significant number of fibres prior to RFS induced potentiation. The estimated percentage overlap measured 5 minutes after RFS did not show any significant increase (3 and 6 % respectively; n=5) and remained constant at this level for all remaining measurements up to 30 minutes post induction.

The spatial extent of the spread of LTP from the site of RFS to distant synapses was next examined. In a group of 8 cells, the level of input specificity between P_0, which received RFS, and P_1, which did not, was assessed by calculating the ratio of the percentage potentiation in each pathway (P_0/P_1) measured 20 minutes after RFS. An increase in P_0/P_1 ratio above a value of 1 will reflect an increase in the degree of input specificity. At electrode separations ranging between 40 and 170 μm there was no evidence to suggest that LTP became more input specific as the electrode separation increased (Figure 3.8; $R_{SPEARMAN}$=0.37, P>0.05). On the assumption that the electrode separation did not affect the degree of input specificity there was also no evidence that the size of the initial P_0 EPSC amplitude (range 190-300 pA) influenced the degree of input specificity over this range of separations (Figure 3.8, R=0.26, P>0.05).

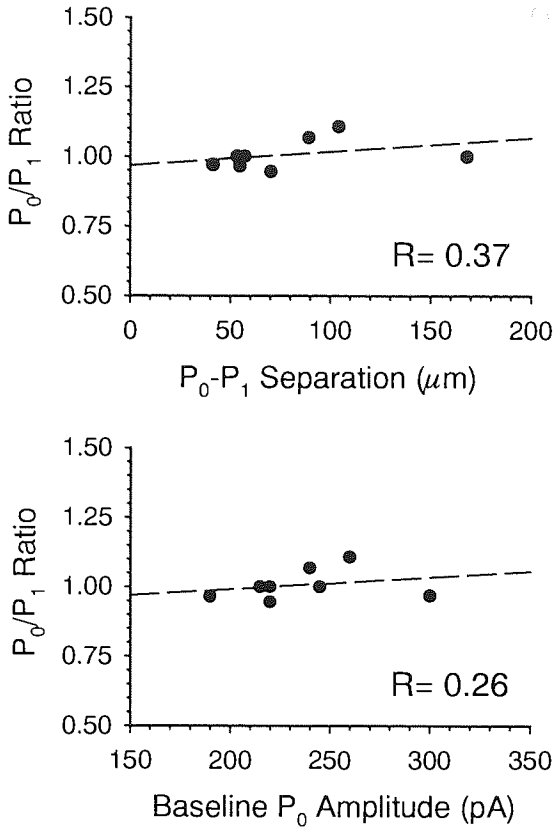


Figure 3.8 The ratios of the percentage level of potentiation compared to baseline levels observed in P_0 compared to that in P_1 are plotted against the separations between electrodes for a group of 8 cells. The dotted line represents the fitted linear regression. For the same set of cells the P_0/P_1 ratio is plotted against the initial mean amplitude of P_0 responses.

3.3.2 The Role Of Nitric Oxide In The Induction And Maintenance Of Heterosynaptic LTP

It has recently been shown that NO release from the molecular layer of the cerebellar cortex can be potentiated following tetanic stimulation and that this potentiation requires PKA activation (Kimura *et al.*, 1998). In view of the fact that NO has been implicated in the spread of synaptic depression at PF-PC synapses (Hartell, 1996) and may be released as a result of high frequency PF activation (Kimura *et al.*, 1998), the possibility that NO was also responsible for the spread of RFS-induced LTP was studied.

With 10mM BAPTA inside the recording pipette and with the neuronal NOS inhibitor 7-NI (Moore *et al.*, 1993) in the bathing media at a concentration of 5μM, RFS failed to induce synaptic potentiation in either pathway (Figure 3.9). The amplitudes of P_0 and P_1 EPSCs measured 20 minutes after RFS were statistically different from those measured without NOS inhibition (Figure 3.13), reaching 100.4 ± 7.8 and 100.6 ± 6.3% of baseline levels respectively ($P<0.05$, n=6, Mann-Whitney-U test). As illustrated in Figures 3.9 and 3.13, no reduction in the PPR was observed in either pathway (99.4 ± 3.3% and 101.4 ± 5.0%, $P<0.05$, n=6, Mann-Whitney-U test).

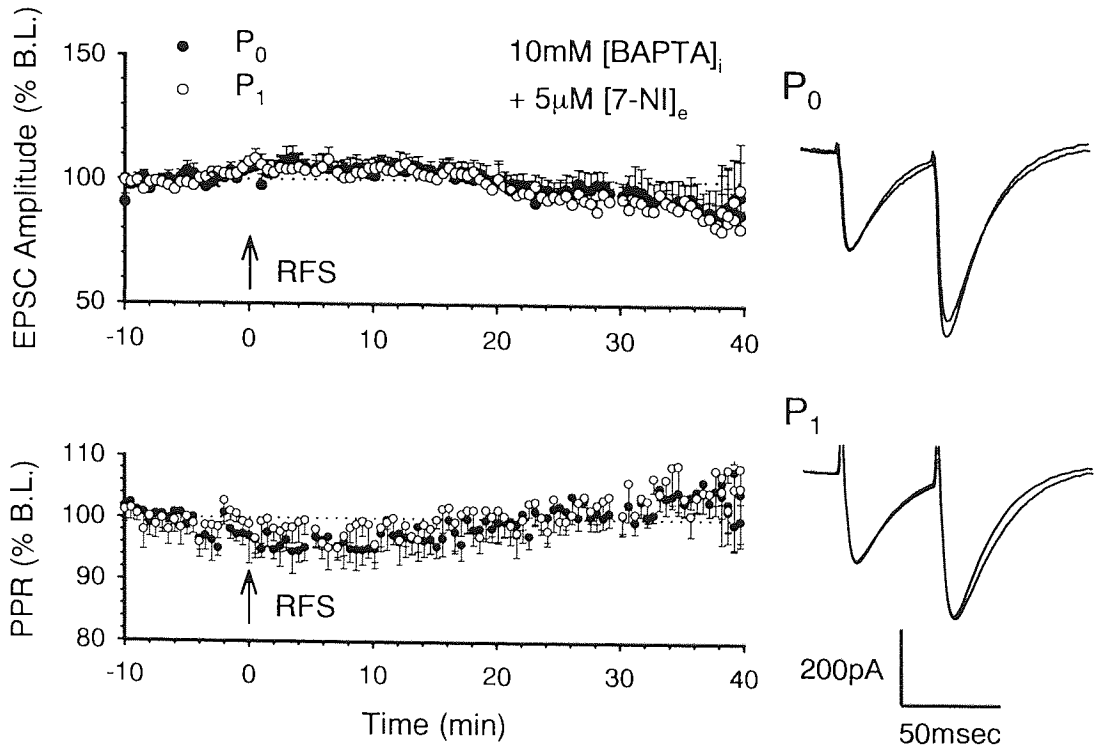


Figure 3.9 Inhibition of NOS with 7-NI in the extracellular perfusate prevented LTP in pathways P_0 and P_1. The lower graph shows the associated mean changes in PPR over time. Data are presented as in Figure 3.3.

A similar result was obtained in cells filled with 0.5 mM EGTA when RFS was accompanied by cell hyperpolarization in the presence of 5μM extracellular 7-NI (Figure 3.10). P_0 (98.7 ± 12%) and P_1 (100.8 ± 9.8%) responses as well as changes in PPR (100.9 ± 1.9% and 99.8 ± 0.9%) were significantly reduced after 20 minutes

compared to those measured in the absence of 7-NI ($P<0.05$, n=6, Mann-Whitney-U test, Figure 3.10).

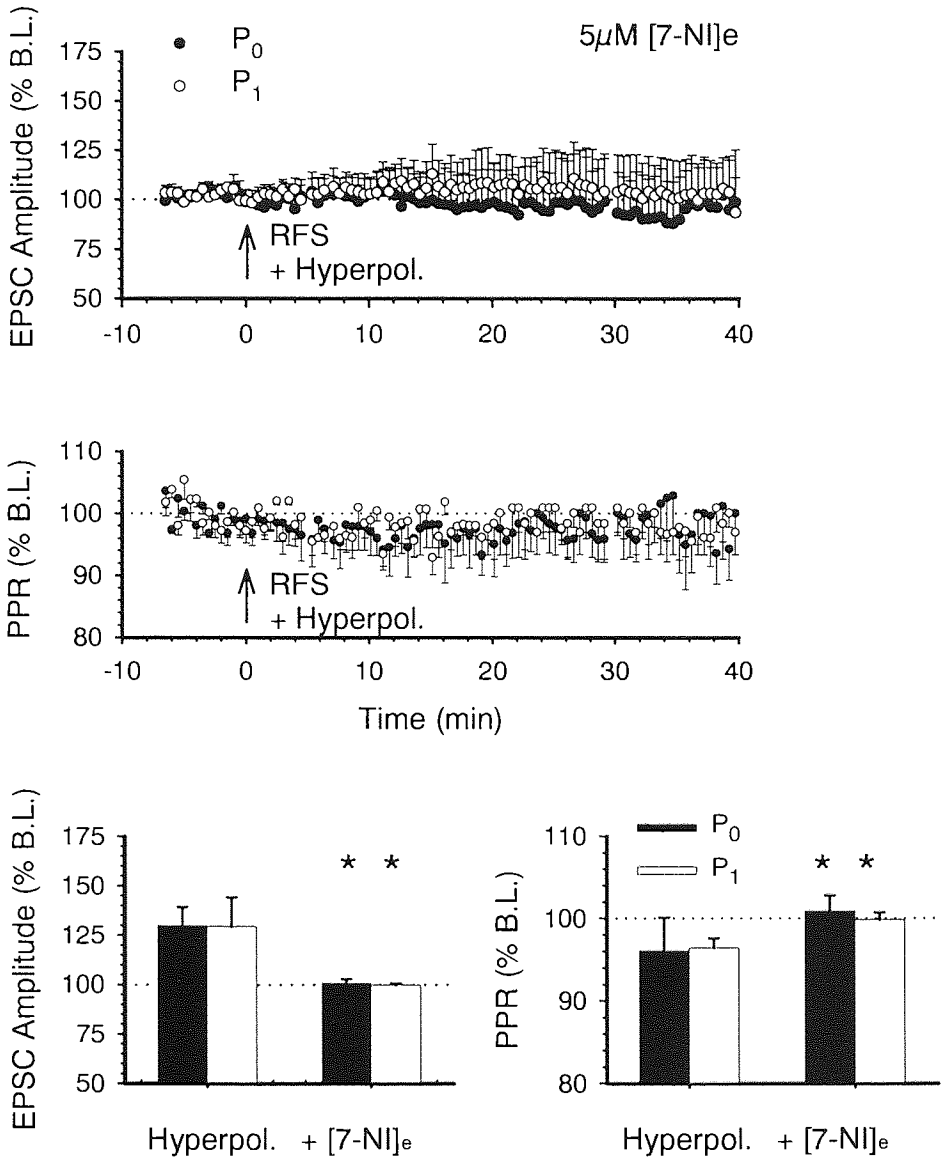


Figure 3.10 The effects of RFS to P_0 combined with cell hyperpolarization in the presence of 5 μM extracellular 7-NI. Data from 6 cells are shown as in Figure 3.3. The bar charts below provide a comparison between P_0 (filled bars) and P_1 (open bars) responses recorded in the absence and presence of 7-NI 20 minutes after RFS. Paired asterisks represent a statistical significance of $P<0.01$ between test and control conditions (Mann-Whitney U-test, n=6). Single asterisks, $P<0.05$.

Since these results contradicted an earlier study in which N^G-Nitro-L- arginine (L-NARG) failed to block LTP (Salin *et al.*, 1996), the effects of another more membrane permeable, non-selective NOS inhibitor N^G-Nitro-L- arginine methyl ester (L-NAME)

were studied (Moore *et al.*, 1990). At a concentration of 100 μM, L-NAME also completely blocked LTP and the associated decrease in the PPR (n = 5, Figures 3.11 and 3.13).

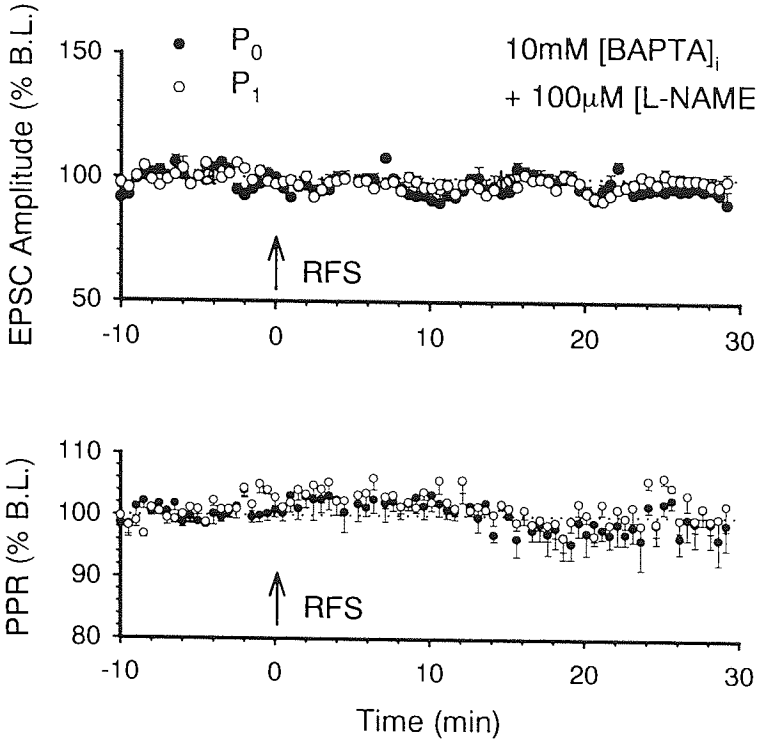


Figure 3.11 Inhibition of NOS with L-NAME prevented the induction of RFS mediated potentiation on both pathways. The PPR did not undergo any changes under these conditions. Data are presented as in Figure 3.3.

It is possible that the failure of the previous study to block LTP with L-NARG most likely reflects poor potency due to its low membrane permeability. Application of 5 μM 7-NI to slices 5 minutes after RFS failed to prevent or reverse potentiation in either pathways (142.0 ± 13.5 and 138.2 ± 20.0% of baseline levels respectively after 20 minutes, n=4, Figure 3.13) and had no effect on the associated decrease in the PPR (91.7 ± 1.9 and 94.4 ± 2.5% of baseline levels respectively after 20 minutes, n=4, Figure 3.13). A representative example illustrating the time course of the effect is shown in Figure 3.12.

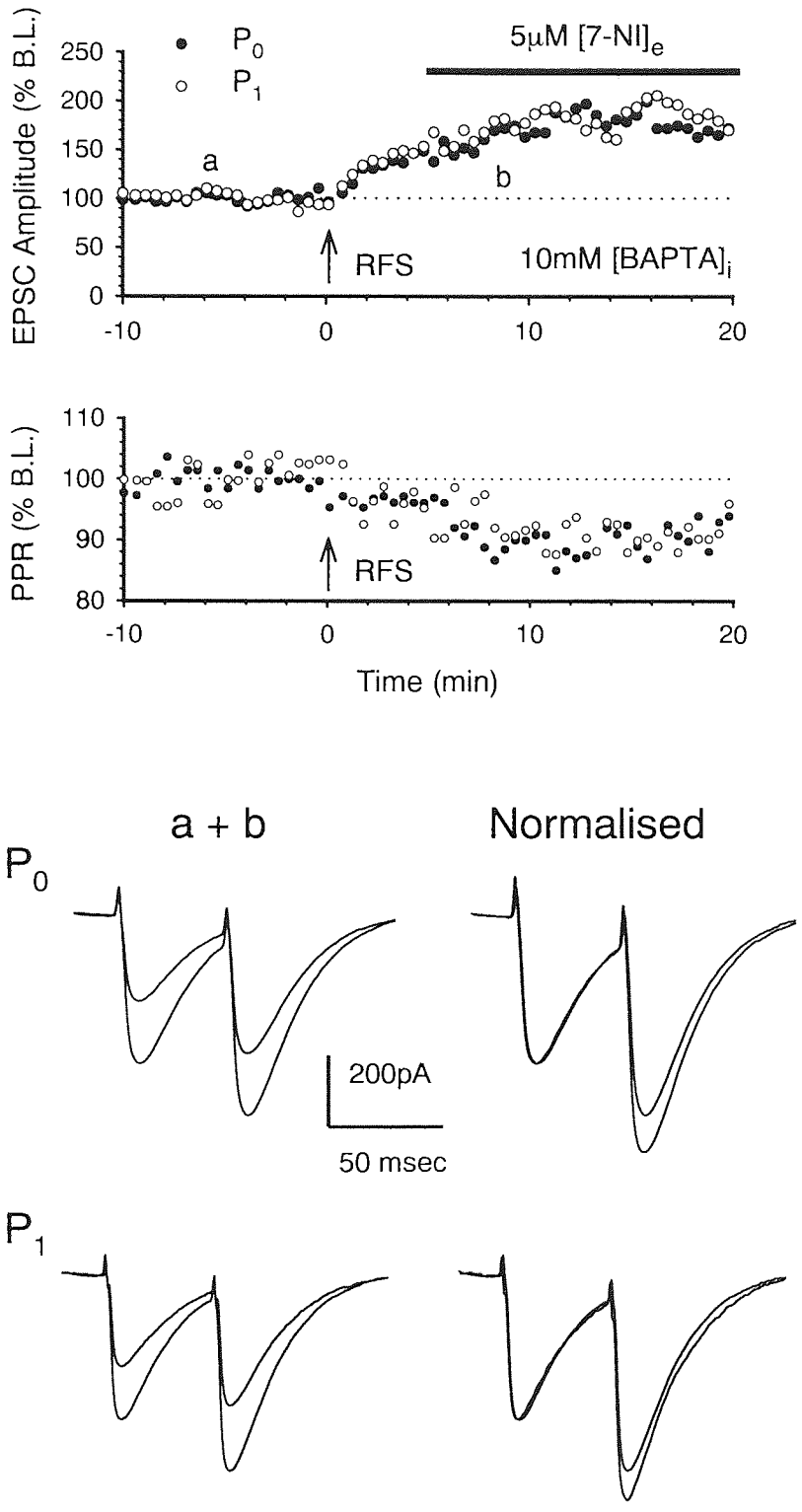


Figure 3.12 In this and three other examples application of 5μM 7-NI to the bathing medium 5 minutes after RFS failed to reverse or reduce the extent of LTP in either P_0 or P_1 nor did it affect the associated reduction in PPR. Below are representative P_0 and P_1 traces recorded at times a and b.

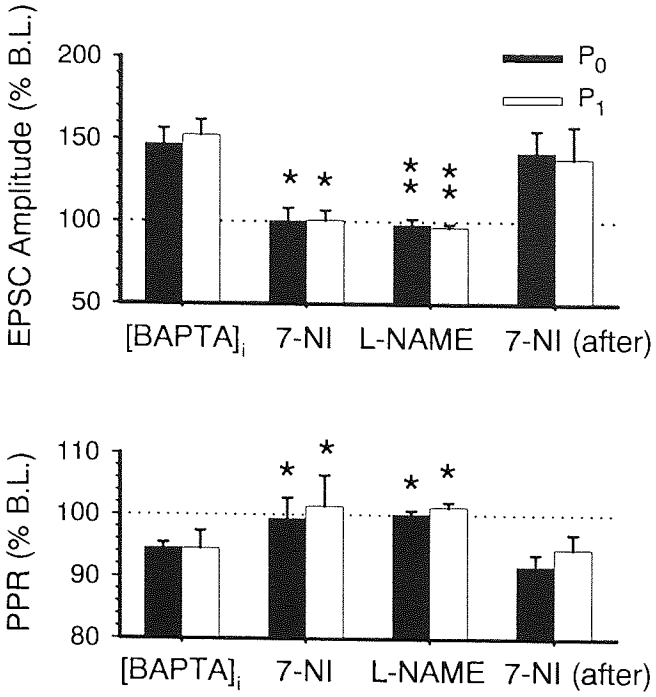


Figure 3.13 A comparison of P_0 and P_1 responses recorded under standard control conditions of 10 mM intracellular BAPTA and in the presence of 7-NI and L-NAME. Application of 5 μM 7-NI to the bathing medium 5 minutes after RFS had no effect on the extent of LTP in either P_0 or P_1. The associated changes in PPR are shown in the lower graph. The means and standard errors of 6 (7-NI), 5 (L-NAME) and 4 (7-NI application 5 minutes after RFS) cells are shown. Asterisks indicate a statistical difference between test and control conditions (Mann-Whitney U-test, *P<0.05; **P<0.01).

3.3.3 cGMP AND PKG ARE NOT REQUIRED FOR HETEROSYNAPTIC LTP

Addition of the selective guanylate cyclase inhibitor ODQ (Garthwaite *et al.*, 1995) at a concentration of 5μM to either the perfusion medium (Figure 3.14) or to the internal patch solution (Figure 3.15) did not prevent RFS induced potentiation in either pathway. P_0 (132.9 ± 7.6 and 120.9 ± 10.4%) and P_1 (141.2 ± 13.3 and 120.5 ± 4.9%) responses remained significantly elevated above baseline 20 minutes after RFS (Figure 3.15; $P<0.05$, n=6, Wilcoxon signed-rank test). The potentiation was also accompanied by a significant reduction in PPR in both pathways under both experimental conditions ($P<0.05$, n=6; Figures 3.14-3.16). The decrease in the duration of potentiation obtained when ODQ was applied extracellularly may have simply been due to experimental variability since inclusion of ODQ into the recording patch pipette or extracellular inhibition of PKG did not effect the amount or duration of potentiation.

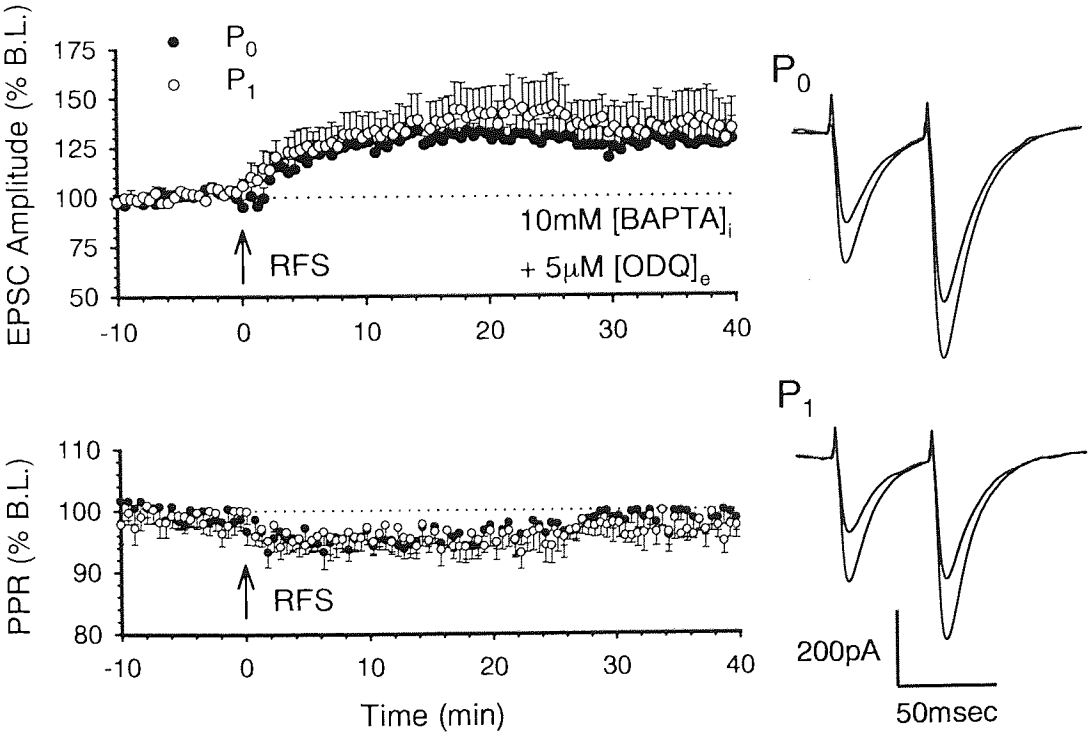


Figure 3.14 Inhibition of guanylate cyclase with 5μM ODQ applied to the extracellular perfusate did not prevent LTP in either P_0 or P_1. The lower graph illustrates the associated changes in PPR over time. Data are presented as in Figure 3.3.

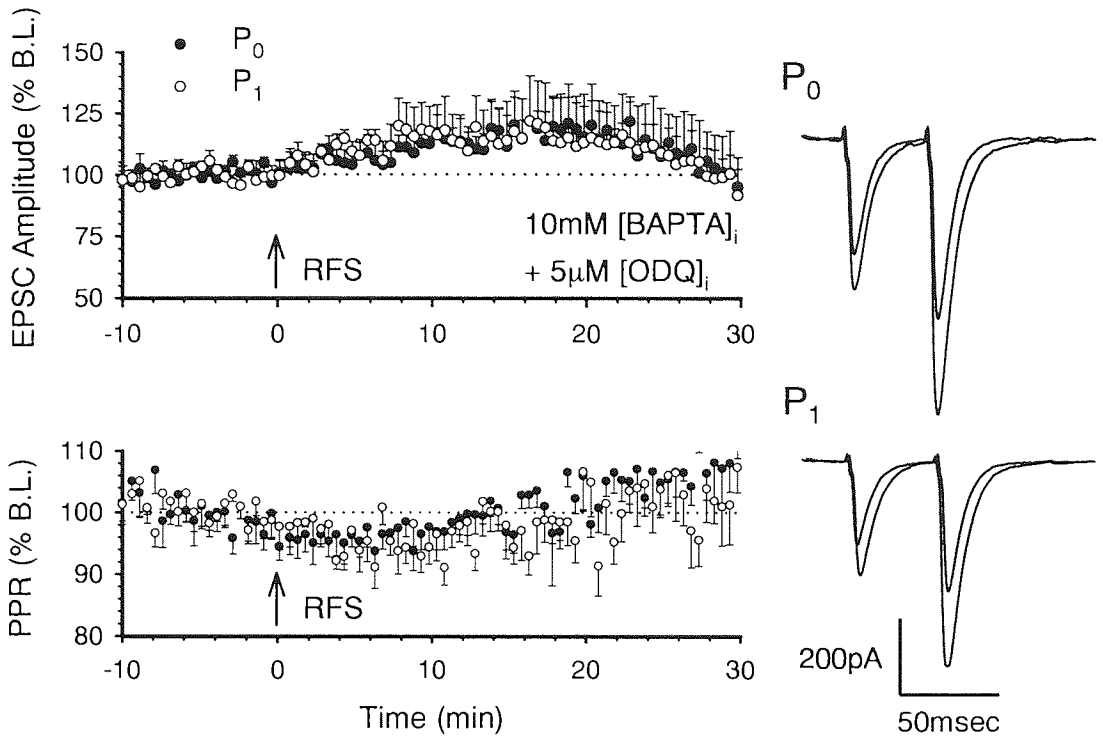


Figure 3.15 Potentiation and the accompanying decrease in the PPR were also apparent when ODQ was included in the internal patch pipette (n=6). Data are presented as in Figure 3.3.

A similar potentiation (118.8 ± 15.2% and 114.1 ± 7.1%) and decrease in PPR (97.2 ± 1.3% and 96.2 ± 0.8%) were induced in both pathways in the presence of extracellular ODQ when cells containing 0.5 mM EGTA were hyperpolarized during RFS (n=6, Figure 3.16).

Intracellular inhibition of PKG with KT5823 (500 nM; Nakanishi, 1989) also failed to prevent potentiation of P_0 (124.48 ± 4.5%, n=6) and P_1 (124.0 ± 6.1%) responses and the associated decreases in PPR (90.5 ± 2.7 and 94.6 ± 0.9%; Figure 3.17). Taken together, these data suggest that in contrast to cerebellar LTD the actions of NO in cerebellar LTP are not mediated by cGMP.

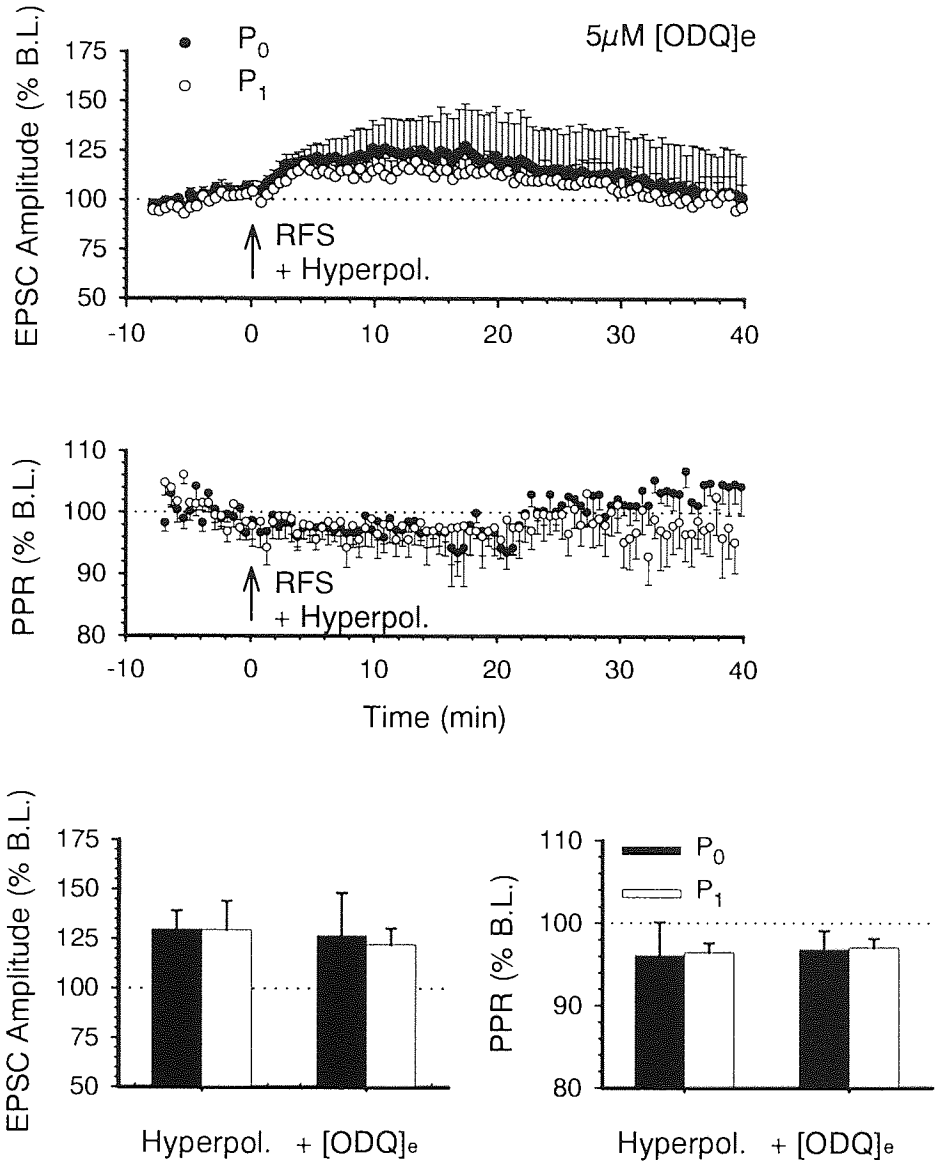


Figure 3.16 Inhibition of guanylate cyclase did not prevent the induction of LTP or the associated decrease in PPR that followed RFS during which cells filled with 0.5 mM EGTA were hyperpolarized. A comparison of PF responses and the PPR measured 20 minutes after RFS in absence and presence of ODQ are shown in the bottom graphs.

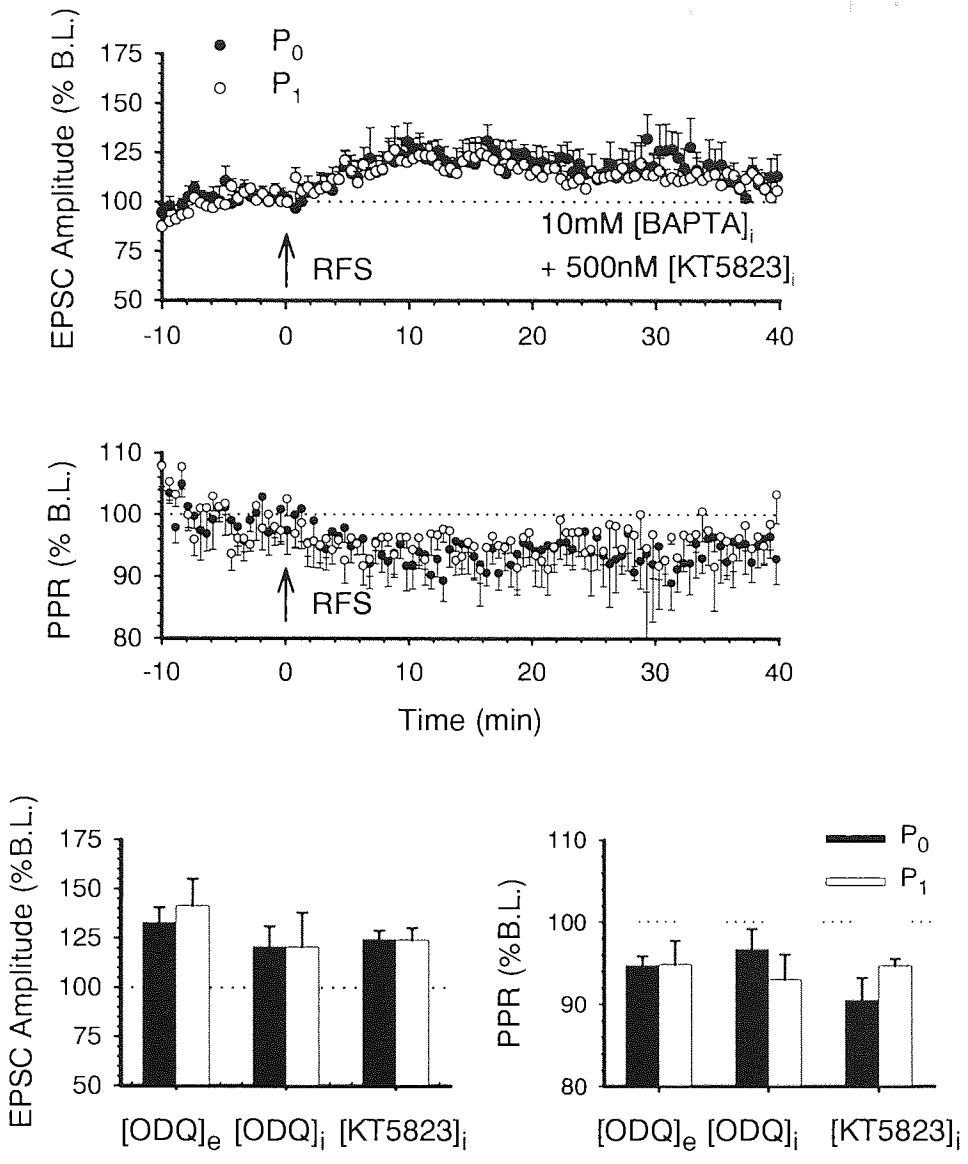


Figure 3.17 Inhibition of protein kinase G with 500 nM KT5823 applied intracellularly had no effect on RFS induced potentiation or the associated decrease in PPR. The bottom graph illustrates a comparison of P_0 and P_1 responses recorded under standard control conditions of 10 mM intracellular BAPTA and in the additional presence of ODQ or KT5823. The associated changes in PPR are shown. In all cases, the means and standard errors of 6 cells are shown.

3.3.4 An Examination Into The Nature Of The Interaction Between cAMP/PKA And NOS

To further examine the mechanism of heterosynaptic potentiation, the adenylyl cyclase activator forskolin (Seamon & Daly, 1986) was used. Forskolin induces a pharmacological form of LTP that resembles RFS induced LTP in that it is PKA sensitive and it occludes further potentiation by tetanic stimulation of parallel fibres (Salin *et al.*, 1996). As shown in Figure 3.18, bath application of 10 μM forskolin for 10 minutes whilst P_0 and P_1 responses were activated at a constant rate of 0.2 Hz led to a gradual increase in EPSC amplitudes that persisted after washout. P_0 and P_1 responses rose significantly to 149.1 ± 17.0% and 152.3 ±16.6 of baseline levels respectively after 20 minutes ($P<0.05$, n=6, Wilcoxon signed-rank test; Figure 3.18). As with RFS induced LTP, this potentiation was accompanied by a significant reduction in PPR in both pathways (Figure 3.18 and 3.19).

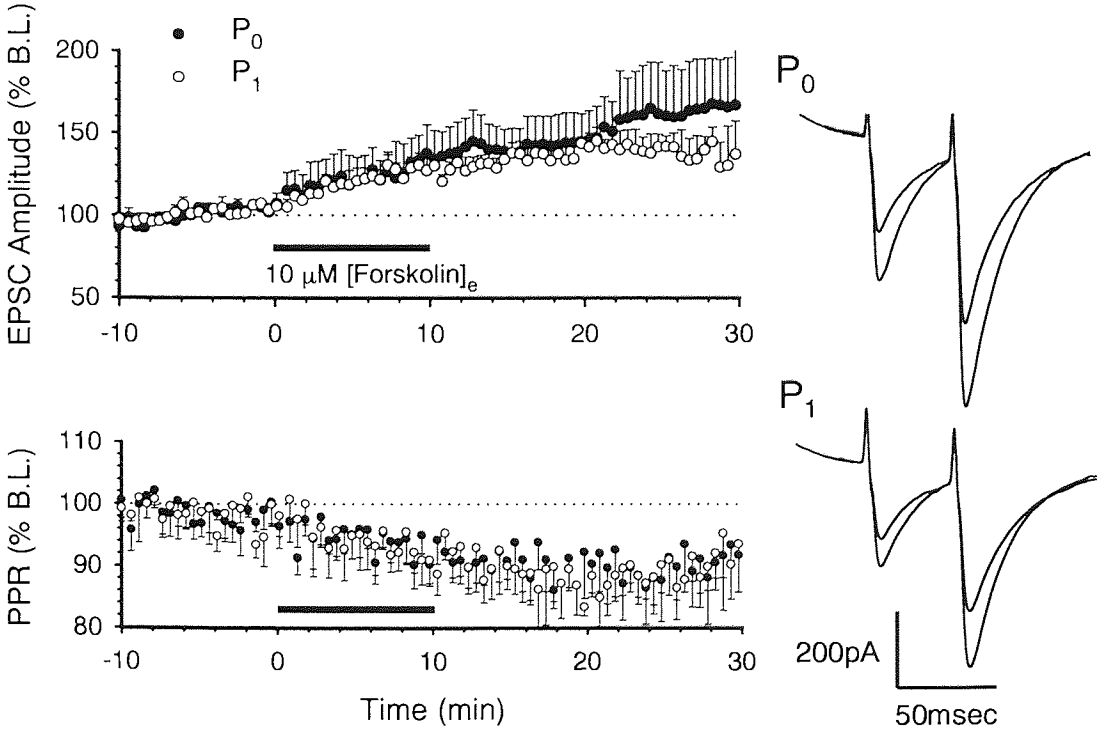


Figure 3.18 10-minute bath application of 10 μM forskolin led to a sustained potentiation of P_0 and P_1 responses and a decrease in PPR. Data are presented as in Figure 3.3.

In view of the fact that both NO and cAMP/PKA are required for LTP, a series of experiments were undertaken to establish whether NO activated cAMP/PKA or *vice versa*. In the presence of 5 μM 7-NI, 10μM forskolin failed to induce potentiation. P_0 and P_1 responses after 20 minutes were 104.4% ± 3.0 and 103.0 ± 6.3% of baseline, significantly different from responses recorded in the absence of NOS inhibition ($P<0.01$, n=6, Mann-Whitney-U test; Figure 3.19). No significant decrease in the PPR was observed compared to control data (97.42 ± 4.21% and 96.10 ± 3.21%, $P<0.05$, n=6, Mann-Whitney-U test, Figure 3.19).

This result might indicate that cAMP/PKA activates NOS but does not rule out the alternative possibility that both systems are required for a long-lasting potentiation. The possibility that NO application alone was capable of inducing potentiation was therefore investigated. 10-minute bath application of the NO donor spermine NONOate (Maragos *et al.*, 1991) resulted in an increase in PF responses (150.8 ± 13.7 and 152.4 ± 9.1%, n=6) measured 20 minutes after NO application (Figure 3.20). Spermine NONOate induced potentiation was accompanied by a reduction in PPR (94.1 ± 2.9 and 94.3 ± 4.0%, n=6, Figure 3.20). These observations suggest that cAMP/PKA is required for NOS activation and that the two systems most likely work in series rather than in concert.

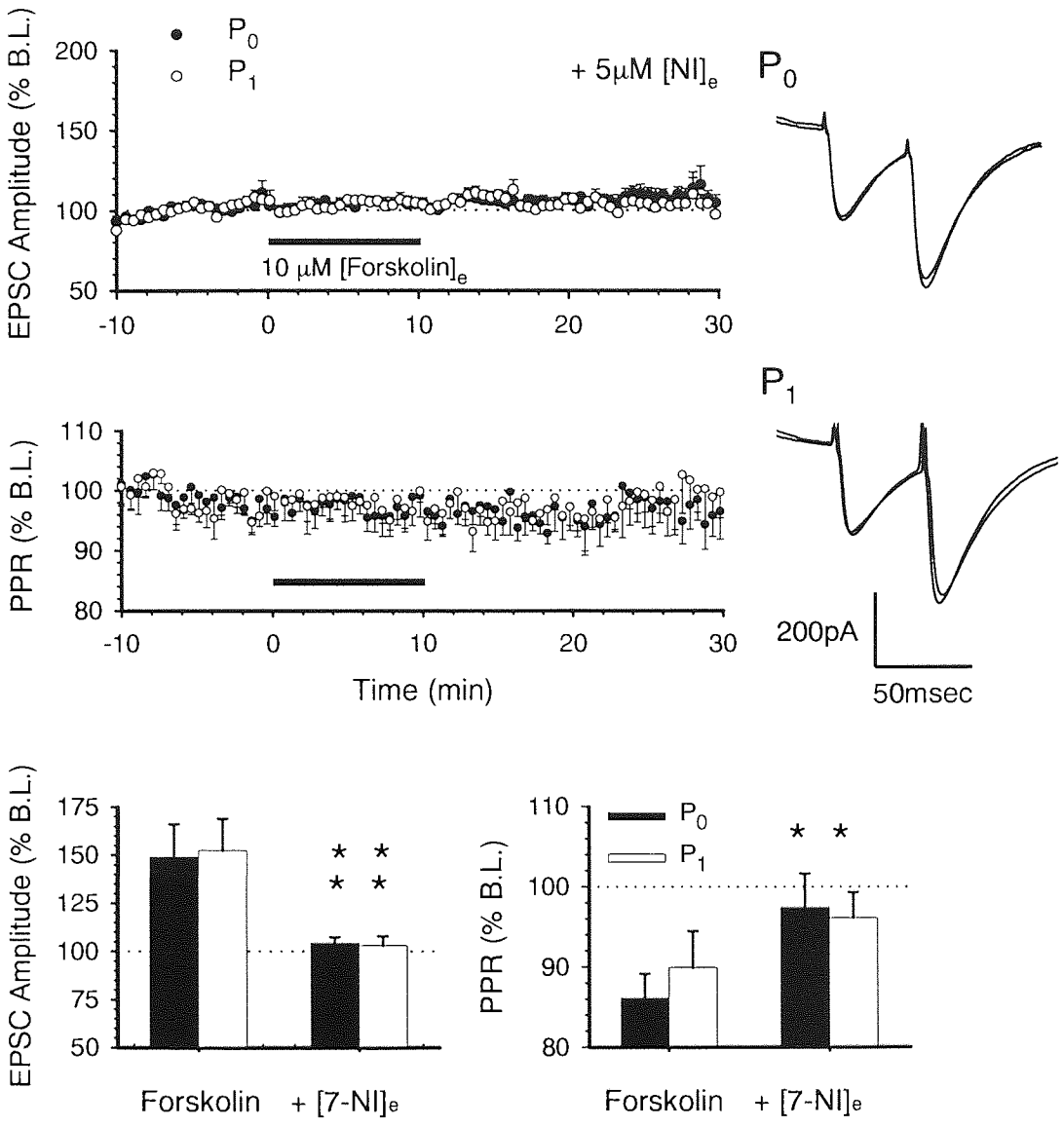


Figure 3.19 Inhibition of NOS with extracellular 7-NI prevented forskolin induced LTP and the associated decrease in PPR. A comparison of P_0 and P_1 responses recorded in the presence of forskolin and additionally with 7-NI is demonstrated in the bottom graph. Paired asterisks represent a statistical significance of $P<0.01$ between test and control conditions (Mann-Whitney U-test). Single asterisks, $P<0.05$. (D) The associated changes in normalised PPR are illustrated. The means and standard errors of 6 cells are shown.

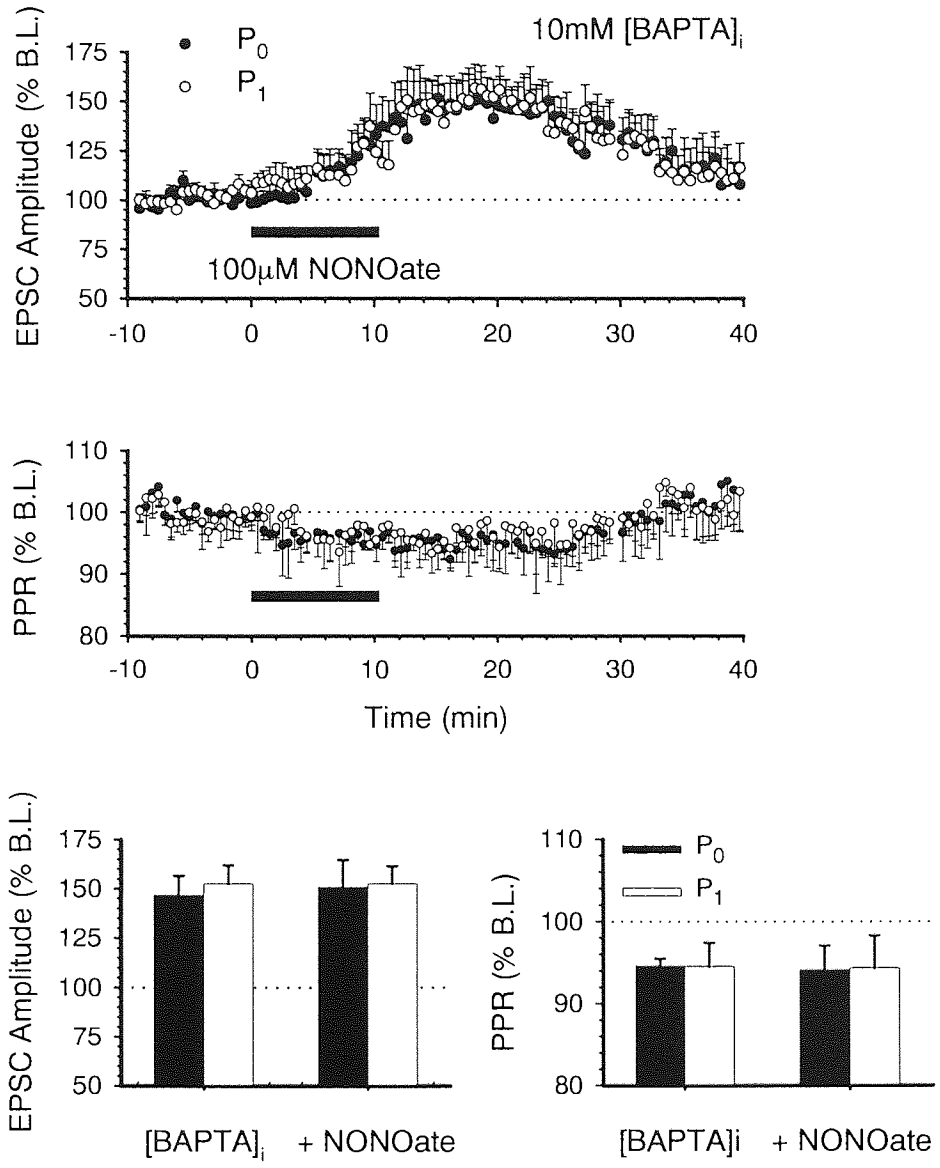


Figure 3.20 10 minutes bath application of the NO donor spermine NONOate induced an increase of responses and a decrease in PPR in both pathways. The lower graphs illustrate a comparison of P_0 and P_1 responses and the changes in PPR measured 20 minutes after RFS under control conditions of 10mM BAPTA and 20 minutes after NONOate application.

3.3.5 The Site Of Origin Of Cerebellar LTP

The data described so far are consistent with the view that forskolin is capable of inducing potentiation and that this potentiation is NOS sensitive. Potentiation was consistently accompanied by a decrease in PPR indicating a presynaptic site of origin. Since changes in PPR are suggestive rather than prove a presynaptic site of action further attempts to clarify the site of potentiation were made by investigating the frequency and amplitudes of mEPSCs before and after forskolin application in the presence of 1 μM TTX. Figures 3.21 and 3.22 illustrate that application of forskolin led to a significant increase in the frequency of mEPSCs over 4 minute epochs measured 15 minutes after application compared to similar baseline periods prior to application ($P<0.01$, Kolmogorov Smirnov Test). Forskolin application was also associated with a small increase in the amplitude of responses shown by a rightward shift in the cumulative probability plot. Control experiments revealed no change in mEPSC frequency or amplitude over the same time course (Figure 3.21).

Next, experiments were undertaken to test whether extracellular application of 7-NI was capable of preventing forskolin mediated increases in the frequency of mEPSCs. Figure 3.21 and 3.22 reveal that forskolin failed to significantly increase mEPSC frequency in the presence of 7-NI. Moreover, application of spermine NONOate mimicked the effect of forskolin alone (Figures 3.21 and 3.22). These data lend further support to the notion that forskolin induced LTP is of presynaptic origin and that potentiation requires the presynaptic activation of NOS and the production of NO.

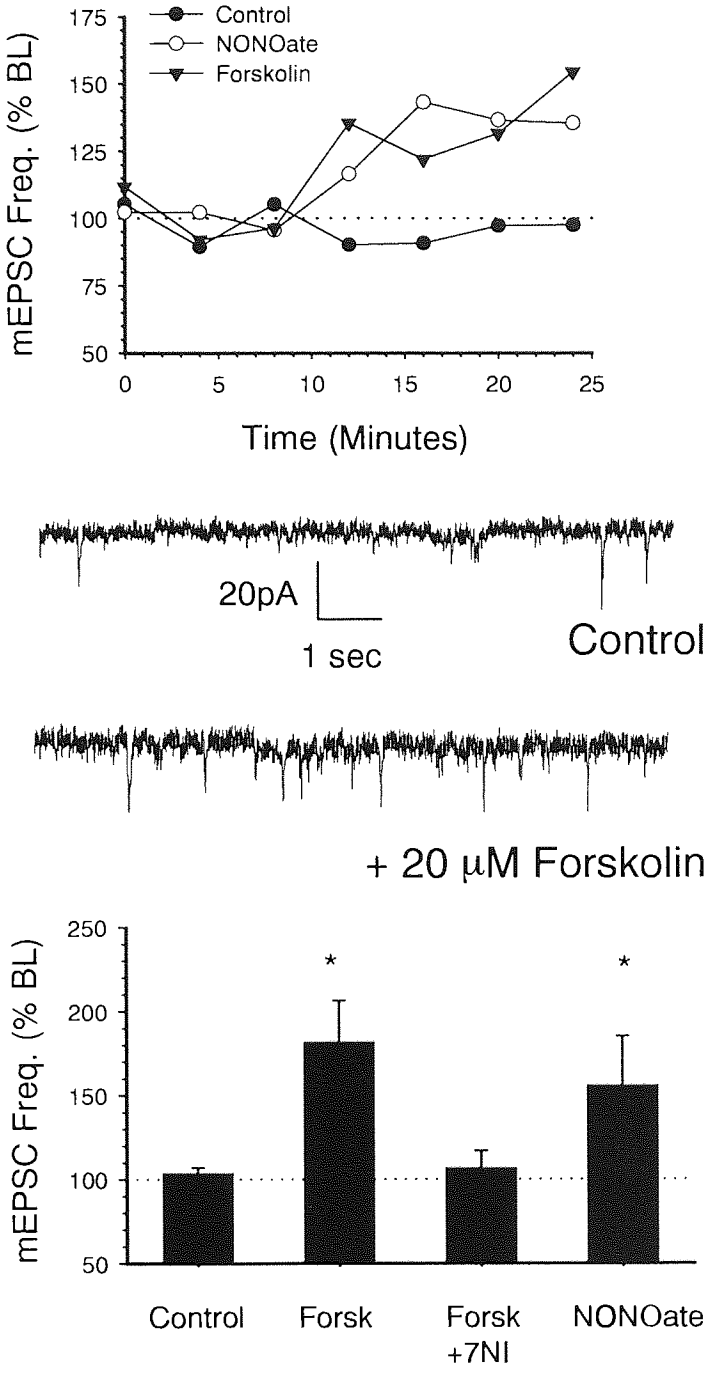


Figure 3.21 Changes in mEPSC frequency over time expressed as a percentage of baseline levels under control conditions of 10mM intracellular BAPTA. The effects of application of 10μM Forskolin and 100μM NONOate are shown. The bottom graph shows the means and standard errors of mEPSC frequency recorded under standard conditions, in the presence of forskolin, in the additional presence of 7-NI and after NONOate application. Asterisks represent a statistical difference ($P<0.01$) between control and test conditions (Kolmogorov Smirnov Test).

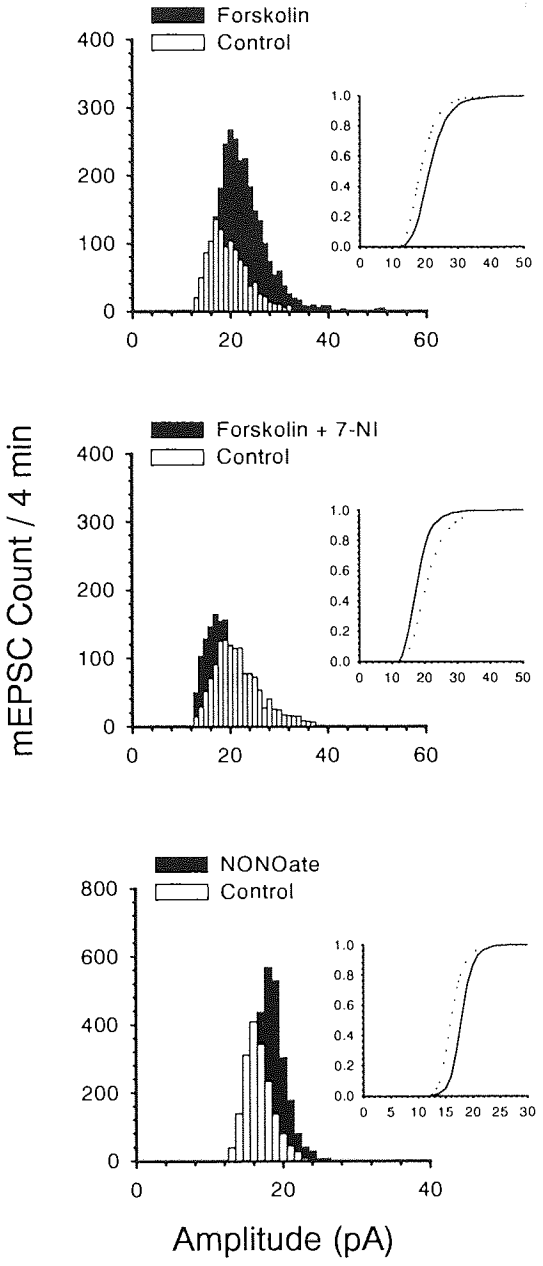


Figure 3.22 The frequency distributions of mEPSCs over two four minute periods, prior to (open bars) and 15 minutes after application of forskolin (closed bars) in absence and presence of 7-NI and the frequency distributions of mEPSCs recorded prior to and 15 minutes after application of 10 μM spermine NONO-ate are shown. Insets show cumulative probability curves generated from the same data sets. Dotted lines illustrate the cumulative distribution of mEPSC amplitudes under control conditions. Solid lines show the effect after drug application (see section 3.3.5).

3.3.5 Measurement Of Pre-Synaptic Release And Uptake With The Styryl Dye FM1-43

The results presented so far suggest that RFS of a limited number of PF synapses not only enhances transmitter release at those active synapses, but that transmitter release at distant, non-activated synapses is also increased. As an alternative approach to examine the spatial extent of this spread during the induction of LTP the styryl dye FM1-43 was used to monitor exocytotic de-staining following RFS. Slices were incubated with 10 μM FM1-43 and then either washed for at least two hours to wash off nonspecific staining of cell – surface membranes or perfused with 50 μM sulforhodamine. Both the emission and absorption spectra of suforhodamine (solid traces, Figure 3.23) fall within the emission spectrum of FM1-43 (dashed trace, Figure 3.23). Therefore an emission filter was used with a bandpass at 530 ± 20 nm, a range of wavelengths over which FM1-43 emits but suforhodamine does not, to quench unbound FM1-43 (Pyle *et al.*, 1999).

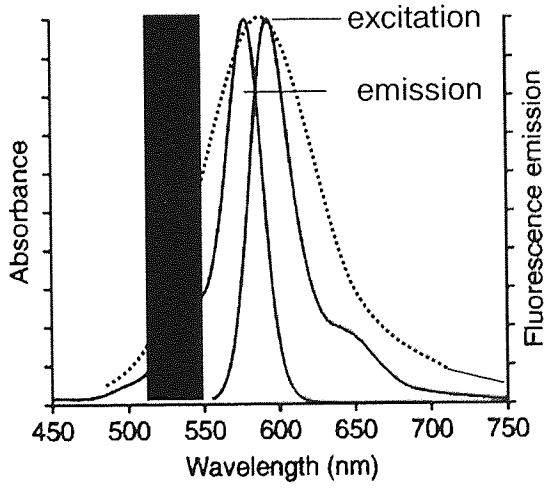


Figure 3.23 The emission spectrum of FM 1-43 (dashed trace) is superimposed on excitation and emission spectra of S-Rh (solid traces). The shaded bar illustrates the emission filter of 530 ± 20 nm.

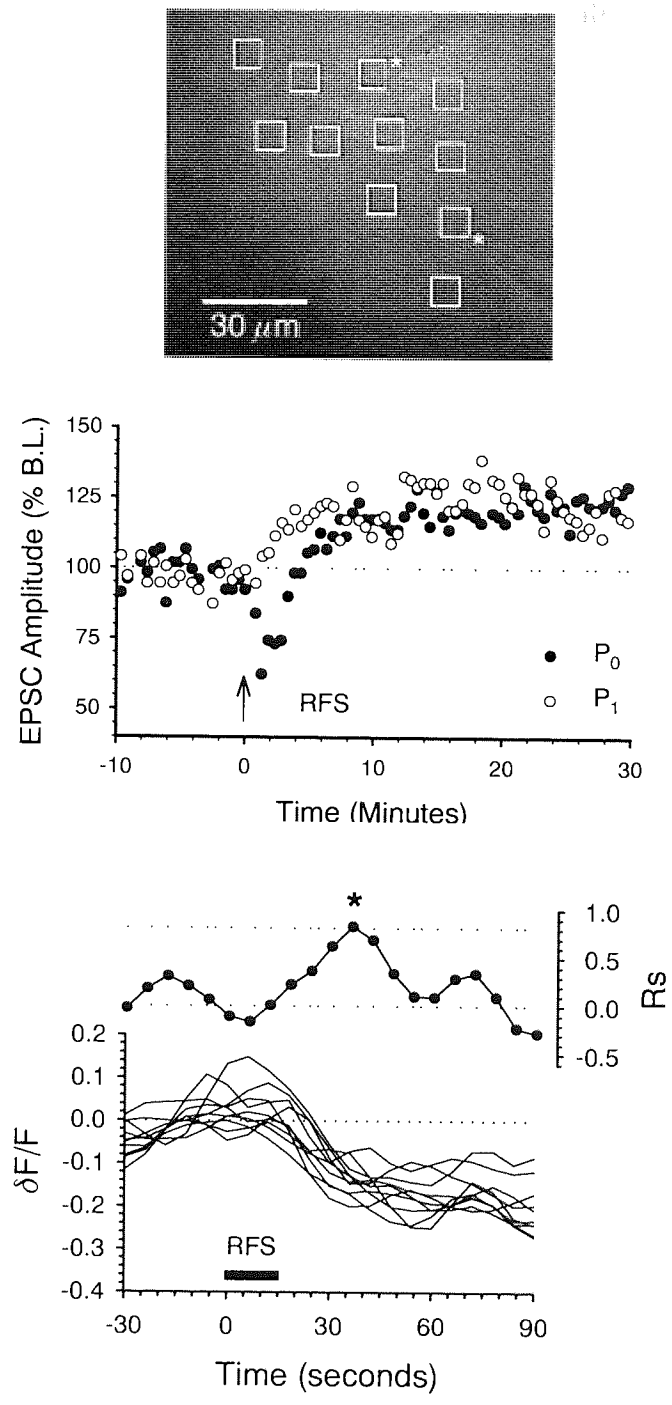


Figure 3.24 Fluorescent image of a slice loaded with 10 μM FM1-43 for 90 seconds in the presence of 50mM KCl and later perfused with 50 μM sulforhodamine. This image illustrates the relative positions of two stimulating electrodes (P_0 and P_1) placed at a separation of 56 μm within the molecular layer and the positions of 10 ROIs. The graph in the middle illustrates the electrical response to RFS applied to P_0 after a ten-minute baseline period. Fluorescence images captured before and after RFS revealed that RFS was accompanied by a reduction in FM1-43 fluorescence shown in the bottom graph. Plotted above are the Spearman Rank correlation coefficients (Rs) calculated over time. These were obtained by correlating the size of the fluorescence reduction for a given ROI with its distance from the point of stimulation (P_0). A statistically significant correlation was apparent within 30 seconds of RFS ($P<0.05$).

Whereas the presence of sulforhodamine did not appear to qualitatively affect the results it did enhance the sensitivity of the measurements, presumably because of our increased ability to resolve punctate like structures from non-specific, non-synaptic background fluorescence (Pyle *et al.*, 1999). Picrotoxin was used throughout at a concentration of 20 μM. Figure 3.24 provides a representative example from a total of 4 separate slices of the spatial and temporal pattern of FM1-43 de-staining within the molecular layer as a consequence of 8Hz RFS. In accordance with our earlier observations, RFS to P_0 induced a long-term potentiation that spread to synapses within the range of a second electrode placed, in this case, 58 μm away (P_1). RFS was associated with a concomitant reduction in FM1-43 fluorescence (Figure 3.24).

Measurements of the relative changes in fluorescence normalised to the initial fluorescence level for each ROI revealed a strong inverse correlation between the extent of the de-staining and the distance from the point of RFS (Figure 3.25). The greatest correlation occurred within 30 seconds of stimulation.

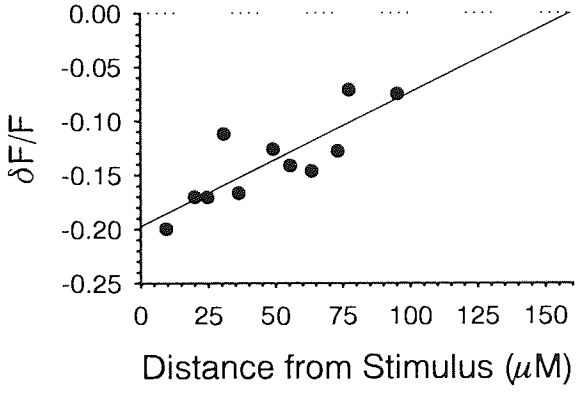


Figure 3.25 The individual changes in fluorescence for each ROI at the point of maximum significance marked with an asterisk in the lower part of Figure 3.24 are plotted against their distance from the point of stimulation. The extrapolated regression line predicts that 15 second RFS to P_0 may influence FM1-43 de-staining as far away as 150 μm.

Interestingly, following stimulation, fluorescence levels generally recovered to baseline levels and in some cases even exceeded baseline levels (data not shown). Since sulforhodamine selectively masks the presence of extracellular dye, it is possible that this recovery of fluorescence reflects reuptake of released FM1-43 that was quenched when released to the extracellular compartment. Therefore a long-term increase in the rate of endocytosis following RFS might take place that accounts for this re-staining.

Although it is not possible to concurrently monitor synaptic responses and the induction of LTP in the presence of AMPA receptor blockade, similar patterns of FM1-43 de-staining were observed in the presence of 10 μM CNQX (n=4) or picrotoxin. Therefore, neither excitatory nor inhibitory synaptic transmission contributes to de-staining. De-staining was also produced by perfusion of slices with solutions containing 50 mM potassium (n=6), 10 μM forskolin (n=8) or 100 μM spermine NONOate (n= 5 slices; Figure 3.26). The effects of potassium and forskolin were completely blocked by the inclusion of cadmium into the perfusate, however NONO-ate induced de-staining was unaffected (Figure 3.26). The effects of forskolin were also blocked by 7-NI. Together, these data suggest that potassium and forskolin induced exocytosis are calcium dependent whereas NO-induced exocytosis is not. The data concur with our electrophysiological results in that forskolin induced transmitter release depends upon NOS activity and taken together they suggest that the actions of NO on transmitter release are downstream of calcium influx, cAMP production and PKA activation. This finding is consistent with recent reports in which NO was found to stimulate calcium-independent transmitter release from synaptosomes (Meffert *et al.*, 1994) by modulating the affinity of proteins involved in transmitter release, effectively promoting vesicle docking/fusion (Meffert *et al.*, 1996).

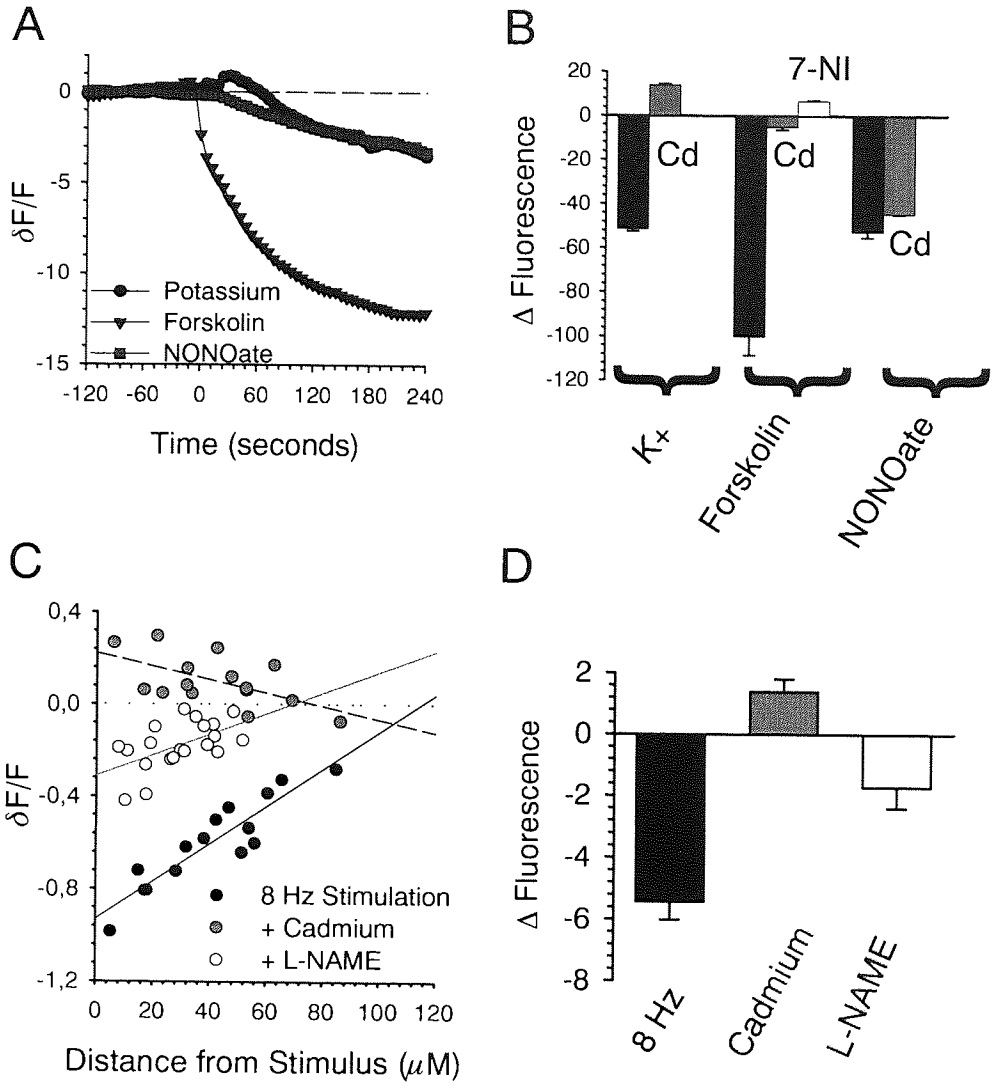


Figure 3.26 (A) The effects of bath applications of 50 mM potassium, 10 μM forskolin and 100 μM NONOate on cerebellar slices incubated FM1-43 are shown. Data from up to 20 separate ROIs were normalised to the initial fluorescence and expressed as δF/F. (B) The absolute changes in fluorescence taken 4 minutes after application of each of these compounds are shown (black bars) together with their effects in the additional presence of cadmium (Cd, grey bars) or 7-NI (white bars). (C) The relationships between the extent of the mean fluorescence change in ROIs placed at random within the molecular layer from the point of electrical stimulation are shown for three examples carried out under control conditions (black circles) and at identical stimulus strengths in the presence of cadmium (grey circles) and L-NAME (open circles). Linear regression lines have been fitted to the data. (D) The absolute changes in fluorescence for data taken from at least 4 slices in each case during 8Hz stimulation and in the presence of cadmium or 100 μM L-NAME are shown.

RFS mediated de-staining was also prevented by the inclusion of cadmium into the bathing medium (Figures 3.26; n= 5 slices). No correlation between the extent of de-staining in a given ROI and its distance from the site of stimulation was apparent, even at high stimulus intensities. In contrast, L-NAME reduced the overall level of de-staining over a similar sized area of molecular layer compared to control experiments undertaken at identical stimulus strengths. However, a clear inverse correlation with distance from the point of stimulation was still apparent (Figure 3.26). By extrapolating the lines of best fit to zero, these data indicate that L-NAME did not prevent de-staining associated with transmitter release but it did reduce the overall spread of de-staining at a given intensity by as much as 50 μm.

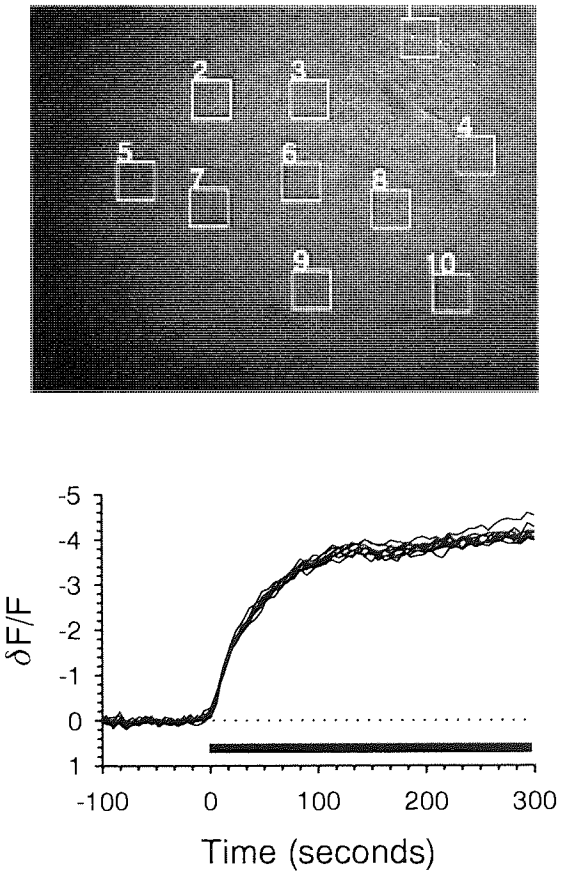


Figure 3.27 The fluorescence image on the top illustrates the ten ROIs distributed over the slice. Bottom graph: Application of 100μM NONOate resulted in some cases in an increase in FM1-43 fluorescence The black bar represents the duration of NONOate application.

Interestingly, in some experiments an increase rather then a decrease in the level of fluorescence was observed following pharmacological activation (Figure 3.27). This may be explained by the fact that sulforhodamine does not remove surface bound FM1-43 it merely quenches it, therefore extracellular FM1-43 dye might in some cases be re-taken up. To test if the level of depolarization of the cells determines if endocytosis or exocytosis predominates 20mM potassium was next applied to a slice on which NONOate application has previously resulted in an increase in fluorescence (Figure 3.27). The initial increase in fluorescence was followed by a sustained decrease suggesting that the cells may not have fully loaded during the incubation period and are therefore still on balance taking up FM1-43. When NONOate was additionally applied a steeper decrease was observed which recovered after washout of NONOate (Figure 3.28).

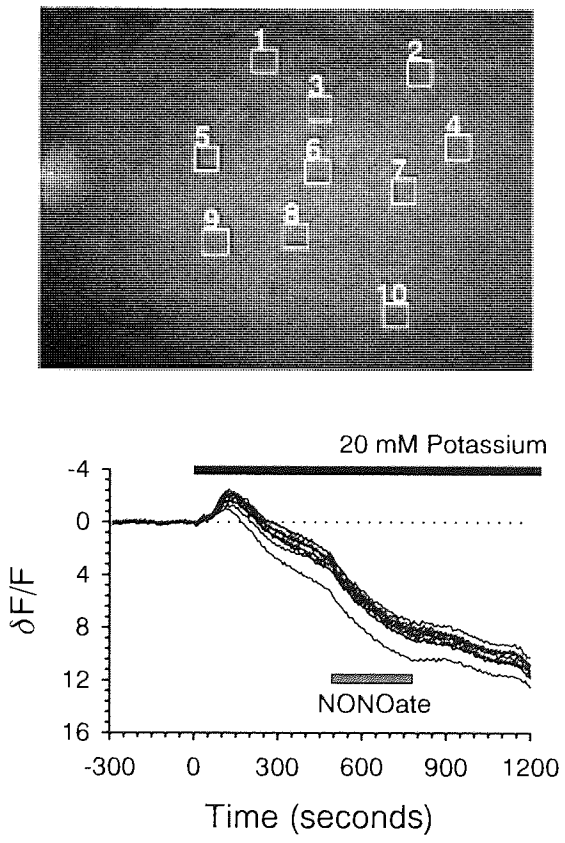


Figure 3.28 Application of 20mM potassium at time 0 resulted in an initial increase followed by a reduction in FM1-43 fluorescence, which was enhanced after application of NONOate at 400 seconds and reduced after washout of NONOate at 750 seconds.

3.4 Discussion

The results presented in this chapter concur with previous data obtained in cerebellar slices (Salin *et al.*, 1996; Chen & Regehr, 1997) and in culture (Linden, 1997; Linden, 1998) in three ways. First, under conditions of reduced post-synaptic calcium activity, raised frequency stimulation to PFs induced a long-lasting potentiation of synaptic responses at the PF-PC synapse. Second, LTP was associated with a reduction in paired pulse facilitation, suggesting a presynaptic locus. Third, LTP was blocked by inhibition of PKA and mimicked by forskolin, which promotes cAMP production by activating adenylate cyclase. In addition, our data reveal a number of other important new properties of cerebellar LTP. Most notably synaptic potentiation, induced even under these conservative stimulus conditions, was not restricted to the discrete set of synapses that received tetanic activation. Without exception, increases in synaptic strength were also observed at synapses several tens of microns distant. Potentiation at both P_0 and P_1 sites was accompanied by a reduction in paired pulse facilitation and an increase in FM1-43 de-staining, suggesting a presynaptic locus in each case. Long-term synaptic potentiation and FM1-43 de-staining were mimicked by applications of forskolin or the NO donor spermine NONOate. That these pharmacological forms of LTP were also associated with NOS sensitive increase in mEPSC frequency further supports a presynaptic site of origin for cerebellar LTP. In contrast to heterosynaptic LTD (Hartell, 2000), neither cGMP nor PKG were required for heterosynaptic LTP. These data show for the first time that cerebellar LTP, like LTD (Hartell, 1996; Reynolds & Hartell, 2000; Hartell, 2000; Wang & Linden, 2000), may not remain input specific and that nitric oxide plays a crucial role in the induction of cerebellar LTP and its lateral spread to distant PF synapses impinging on the same cell.

Although there are an increasing number of reports of synaptic plasticity spreading beyond individual synapses (Vincent & Marty, 1993; Reynolds & Hartell, 2000; Schuman & Madison, 1994; Fitzsimonds *et al.*, 1997), it is important to first exclude other anomalous explanations that might account for the apparent loss of input specificity that accompanied potentiation in our model. The most obvious of which is that our two PF inputs, P_0 and P_1, were not independent but essentially comprised the same or substantially overlapping sets of PF inputs. Using a modified paired pulse facilitation technique to test for pathway independence no evidence of pathway overlap was found in any example prior to LTP induction over the entire range of electrode separations (30-100 μm) and stimulus intensities used. Moreover, little or no evidence to suggest that pathway overlap increased subsequent to LTP induction was obtained. Therefore, it is reasonable to assume that the spread of LTP represents genuine, heterosynaptic potentiation.

Several pieces of evidence, in this and in earlier reports, support the view that potentiation at the PF-PC synapse is primarily of presynaptic origin. LTP induced through raised frequency PF activation is accompanied by a reduction in paired pulse facilitation in slices (Salin *et al.*, 1996). In culture, RFS induces a form of LTP in granule cell-PC pairs that is accompanied by a reduction in the subsequent EPSCs failure rate as well as a decrease in paired pulse facilitation (Linden, 1998). Potentiation of transmitter release can also be detected in neighbouring glial cells. Pharmacological activation of the cAMP/PKA cascade with forskolin (Chen & Regehr, 1997) or application of the NO donor spermine NONOate induced a form of potentiation that shares a number of properties similar to synaptically induced LTP and leads to an increase in mEPSC frequency.

There are two ways in which transmitter release may effectively be enhanced. Either the probability (p) of transmitter release for a given presynaptic stimulus may increase and/or the number (n) of release sites may rise (Atluri & Regehr, 1998; Chen & Regehr, 1997; Manabe *et al.*, 1993; Zucker, 1973; Zucker, 1989). In the latter case, this could result from an increase in release sites from the same number of activated fibres or through an increase in the number of fibres activated, i.e. a reduction in firing threshold. This last possibility was directly tested by comparing the degree of pathway overlap before and after LTP induction in both P_0 and P_1 pathways. Although the levels of potentiation at P_0 and P_1 were essentially identical, little evidence of any significant increase in pathway overlap after LTP induction was found compared to that before. This is consistent with observations that tetanic or forskolin induced LTP are not accompanied by increases in the size (Salin *et al.*, 1996) or the waveform of the PF volley (Chen & Regehr, 1997). However, whether cAMP acts preferentially to increase the probability of release and/or the number of release sites at this or at other synapses in the CNS remains a matter of debate (see for example Trudeau *et al.*, 1996; Chavis *et al.*, 1998).

3.4.1 ORIGIN OF HETEROSYNAPTIC CEREBELLAR LTP

Although a change in the degree of pathway overlap following LTP induction was not evident, a decrease in paired pulse facilitation at synapses that underwent potentiation up to 100 μm from the site of RFS was detected, leading to the conclusion that a widespread change in the probability of release most likely provides the causative mechanism for heterosynaptic potentiation. Interestingly, the reduction in paired pulse facilitation was not maintained for the duration of the potentiation. This might suggest, as with cAMP-dependent forms of LTP in other areas of the brain that early and late

phases of potentiation may exist in the cerebellar cortex that mediate synaptic enhancement through different mechanisms. Further evidence for a presynaptic locus for heterosynaptic LTP comes from our observation that FM1-43 de-staining during LTP induction did not remain restricted to the site of RFS but it was also evident in regions of the molecular layer over 100 μm from the point of stimulation.

3.4.2 Cellular Mechanism of Heterosynaptic LTP

LTP at the PF-PC synapse is prevented when external calcium is lowered during tetanic stimulation (Salin *et al.*, 1996). It has been argued that the involvement of cAMP and PKA in LTP stems from the calcium sensitivity of adenylyl cyclases. At least two calcium/calmodulin sensitive isoforms of adenylyl cyclase, types I (AC1) and VIII (AC8), are expressed in the cerebellar cortex (Xia *et al.*, 1991). Whilst activity of both isoforms is required for late phase LTP in the hippocampal mossy fibre pathway (Wong *et al.*, 1999), a single knockout of AC1 appears to be sufficient to prevent LTP in granule cell-Purkinje cell pairs in culture (Storm *et al.*, 1998). Our current observation that NOS is also critically required for the induction but not the maintenance of LTP now provides an additional mechanism that might contribute not merely to the presynaptic calcium sensitivity of LTP in the cerebellar cortex, but also to its laterograde spread.

There are a number of ways in which calcium, NO and cAMP/PKA could interact to produce LTP. It is already known, however, that forskolin induced LTP is not accompanied by an increase in influx or basal levels of calcium in presynaptic PF terminals (Chen & Regehr, 1997). It is unlikely then that a sustained or prolonged calcium signal sub-serves the expression of LTP, either directly or indirectly through

prolonged activation of NO or cAMP/PKA. Therefore, given that both neuronal NOS and AC1 are both calcium sensitive, it is more probable that the transient calcium influx following RFS triggers either a serial interaction of NO and cAMP/PKA or alternatively, these two messenger pathways might both be required to produce a potentiation of transmitter release.

Forskolin induced potentiation was completely blocked by 7-NI. As forskolin is unable to induce potentiation if NOS activity is blocked the possibility that NO merely activates cAMP/PKA can be discarded. Therefore, a contrary scenario whereby cAMP/PKA facilitates NO production or one in which both messengers are required for LTP is more probable. There are a number of lines of evidence to suggest that cAMP/PKA might activate NO. Forskolin has been shown to potentiate NO release from the molecular layer of the cerebellar cortex and tetanus induced potentiation of NO release is sensitive to PKA inhibition (Kimura *et al.*, 1998). Although nNOS has several phosphorylation sites that are recognized by kinases (Bredt *et al.*, 1992), including PKA and PKC (Okada, 1992) direct evidence of PKA modification of NOS activity is limited (Inada *et al.*, 1998; Inada *et al.*, 1999) and controversial (Bredt *et al.*, 1992; Brune & Lapetina, 1991). PKA could stimulate NO production through an indirect mechanism of which several have been described (see for example Polte & Schroder, 1998; Dubey *et al.*, 1998) or alternatively, both PKA and NO might be necessary for LTP. However, two further pieces of evidence suggest that this may not be the case. First, the NO donor spermine NONO-ate mimicked the actions of forskolin in raising the frequency of mEPSCs, illustrating that NO alone can modify presynaptic transmitter release. Second, given that cAMP production in PFs is frequency and calcium dependent, and assuming that cAMP is a poor candidate for a diffusible messenger, cAMP levels would not be

expected to be elevated at distant synapses yet potentiation is still apparent. The consequence of this argument is that NO alone should produce potentiation under conditions of reduced post-synaptic calcium influx and indeed this was found to be the case (Figure 3.20).

Since forskolin induced FM1-43 de-staining was calcium dependent whereas spermine NONO-ate induced de-staining was not and given the calcium dependency of AC1 and nNOS, it is reasonable to conclude that whilst presynaptic calcium influx is required for LTP, NO, once produced, can modulate transmitter release via a calcium-independent process. This would account for the spread of LTP to synapses that were not specifically activated during LTP induction and in whose terminals calcium would not be expected to have risen above that expected during basal activity.

Application of 7-NI after LTP induction did not reverse potentiation, at least within a 5-minute window of RFS. Therefore the expression of potentiation is not due to a sustained elevation of NO production but to a relatively short-lived increase. Moreover, LTP was not sensitive to inhibition of either extracellular or intracellular guanylate cyclase or to inhibition of PKG, indicating that the actions of NO in mediating potentiation do not take place via cGMP or PKG. This is consistent with the proposed presynaptic origin of potentiation and the post-synaptic location of guanylate cyclase (Ariano *et al.*, 1982) but different from LTP in the CA3 region of the hippocampus in which the actions of NO are thought to be dependent, at least in part on cGMP.

Based upon the findings reported here, the following working model is proposed. RFS causes a transient increase in calcium levels in PF terminals. This triggers an increase in

adenylate cyclase activity, leading to the production of cAMP and activation of PKA. The increase in calcium may simultaneously activate NOS in PF terminals (although this is not the only potential source of NO) and in the presence of PKA, NOS activity is facilitated. NO potentiates transmitter release not only at the site of generation but it diffuses over distances of tens of microns to produce presynaptic potentiation at distant synapses. Although there are a number of reports of NO acting as an anterograde or retrograde mediator of LTD and LTP (Schuman & Madison, 1994; Holscher, 1997; Hartell, 1996; Arancio *et al.*, 1996; Hartell, 2000; Holscher, 1997), this is, to the best of our knowledge, the first example of NO acting additionally as a "laterograde" facilitator of presynaptic transmission.

This hypothesis predicts that potentiation at the site of RFS and at distant sites will differ in that PKA will be activated only at those synapses that receive high frequency stimulation. One could conceive, therefore, of a mechanism by which cAMP might trigger, as well as a temporary, NO-dependent potentiation, a series of events that lead to longer term, transcriptional or phosphorylation based changes in presynaptic signaling. In other models of presynaptic, cAMP-dependent LTP, molecules such as cAMP response element binding protein (CREB) and tissue plasminogen activator (tPA) have been shown to contribute to longer-term facilitation of transmission (Casadio *et al.*, 1999; Baranes *et al.*, 1998). Whilst there is, as yet, no direct evidence for their involvement in cerebellar LTP, a recent study has demonstrated a role for CREB in a late phase form of LTD expressed post-synaptically in cultured Purkinje cells (Ahn *et al.*, 1999). It is conceivable that NO might play a role in an early phase of LTP that could be consolidated in a more input or synapse specific way at a later stage. NO could act to increase the spatial dimension of synaptic plasticity in the short term

before longer term, more spatially discrete events requiring protein synthesis can take effect.

CHAPTER 4

AN INVESTIGATION INTO THE CELLULAR MECHANISMS OF LTD INDUCED BY RAISED FREQUENCY PF STIMULATION.

4.1 INTRODUCTION

LTD of excitatory synaptic transmission between PFs, the axons of granule cells, and Purkinje cells is the most widely studied and best understood form of synaptic plasticity within the cerebellar cortex. LTD can be induced by repetitive and coincidental activation of PFs with CFs, which arise from neurones located in the inferior olive (Ito *et al.*, 1982). As outlined in the general introduction, PFs and CFs each contribute essential cellular components required for LTD induction. PFs activate AMPA (Konnerth *et al.*, 1990) and metabotropic glutamate receptors (Batchelor *et al.*, 1994), while CF activation induces a global calcium influx through voltage gated calcium channels (Ross & Werman, 1987). If these three molecular elements are activated in synchrony, LTD ensues through a complex process involving activation of protein kinase C (Crepel & Krupa, 1988; Hartell, 1994) and tyrosine protein kinase (Boxall *et al.*, 1996). Several studies have suggested that in addition to these three basic requirements NO, cGMP and PKG also contribute to the induction of cerebellar LTD (Daniel *et al.*, 1993; Hartell, 1994; Lev Ram *et al.*, 1995). While the contribution of the NO/cGMP/PKG pathway in LTD induced in cerebellar slices is now generally accepted there is no evidence to suggest that it also plays a role in LTD induced in cultured systems (for detailed discussion see Chapter1).

It has recently been demonstrated that certain conditions of PF activation can alone satisfy the conditions required for synaptic depression at the PF-PC synapse (Hartell, 1996d; Eilers *et al.*, 1997). Activation of approximately 10-20 PFs is sufficient to

depolarise the cell enough to open post-synaptic voltage gated calcium channels locally and elevate calcium close to the site of stimulation (Hartell, 1996b). This finding further illustrates that an association of PF and CF synaptic inputs is not absolutely necessary for LTD (Crepel & Jaillard, 1991). However, a recent study demonstrates that LTD is effectively induced when low intensity PF stimulation is paired with CF activation while pairing PF stimulation with cell depolarisation only leads to LTD when PFs are activated at intensities that give rise to EPSCs with amplitudes greater than 1000pA (Reynolds & Hartell, 2000). Since cell depolarisation proved to be more efficient in mobilising a global calcium increase than CF activation the possibility that the CF may contribute an additional element was suggested (Reynolds & Hartell, 2000). One possible candidate is CRF, which has been proposed to be released from the CF and then via activation of PKC facilitate LTD (Miyata *et al.*, 1999). Since PF stimulation at raised frequencies and raised intensities has been shown to lead to NO/cGMP/PKG dependent LTD (Hartell, 1996b; Hartell, 2000) and since NO release is facilitated with increasing frequencies of molecular layer stimulation, (Shibuki & Kimura, 1997) it is also possible that NO, when released in sufficient quantities, may additionally compensate for the lack of CF activation.

Activation of approximately 10-20 PFs produced a localised influx of calcium, restricted to spiny dendritic branchlets close to the site of activation (Eilers *et al.*, 1995). Nevertheless, the LTD that emerged did not remain input specific but spread tens of microns to synapses outside the region of calcium elevation (Hartell, 1996d). This heterosynaptic depression was mediated by NO, which was generated as a result of elevated PF activation and which facilitated heterosynaptic LTD induction through the production of guanylate cyclase and PKG in the Purkinje cell (Hartell, 1996d; Hartell,

2000). Although the levels of PF activation required for LTD_{PF} are well within the levels used by most research groups in the field, the physiological relevance of this alternative form of LTD remains to be established.

It was recently shown that repetitive PF activation leads to a transient and localised increase in post-synaptic calcium levels due to IP_3 mediated calcium release from internal calcium stores (Finch & Augustine, 1998; Takechi *et al.*, 1998). Furthermore, repetitive, localised release of caged IP_3 produced spatially restricted calcium release and led to a LTD of PF responses that was limited to synapses where a raised calcium concentration was observed (Finch & Augustine, 1998). The fact that LTD induced by the release of caged IP_3 does not require PF activation may explain its input specificity since NO, which may be released upon PF stimulation, contributed to the spread of LTD induced by raised frequency and raised intensity stimulation to distant synapses.

Higher frequencies of molecular layer activation have been shown to potently release NO (Shibuki & Kimura, 1997) and trigger cGMP production in Purkinje cells (Hartell & Okada, 1998). Moreover, tetanic PF activation can cause a long-term potentiation of NO release (Kimura *et al.*, 1998). Since PF activation at a rate of 8Hz under conditions of reduced post-synaptic calcium activity led to NOS dependent LTP and given that simultaneous NO production and elevation of intracellular calcium can induce long-term depression (Lev Ram *et al.*, 1995), the possibility that RFS of PFs may be capable of inducing post-synaptic depression as well as presynaptic potentiation was investigated. In addition I examined whether NO released by PF activation could influence GABAergic transmission between inhibitory interneurones and Purkinje cells.

4.2 METHODS

Experiments and data analysis were carried out according to the methods detailed in Chapters 2 and 3 with the exception that 0.5 mM EGTA was substituted for 10 mM BAPTA in the intracellular pipette solution in order to impose a less strict control of post-synaptic calcium activity. Furthermore, some experiments were repeated in the absence of the GABA antagonist picrotoxin (Qian & Dowling, 1994) in order to examine the effects of GABAergic inputs on synaptic transmission induced by RFS to P_0. In separate experiments the effects of 8Hz PF stimulation on transmission at inhibitory synapses between interneurones and PCs were investigated. In these experiments one of two stimulating electrodes was placed in the molecular layer to stimulate PFs off-beam, which also synaptically activates inhibitory interneurones (Ekerot & Kano, 1985). Transmission at these synapses is mediated by $GABA_A$ receptors, which open chloride channels. Since the driving force of chloride is low at –70mV at the intracellular chloride levels present in the patch electrode compared to the ACSF, cells were briefly held at –90mV during stimulation of the inhibitory input. Under these conditions the inhibitory current is inward.

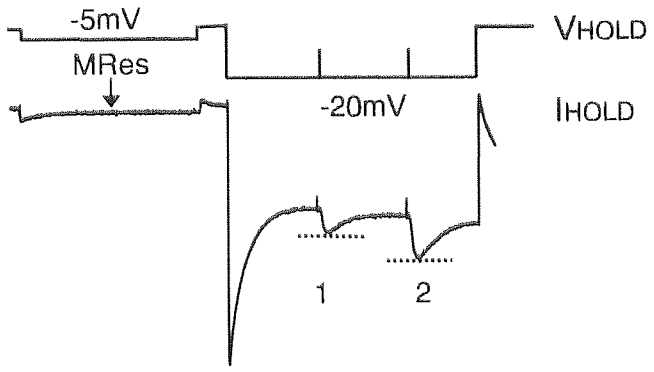


Figure 4.1 Protocol for measuring IPSCs as a result of "off beam" stimulation. The current response (IHOLD) to a 5 mV hyperpolarizing voltage step (VHOLD) from a holding potential of –70 mV is shown, followed by paired pulse activation of PFs superimposed on a hyperpolarisation to –90 mV designed to enhance the outward current following "off beam" stimulation. The bottom trace represents the average of 6 successive sweeps. Data are shown as in Figure 3.1

4.3 RESULTS

4.3.1 THE EFFECTS OF HOMOSYNAPTIC RFS ON PF-PC SYNPATIC TRANSMISSION UNDER CONDITIONS OF LOW POST-SYNAPTIC CALCIUM BUFFERING.

In the presence of 0.5 mM EGTA in the recording patch pipette, 15 seconds RFS to P_0 resulted in an immediate but brief increase in the amplitudes of PF-EPSC responses in both pathways. After approximately 10 minutes responses declined towards baseline levels and a long lasting depression of responses relative to baseline levels gradually occurred (Figure 4.2). This robust decrease in synaptic efficacy reached values of 73.1 ± 8.8% and 74.2 ± 11.0 % of baseline values at test (P_0) and control (P_1) sites respectively, 20 minutes after the induction protocol (n=6). The initial short-term potentiation (STP) was consistently accompanied by a decrease in the PPR, which, as discussed in Chapter 3, is considered to reflect an increase in presynaptic transmitter release probability. As the LTD replaced potentiation, however, the PPRs returned towards baseline levels and further increased to 108.7 ± 7.3% and 105.8 ± 1.5% respectively (n=6, Figures 4.2 and 4.5).

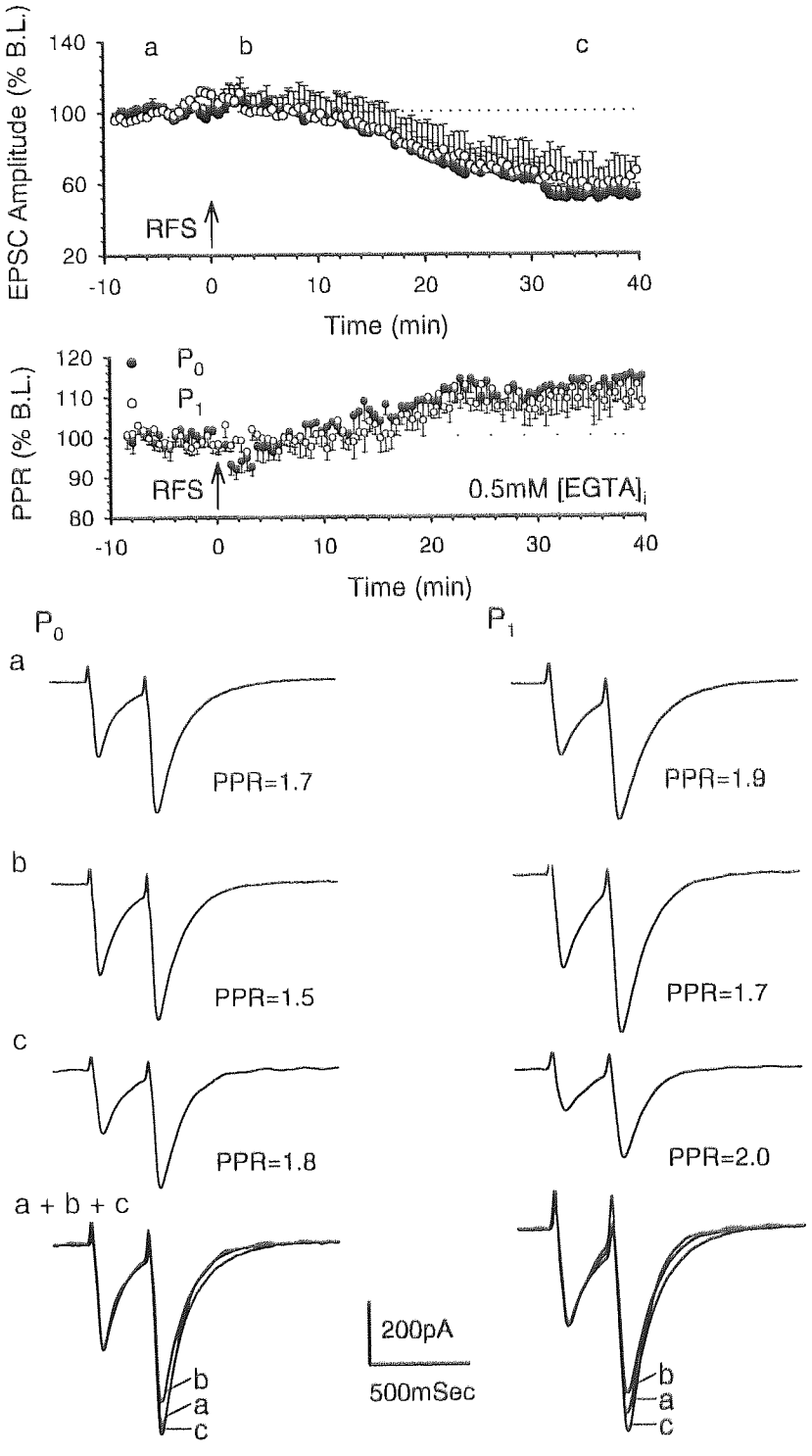


Figure 4.2 The effects of RFS to P_0 in the presence of 0.5 mM $[EGTA]_i$ are shown in the upper graph. EPSC amplitudes are expressed as a percentage of the mean baseline level prior to RFS. Standard errors are also shown. Shown below are the mean changes in PPR. The results are collated from 6 separate cells. The lower graph provides a representative example of EPSCs elicited by stimulation of P_0 (left) and P_1 (right) at times a, b and c indicated in the upper graph. The first pulses were normalised to illustrate the accompanying reduction in PPR (a + b + c). Each trace is the average of 6 consecutive sweeps.

Although RFS was applied only to the test input (P_0), the STP and LTD that emerged, together with the associated changes in PPR, were apparent in both inputs even though the control input (P_1) was not directly activated at high frequency. As with the experiments described in Chapter 3, the membrane resistance, holding currents and series resistances of all cells were monitored throughout. Cells were discarded from the analysis if there were any significant changes in these parameters during experiments. Therefore LTD was not associated with nor did it depend upon changes in any of these parameters. To further ensure that the heterosynaptic depression of PF responses was not due merely to cell deterioration, P_0 and P_1 were activated alternately at a rate of 0.2 Hz for 40 minutes. Over this period, no changes in either EPSC amplitudes or PPR in either pathway were observed (Figure 4.3). Therefore, it is reasonable to suggest that the observed depression was not due to cell deterioration and was dependent upon RFS.

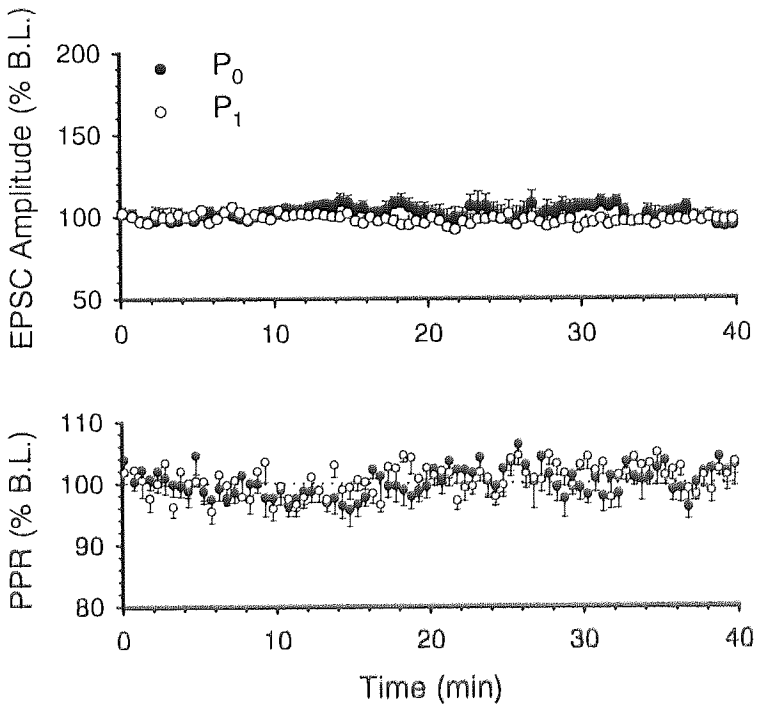


Figure 4.3 No long-term changes in EPSC amplitude or PPR (below) were observed during alternate activation of P_0 and P_1 at a rate of 0.2 Hz over a period of 40 minutes.

4.3.2 The Role Of cAMP And PKA In RFS Induced Potentiation And Depression.

Since presynaptic potentiation induced by 8Hz stimulation under conditions of reduced post-synaptic calcium activity was mediated by cAMP and PKA (see Chapter 3), the possibility that STP and/or LTD also depended upon cAMP production and PKA activation was next tested. Inhibition of PKA by inclusion of H-89 in the bathing media at a concentration of 0.2μM prevented the appearance of any STP but had no significant effect on the later phase of depression in P_0 (75.09 ± 8.1%) and P_1 (82.56 ± 9.47%) or the PPR (101.14 ± 2.47% and 102.05 ± 2.2%) measured 20 minutes after RFS compared to data recorded in standard ACSF ($P<0.05$, n=6, Mann-Whitney U-test, Figures 4.4 and 4.6).

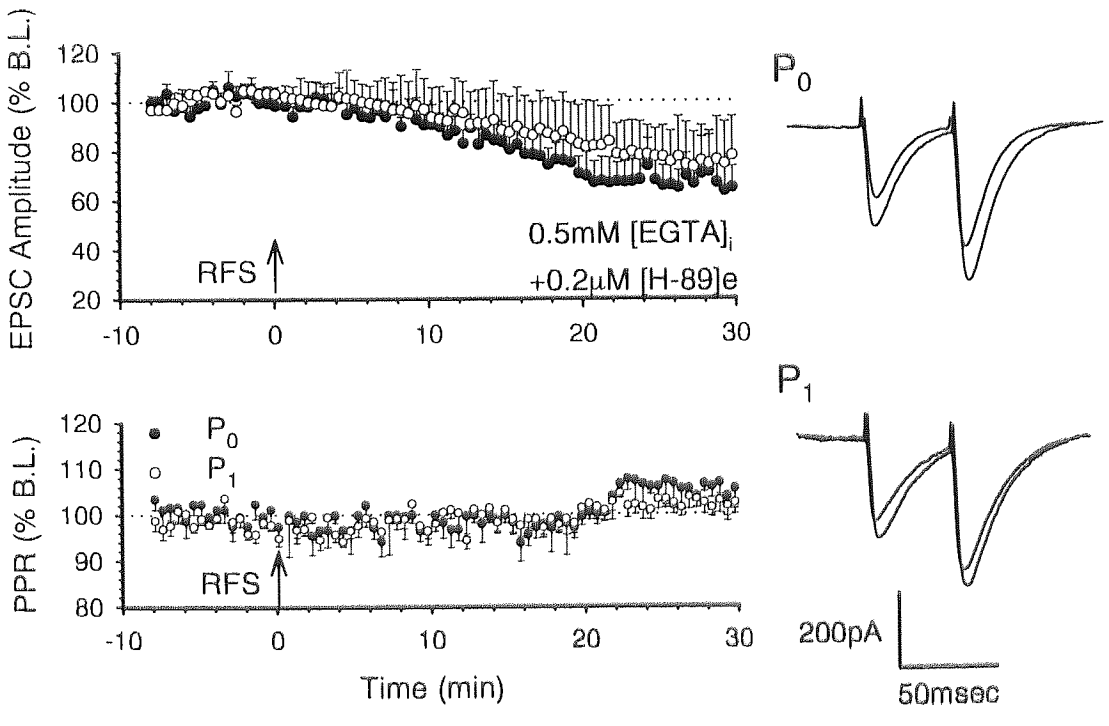


Figure 4.4 PKA activity is required for the induction of STP but not LTD. Addition of 0.2 μM H-89 to the perfusion medium had no effect on the level of depression in either pathway, but prevented the induction of STP. Shown below are the changes in PPR.

Next the effects of RFS to P_0 in the presence of Rolipram, an inhibitor of type IV, cAMP-specific PDE (Beavo, 1988) were examined. Rolipram provides a means of increasing the overall level of endogenous cAMP activity in response to synaptic activation. In the extracellular presence of rolipram (5μM) RFS led to a marked increase in synaptic transmission in both pathways that proved to be significant after 20 minutes (122.82 ± 18.15% and 139.82 ± 25.5% of baseline levels respectively ($P< 0.05$, n=6, Mann-Whitney U-test, Figure 4.5) even though no measures to attenuate post-synaptic calcium were made. This potentiation declined over time and was accompanied by a marked but transient, heterosynaptic decrease in the PPR (87.3 ± 1.8% and 89.68 ± 1.36% of baseline levels respectively after 20 minutes, n=6, Figures 4.5 and 4.6).

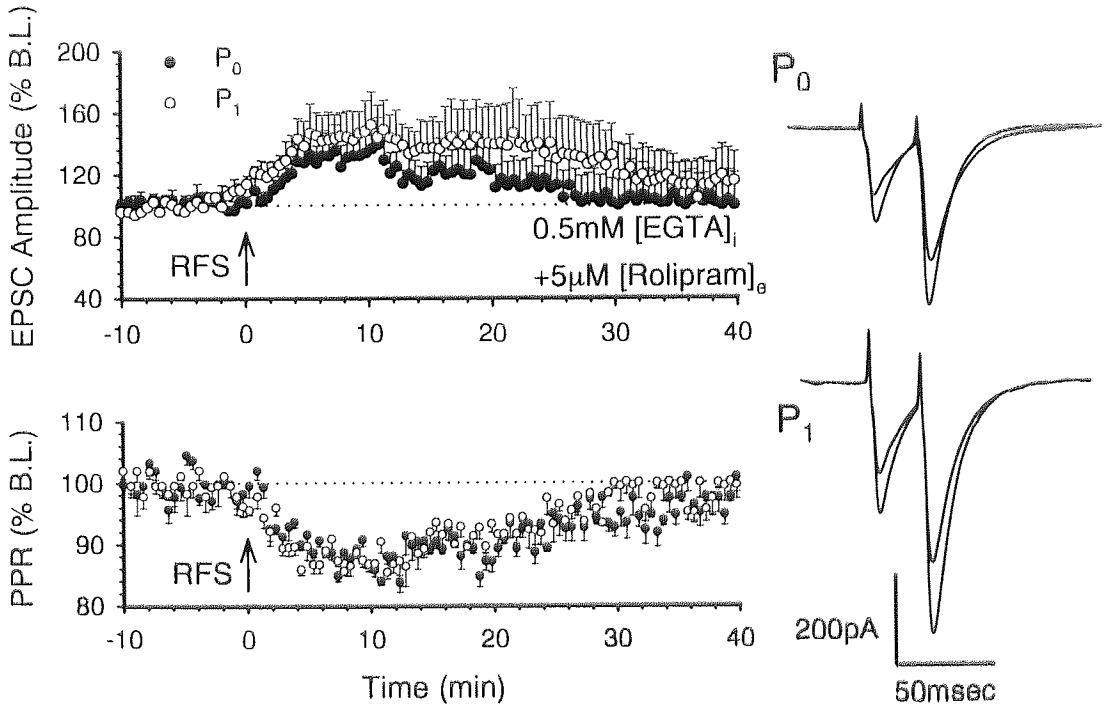


Figure 4.5 The level and duration of potentiation as well as the decrease in PPR were enhanced when the PDE IV inhibitor rolipram was added to the perfusate.

Although EPSC amplitudes remained significantly greater than those recorded in the absence of rolipram for over 40 minutes (Figure 4.5), PF responses declined towards baseline levels and some overall depression was evident within 60 minutes of RFS. This

suggests that rolipram rather enhanced the amount and duration of potentiation than blocked LTD. These results are consistent with the observation that cAMP and PKA activity are involved and required for the induction of presynaptic potentiation (Salin *et al.*, 1996; Jacoby & Hartell, 1999and see Chapter 3) but they have no significant effect on heterosynaptic RFS induced depression.

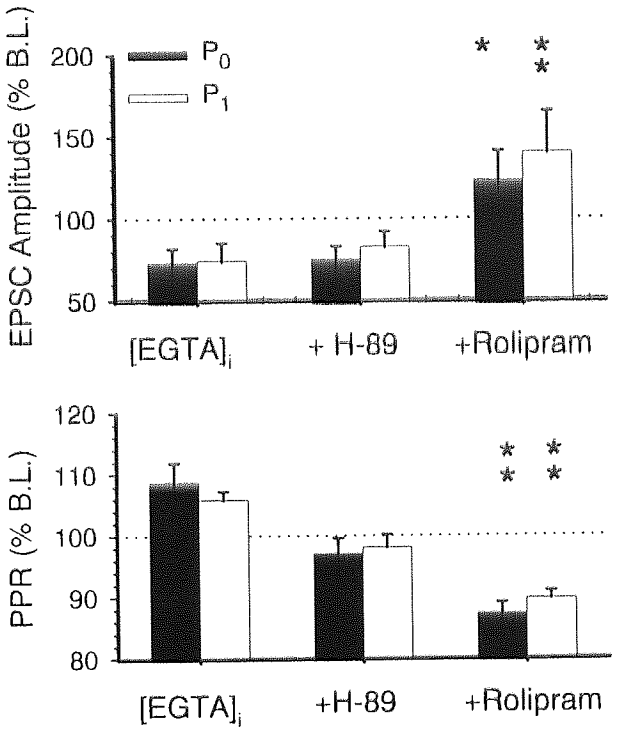


Figure 4.6 A comparison of P_0 and P_1 responses and the associated changes in PPR under control conditions of 0.5 mM EGTA and in the additional presence of 0.2μM H-89 and 5μM rolipram. Data were collected 20 minutes after RFS and represent the means and standard errors of 6 cells in each experimental group. Paired asterisks represent a statistical significance of P<0.01 between test and control conditions (Mann-Whitney U-test). Single asterisks, P<0.05.

4.3.3 The Role Of The NO/cGMP/PKG Cascade In RFS Induced Potentiation And Depression.

Since NO was required for the induction of heterosynaptic potentiation induced by 8Hz PF stimulation and since the NO/cGMP/PKG cascade has been demonstrated to contribute to non-input specific LTD induced by raised frequency, raised intensity stimulation (Hartell, 1996c; Hartell, 2000) whether LTD induced by 8Hz PF stimulation

also depended on the production of NO and cGMP was next examined. Disruption of NO production by addition of the NOS inhibitor 7-NI (5 μM) to the bathing media prevented the induction of STP as well as LTD in both pathways (Figure 4.7). P_0 (100.29 ± 13.82%) and P_1 (102.97 ± 9.72%) responses, as well as the accompanying changes in PPR (100.38 ± 3.02% and 101.24 ± 3.92%), were significantly different after 20 minutes ($P<0.01$, n=6, Mann- Whitney U-test, Figures 4.7 and 4.10) from data recorded in the absence of 7-NI.

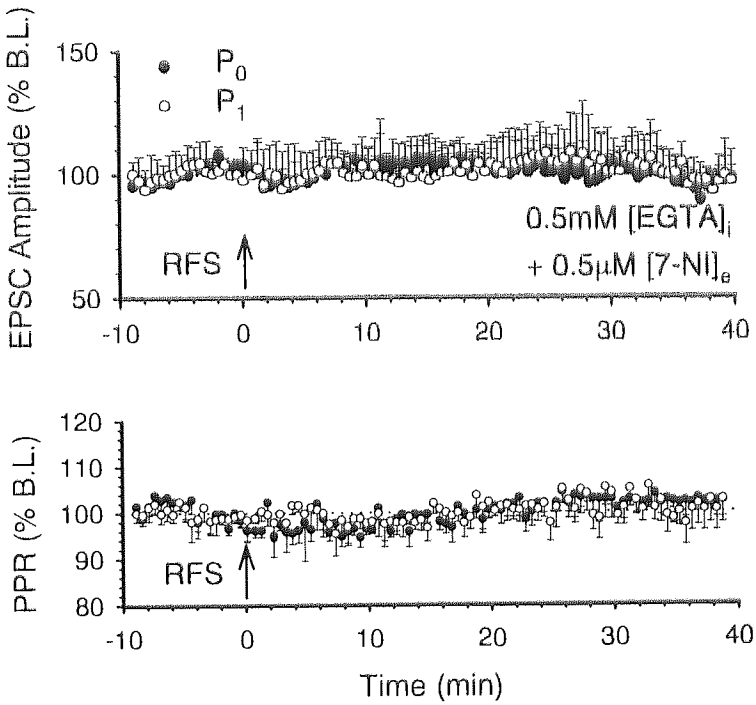


Figure 4.7 Inhibition of NOS with 7-NI applied to the perfusate at a concentration of 5μM prevented the induction of STP and LTD in both pathways. Shown below are the mean changes in PPR.

The prolonged potentiation that was evident in the additional presence of rolipram as well as the subsequent depression that appeared 40 minutes after RFS was applied were prevented in both pathways (98.01 ± 5.15% and 100.47 ± 6.04%) when NOS was inhibited by 7-NI and no changes in PPR were observed in either P_0 (98.87 ± 3.84%) or P_1 (100.13 ± 4.60%; $P<0.05$, n=6, Mann- Whitney U-test, Figure 4.8).

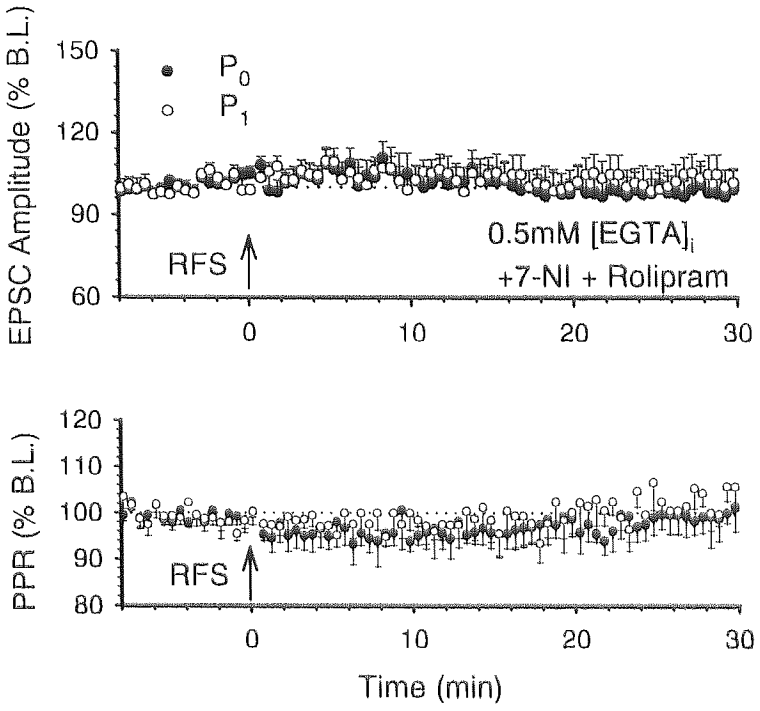


Figure 4.8 The potentiation that was apparent in the presence of 5μM rolipram was prevented when 5μM 7-NI was additionally included in the bathing medium. Shown below are the associated changes in PPR.

Inclusion of the guanylate cyclase inhibitor ODQ at a concentration of 5μM into the pipette solution prevented LTD in both pathways within 20 minutes (130.75 ± 23.89% and 118.71 ± 11.58% of baseline levels, P<0.01, n=6, Mann-Whitney U-test). In contrast to the effects of 7-NI, however, a clear underlying but short lasting potentiation of P_0 and P_1 responses emerged. This potentiation of responses was accompanied by a significant decrease in PPR in P_0 (87.66 ± 5.07%) and in P_1 (92.26 ± 4.01% of baseline level measured 20 minutes after RFS, P<0.01, n=6, Mann-Whitney U-test, Figures 4.9 and 4.10).

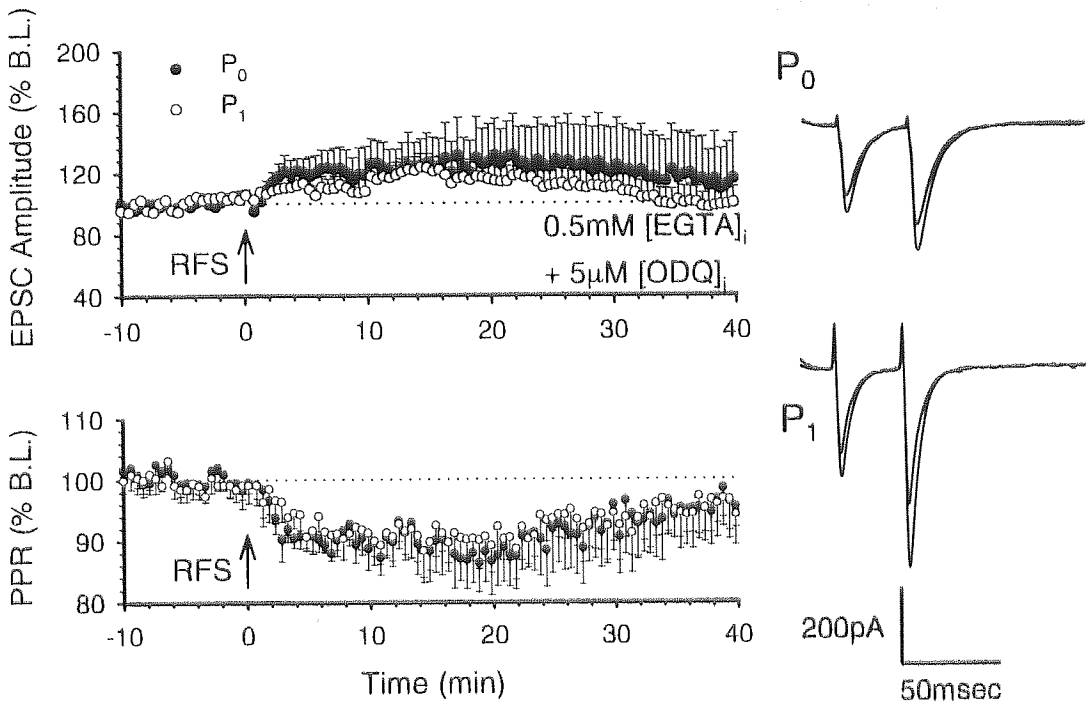


Figure 4.9 Inclusion of 5μM ODQ in the internal pipette solution prevented the induction of herterosynaptic depression but induced potentiation of PF responses in both pathways. Shown below is the associated decrease in PPR.

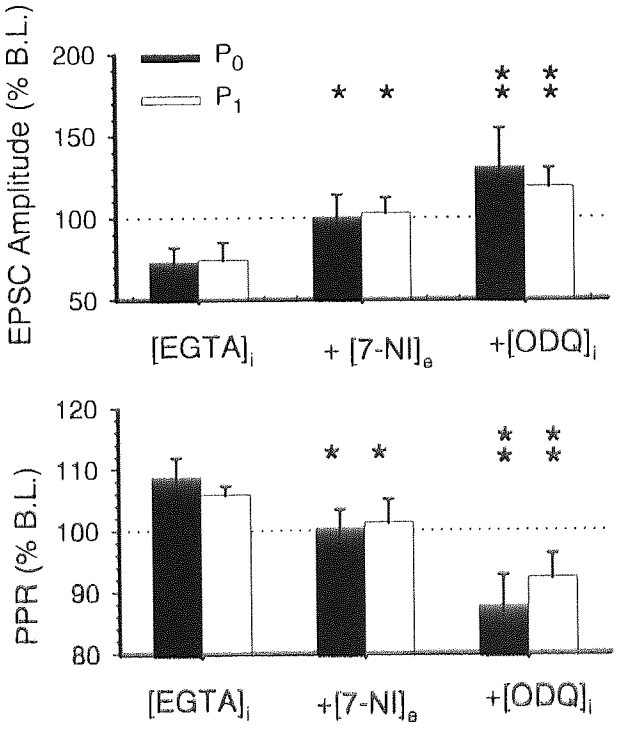


Figure 4.10 The means and standard errors of 6 cells measured 20 minutes after RFS was applied in each experimental group. Paired asterisks represent a statistical significance of P<0.01 between test and control conditions (Mann-Whitney U-test). Single asterisks, P<0.05.

These data are consistent with previous observations that LTD at both P_0 and P_1 following PF stimulation depends upon the generation of NO and the subsequent post-synaptic production of cGMP (Daniel *et al.*, 1993; Boxall & Garthwaite, 1996) but that LTP depends only upon NO (See Chapter 3).

4.3.4 The Effects Of Modulation Of GABAA Inhibitory Inputs On RFS Induced Plasticity.

The effects of RFS to P_0 in the absence of the GABA receptor antagonist picrotoxin were next investigated. It has previously been shown that induction of LTD by pairing PF and CF activation was not possible in the absence of picrotoxin (Schreurs & Alkon, 1993), which blocks the inhibition of PCs by inhibitory interneurones. Under these conditions LTD was replaced by potentiation in both pathways. EPSC amplitudes (116.61 ± 7.58% and 128.73 ± 8.39% of baseline levels respectively) as well as changes in PPR (95.87 ± 2.3% and 95.74 ± 1.77%) were significantly different compared to measurements taken in presence of picrotoxin ($P<0.05$, n=6, Mann-Whitney U-test, Figure 4.11).

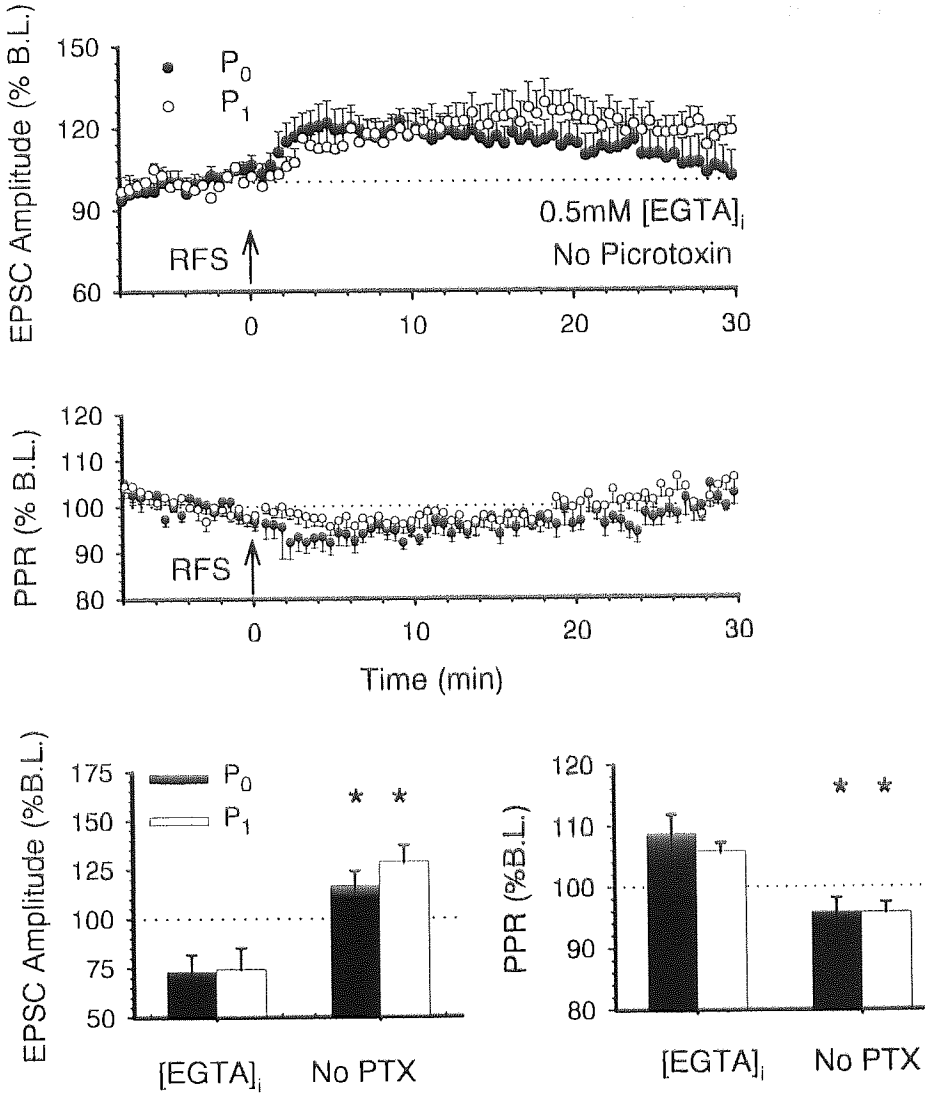


Figure 4.11 In the absence of Picrotoxin RFS to P_0 induced potentiation in both pathways. Shown below are the changes in PPR. The lower graph illustrates a comparison of P_0 and P_1 responses in the absence and presence of 20μM Picrotoxin. Asterisks indicate a statistical difference between test and control conditions (Mann-Whitney U-test, *P<0.05).

The data presented in Chapter 3 and so far in this chapter indicate that the plasticity that emerges as a result of homosynaptic RFS, regardless of direction or origin, spreads to other excitatory PF inputs to the same cell. We next examined whether RFS of PFs was also capable of modifying inhibitory inputs to Purkinje cells. Two types of inhibitory interneurones can be found in the molecular layer. Stellate cells are located in the outer two thirds of the molecular layer; their short axons form synapses with the dendrites of

PCs. The second type are basket cells which lie in the inner third of the molecular layer their axons have two types of collaterals one of which are long, descending collaterals which contact the soma of distant PCs, while their short, ascending collaterals form less powerful synapses with the dendrites of PCs (Figure 4.12). One of the two stimulating electrodes was placed in the molecular layer to elicit IPSCs originating from inhibitory interneurones. The other electrode was positioned to elicit "on beam" EPSCs as usual (Figure 4.12).

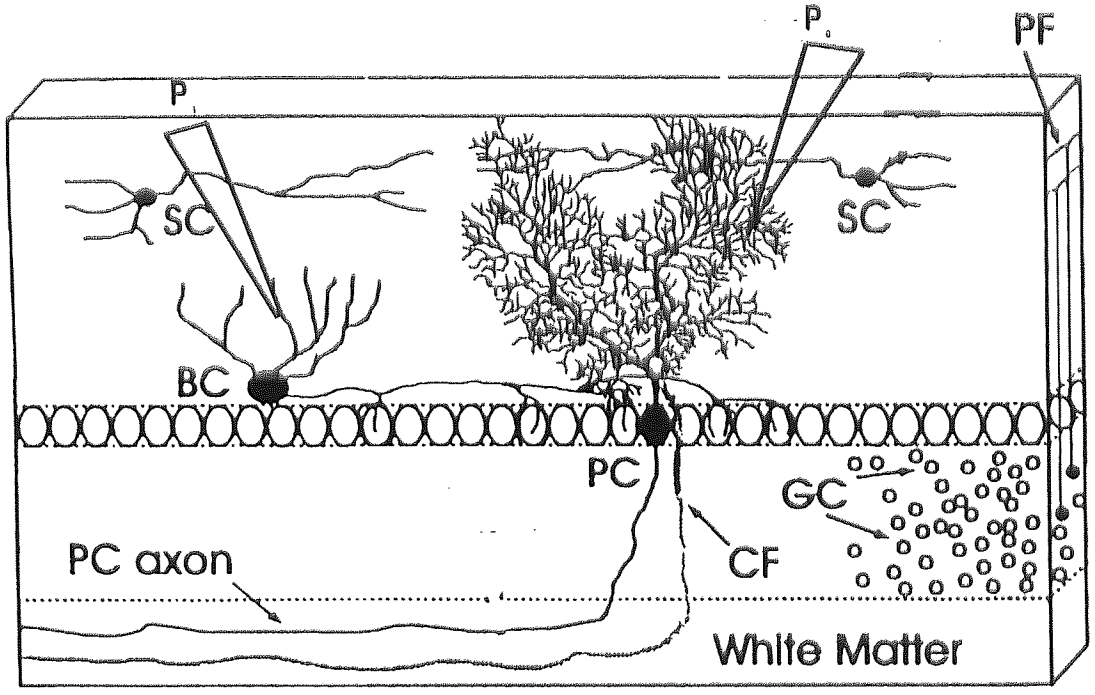


Figure 4.12 Schematic diagram of electrode positioning for experiments in which the effect of RFS of PFs on synaptic transmission at inhibitory synapses was examined. SC: stellate cell; BC: basket cell; GC granule cell; CF: climbing fiber; PF: parallel fiber; PC: Purkinje cell.

As before, the excitatory input, designated as P_0, and the inhibitory input, termed P_1, were stimulated alternatively at a rate of 0.2 Hz. Cells were briefly held at –90mV during stimulation of the inhibitory input (see section 4.2). Once baselines were stable for at least 10 minutes the frequency to the PF input was raised to 8 Hz for 15 seconds. Stimulation to P_1 was stopped during this period of RFS. After that alternate activation at a rate of 0.2 Hz was resumed. Figure 4.13 illustrates representative current-voltage

relationships for "on beam" and "off beam" stimuli. EPSCs and IPSCs were recorded from a Purkinje cell at membrane potentials from –90 to +30 mV in absence (left) and presence (right) of the $GABA_A$ antagonist bicuculline (10 μM). The reversal potential for these currents were –62 and 0 mV respectively. Inhibitory currents elicited by off beam stimulation were completely blocked in the presence of bicuculline (Figure 4.13). When $GABA_A$ receptors were blocked a slight increase in PF-EPSC amplitude was observed. This is consistent with recent work that suggests that GABA-induced currents may help shape EPSCs evoked by on beam stimulation (Figure 4.13).

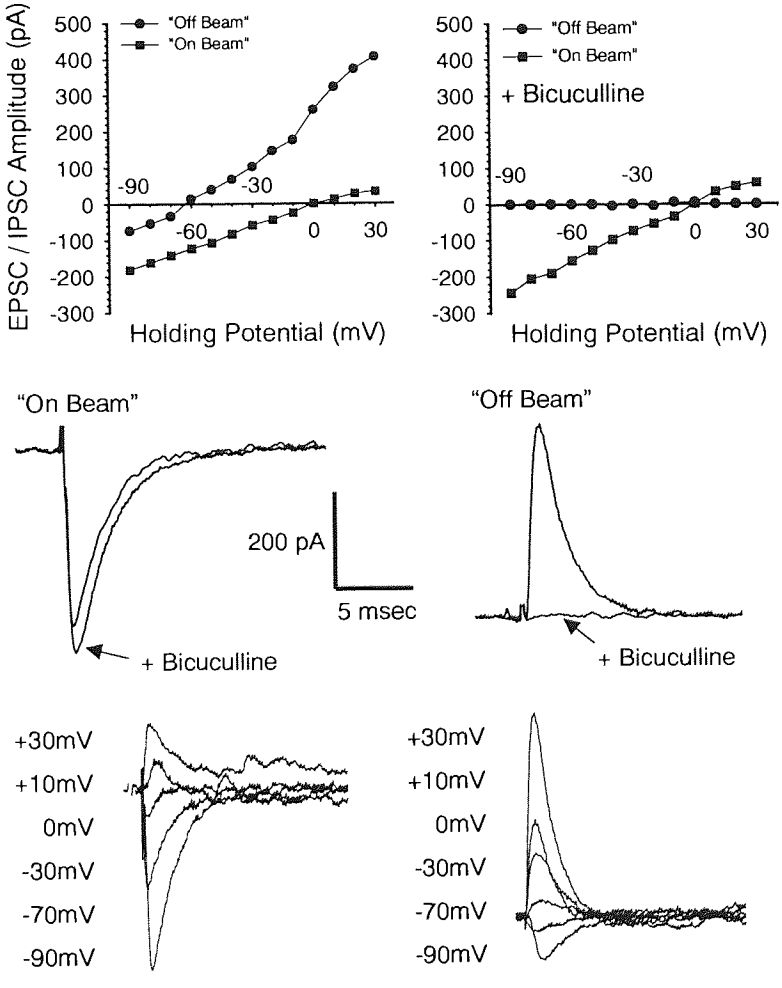


Figure 4.13 The relationship between Purkinje cell holding potential and peak amplitude of evoked EPSCs and IPSCs are shown in presence (left) and absence of 10μM bicuculline (n=5, respectively). Below, the left hand graph shows an EPSC evoked by PF stimulation recorded at a holding potential of –70mV in the absence and presence of bicuculline. An IPSC recorded at 0 mV in the presence and absence of bicuculline is shown on the right. Below are EPSC (left) and IPSC (right) responses recorded over the range of holding potentials shown.

Having established that on beam and off beam stimulation activated largely excitatory and inhibitory responses respectively, the effect of RFS to the excitatory input was then studied. RFS to P_0 led to a significant potentiation of excitatory PF responses above baseline (138.34 ± 15.63%) and a decrease in PPR to 92.18 ± 1.86% ($P<0.05$, n=6, Wilcoxon signed-rank test, Figure 4.14). However, no significant changes in synaptic transmission (101.9 ± 10.48%) or PPR (102.63 ± 2.94%) were observed in P_1 ($P<0.05$, n=6, Wilcoxon signed-rank test, Figure 4.14).

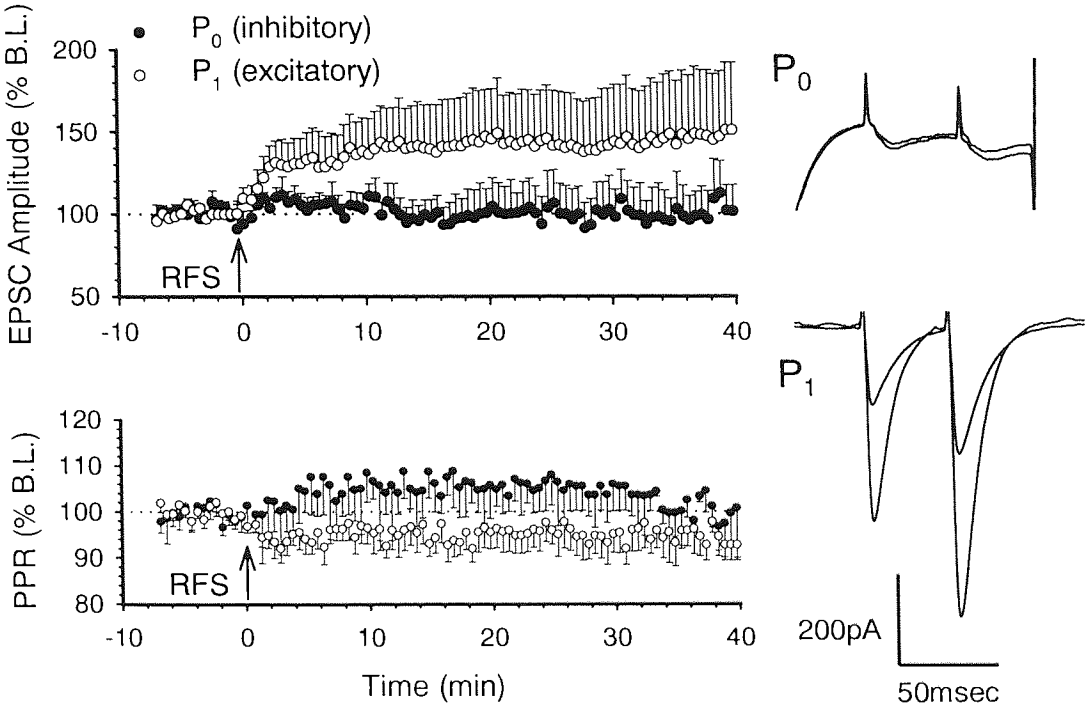


Figure 4.14 The effects of RFS to P_0 on PF-EPSC and inhibitory currents arising from "off beam" activation of inhibitory interneurones are illustrated. Shown below are the associated changes in PPR for each pathway.

4.4 DISCUSSION

The effects of applying RFS for 15 seconds to one of two separate PF inputs impinging on a single Purkinje cell were studied in the presence and absence of the $GABA_A$ receptor chloride channel blocker picrotoxin under conditions where post-synaptic calcium was only partially buffered with 0.5 mM EGTA. The effects that RFS PF

stimulation had on an inhibitory input originating from inhibitory interneurones were also examined. The results of these experiments reveal several characteristics of PF-mediated plasticity in the cerebellar cortex. First, the overall balance of the direction of any emerging synaptic plasticity at the PF-PC synapse is principally determined by conditions that influence post-synaptic calcium activity. As demonstrated in Chapter 3 RFS was followed by LTP when post-synaptic calcium levels were buffered with 10mM BAPTA. When minimal levels of a calcium chelator were used, an underlying potentiation was still evident but it was significantly smaller, shorter-lived and superseded by a robust LTD of PF responses.

In contrast to LTP cerebellar LTD is thought to be expressed entirely post-synaptically (Crepel & Jaillard, 1990; Linden *et al.*, 1991; Lev Ram *et al.*, 1995). Indeed LTD was not accompanied by any significant paired pulse ratio increase indicative of a reduction in transmitter release probability.

Previous work has shown that when the frequency of PF stimulation is raised to 1Hz, in conjunction with an increase in the stimulus strength sufficient to open post-synaptic voltage gated calcium channels, LTD occurs in both the pathway that received the enhanced levels of stimulation and in a separate pathway that did not (Hartell, 1996a; Hartell, 1996b). The data presented here indicate that 8Hz stimulation alone can similarly fulfil the criteria required for LTD but at much lower stimulus intensities. Thus higher frequency stimulation appears to more efficient in mobilising sufficient post-synaptic calcium to trigger LTD. High frequency PF activation has been shown to activate mGluRs (Batchelor *et al.*, 1994) and more recently to trigger spatially restricted calcium transients through IP_3 mediated CICR (Takechi *et al.*, 1998; Finch &

Augustine, 1998). It is entirely plausible that such a mechanism of IP_3 mediated CICR might account for the lower number of PFs required to trigger the homosynaptic LTD observed at the test site following RFS.

We found that LTD following RFS did not remain restricted to those synapses that were specifically activated. In all cases where depression was observed, reductions in synaptic efficacy spread over distances in the order of tens of microns. The spread of LTD did not simply reflect an overlap of activated fibres since the test for independence described in Chapter 3 revealed that P_0 and P_1 did not share a significant number of fibres prior to RFS. Furthermore, heterosynaptic depression was not simply due to cell deterioration over the standard 40 minute recording duration since alternate stimulation of P_0 and P_1 at 0.2 Hz for this duration did not induce any significant changes in synaptic transmission. The fact that pathway specificity was preserved between excitatory and inhibitory pathways provides further evidence that the depression observed was not simply due to post-synaptic deterioration (Figure 4.14).

Since the calcium transients arising from PF stimulation have been shown to remain restricted, provided regenerative spiking does not take place (Finch & Augustine, 1998; Hartell, 1996b; Takechi *et al.*, 1998), and taking into account the relatively low stimulus strengths used in the experiments described in this chapter it is likely that any post-synaptic requirement for calcium elevation need not be generalised. This raises the possibility that only LTD at the site of RFS actual requires an increase in post-synaptic calcium above resting levels. LTD at the control site does not require a specific increase in calcium above resting levels itself but its calcium dependence stems from its dependence on depression at the test site.

Apart from a dependency on post-synaptic calcium, LTD in cerebellar slices also depends upon the production of NO (Shibuki & Okada, 1991; Daniel *et al.*, 1993; Hartell, 1994; Ito & Karachot, 1990). It has been demonstrated that RFS within the molecular layer leads to presynaptic production of NO (Kimura *et al.*, 1998; Hartell, 1996d) whose target may then be the soluble GC located in PCs (Boxall & Garthwaite, 1996). Whether the NO/GC/cGMP/PKG pathway was involved in the induction of the cerebellar LTD that emerged after RFS was therefore investigated when the intracellular calcium concentration was not clamped. Inhibition of the NO-cGMP cascade, at any step, prevented the induction of LTD in both pathways, confirming that homo- and hetero-synaptic depression induced by this protocol also depends on the production of NO and the subsequent generation of cGMP in PCs. This differs from the LTD that arises following raised intensity, raised frequency PF stimulation in that homosynaptic LTD under these conditions is NO/cGMP independent. This may be explained by the presence of a second pathway to LTD, similar to that described in cultured systems, that is calcium dependent but which neither requires nor involves the NO/cGMP/PKG pathway (Linden *et al.*, 1995). This pathway may predominate under more harsh conditions of LTD induction such as raised intensity, raised frequency PF stimulation (Hartell, 1996b) or the iontophoretic applications of glutamate paired with cell depolarization. Moreover, the existence of more than one pathway explains why the LTD that emerges following uncaging of IP_3 in Purkinje cells remains input specific (Finch & Augustine, 1998) since in that case the presynaptic production of NO by RFS of PFs is completely bypassed yet LTD can still take place (Linden *et al.*, 1991).

The finding that H-89, an inhibitor of PKA, reduced the appearance of STP but had no significant effect on the induction of LTD indicates that RFS mediated potentiation, in

contrast to heterosynaptic LTD, does not require the production of cAMP and activation of PKA. Furthermore, modulation of cAMP production by incubation of slices with rolipram, a specific inhibitor of type 4, cAMP specific PDE (Beavo, 1988), increased the extent and duration of potentiation but not LTD. The induction of LTP rather than LTD was also favoured when picrotoxin was absent and $GABA_A$ mediated inputs to Purkinje cells were intact. This confirms the observations of an earlier study in which application of GABA, which is likely to lower post-synaptic calcium levels, triggered potentiation of synaptic transmission (Shibuki & Okada, 1992).

Together, these observations indicate that the direction of plasticity at the PF-PC synapse may well be controlled by the pattern of associated inhibitory inputs. Temporal proximity of GABA-ergic input to high frequency granule cell input may reduce post-synaptic depolarization and calcium influx and tip the balance towards pre-synaptic potentiation. Inhibitory synaptic transmission between GABAergic interneurones and PCs has been shown to undergo a form of potentiation called rebound potentiation. Rebound potentiation is induced by CF activation and depends on calcium influx through voltage gated calcium channels and activation of PKA (Kano *et al.*, 1992; Kano *et al.*, 1996). Furthermore, it has recently been shown that stimulation of the molecular layer, which most likely activated basket cells, in combination with application of noradrenaline or serotonin as well as repetitive activation of monoaminergic fibres, which run through the molecular layer leads to potentiation of $GABA_A$ mediated inhibitory currents (Mitoma & Konishi, 1996; Mitoma & Konishi, 1999). Similar to PF stimulation induced LTP this form of potentiation seems to be heterosynaptic and is underpinned by a presynaptic mechanism involving the activation of PKA (Mitoma &

Konishi, 1999). However, LTP induced by 8Hz PF stimulation had no effect on inhibitory currents arising from inhibitory interneurones.

It is likely therefore, that the direction of plasticity at the PF-PC synapse is crucially dependent on post-synaptic calcium activity. RFS under conditions of reduced post-synaptic calcium activity leads to potentiation of PF responses whereas depression is observed when post-synaptic calcium levels are not clamped. However, the results from this chapter and from chapter 3 indicate that the processes are not opposite but mechanistically distinct phenomena. The induction of non-input specific LTD requires NOS activation, production of cGMP and activation PKG but not production of cAMP and activation of PKA. The induction and spread of both cerebellar LTD and LTP to distant synapses involve NO, which is known to be able to diffuse over large distances. Although NO dependent synaptic plasticity induced by RFS is capable of spreading tens of microns to distant excitatory PF inputs to the same Purkinje cell, plasticity does not spread to inhibitory pathways indicating that synaptic plasticity at inhibitory interneurones –PC synapses does not involve the actions of NO.

CHAPTER 5

GENERAL DISSCUSSION

The main purpose of this study was to examine the effects of 8Hz stimulation to one of two, synaptically separate PF inputs to the same PC under various conditions of post-synaptic calcium activity. We found that synaptic plasticity at the PF-PC synapse can be modified bi-directionally. When post-synaptic calcium levels were buffered with 5-10mM BAPTA RFS triggered LTP. A similar increase in synaptic transmission was also evident when PCs were hyperpolarized to –90mV during RFS. This procedure was designed to reduce the likelihood of calcium influx via voltage gated calcium channels during raised frequency PF stimulation. Potentiation of PF-responses was also apparent in the absence of picrotoxin. Therefore, basal activity of inhibitory GABAergic inputs, which would also tend to hyperpolarize PCs, similarly favours the induction of LTP.

When post-synaptic calcium levels were not buffered with raised levels of calcium chelators or limited by concurrent post-synaptic hyperpolarization, 8Hz PF stimulation led to a small, transient potentiation, which was followed by a sustained depression. This suggests that a reduction of post-synaptic calcium influx through voltage-gated channels blocks the induction of LTD thereby allowing an underlying potentiation to predominate.

Surprisingly, RFS induced synaptic plasticity, regardless of its direction, was not input specific. The spread of LTP or LTD to synapses tens of microns distant from the site of induction was not due to an overlap of activated fibres since pathways were tested for independence before and after RFS was applied. Furthermore, depression was not a result of a general deterioration of recording conditions. Recordings were not included

in the analysis if the membrane resistance or holding currents changed significantly. Moreover, no changes in synaptic transmission strength were detected over a duration of 40 minutes when P_0 and P_1 were activated alternately at a rate of 0.2 Hz.

Various approaches were taken to determine the nature of LTP. Potentiation was consistently accompanied by a decrease in PPR indicating that its induction might have a presynaptic origin. This assumption was further supported by the increase in mEPSC frequency following application of the AC activator forskolin or the NO donor NONOate, substances which were shown to induce LTP pharmacologically. Furthermore, a decrease in fluorescence staining of FM1-43 was observed when LTP was induced either pharmacologically or through electrical stimulation. Several studies have already examined the role of cAMP and PKA in the induction of cerebellar LTP (Salin *et al.*, 1996; Storm *et al.*, 1998). Our findings that H-89, an inhibitor of PKA, prevents RFS induced potentiation and that LTP can be mimicked by application of forskolin are consistent with those earlier reports. Next, the possible involvement of cGMP/PKG in the induction of cerebellar LTP was examined. Inclusion of the GC inhibitor ODQ to either the perfusion medium or the internal pipette solution had no effect on the extent or the duration of LTP. Similarly, RFS induced LTP was still observed when PKG was inhibited by intracellular application of KT5823. The ability of NOS inhibitors to prevent LTP induced by RFS of PFs or by the application of forskolin at the activated as well as at the non-activated site shows that NOS activity is necessary for the induction of heterosynaptic cerebellar LTP.

However, NOS may not be required for the maintenance of RFS induced potentiation since inclusion of 7-NI in the perfusion medium 5 minutes after RFS was applied had

no effect on the extent or the duration of LTP. As inhibition of NOS prevented forskolin induced potentiation it is unlikely that NO acts up-stream to activate AC/PKA but it is rather possible that AC/PKA activate NOS activity. The possibility that both messengers act in concert to result in potentiation seems unlikely since application of the NO donor spermine NONOate led to potentiation and furthermore, preliminary experiments indicate that potentiation can be induced by NONOate application in the additional presence of H-89 a substance that has been shown to block RFS induced potentiation. It would be interesting to examine whether potentiation or depression were delayed if the resumption of 0.2 Hz stimulation in one pathway was delayed. Further experimentation will be required to clarify this issue.

Taking into account the results presented in the preceding chapters we can propose a model in which PF activation leads to a transient calcium increase in presynaptic terminals, which in turn activates AC and NOS. NO production, which may be facilitated when PKA is active, may then lead to a potentiation of presynaptic transmitter release, not only at the induction site, and may be responsible for the lateral spread of LTP to distant synapses. It is not yet clear if synaptic transmission is directly increased via the actions of PKA and NO or whether NOS and PKA activity result in LTP via an indirect mechanism. NO has also been shown to contribute to the late phase of LTP in the hippocampus by acting in parallel with PKA to increase phosphorylation of the transcription factor CREB (Lu *et al.*, 1999). Furthermore, tPA has also been shown to play a role in the induction of the late phase of hippocampal LTP (Baranes *et al.*, 1998). Further experimentation will be required to examine if CREB and/or tPA are also required for cerebellar potentiation.

It was shown previously that raising the frequency of PF stimulation to 1 Hz in conjunction with an increase in the intensity of stimulation leads to a heterosynaptic LTD at the PF-PC synapse (Hartell, 1996a). Therefore we next investigated if 8Hz PF stimulation was capable of inducing LTD when post-synaptic calcium levels were only partially buffered with 0.5 mM EGTA. PF activation under these more physiological conditions resulted in a depression of PF-EPSCs that was not restricted to the induction site but that spread to distant synapses. Since it has been shown that increases in calcium induced by PF stimulation remain spatially restricted (Eilers *et al.*, 1995; Finch & Augustine, 1998; Hartell, 1996a; Takechi *et al.*, 1998) and as the stimulus intensities used were low, it is unlikely that the depression of distant synapses was due to a generalised increase in post-synaptic calcium. It was therefore reasonable to investigate if the diffusible messenger NO was also involved in the induction of RFS mediated LTD. The NO/cGMP/PKG signalling cascade is required for depression induced by raised frequency and intensity PF stimulation (Hartell, 1996b). Addition of the GC inhibitor ODQ to the internal pipette solution prevented the induction of LTD in both pathways thereby unmasking the underlying potentiation. Similarly no depression was observed when NOS activity was prevented by extracellular application of 7-NI. However, under these conditions the induction of both potentiation and depression was prevented. These data demonstrate that LTD induced by 8Hz PF stimulation crucially depends on NOS activity as well as cGMP production while potentiation is still evident when GC activity is prevented. Since cAMP production and activation of PKA were required for cerebellar potentiation a possible role for this messenger in LTD was examined. Inhibition of PKA with H-89 prevented the induction of STP but had no effect on the depression. However, evaluation of cAMP levels by application of the PDE5 inhibitor rolipram resulted in an increase in the extent and duration of

potentiation thereby delaying the onset of the subsequent depression. These results confirm that cAMP/PKA are crucial for the induction of potentiation but do not seem to be required for depression.

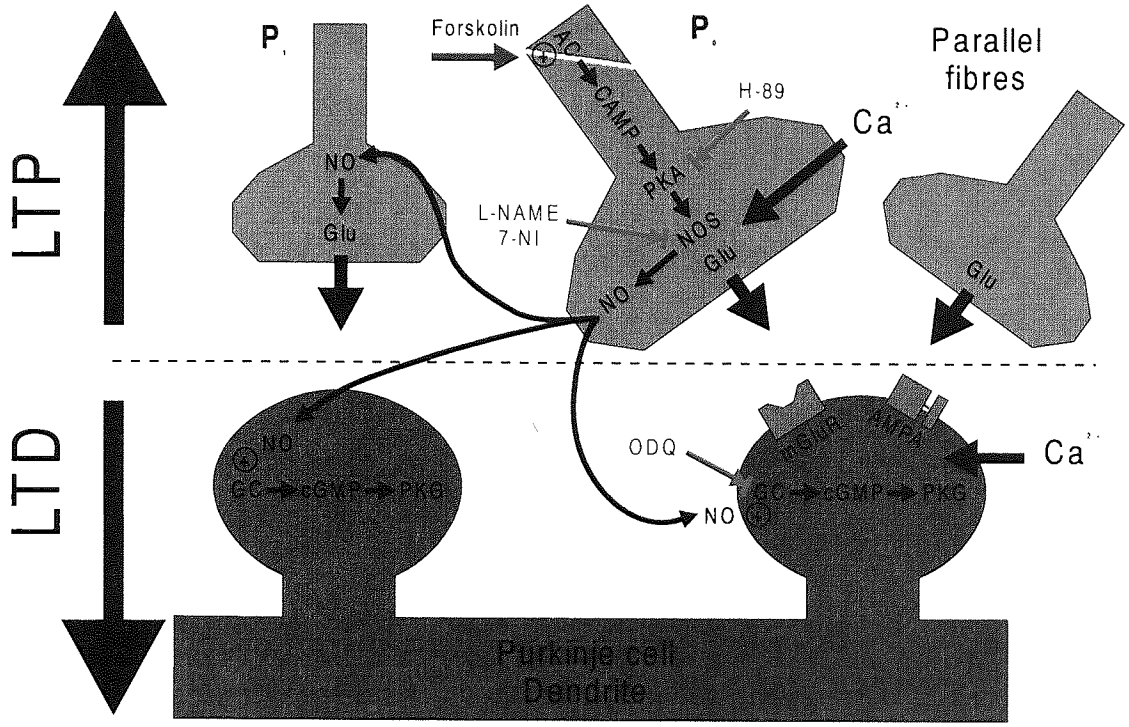


Figure 5.1 The proposed mechanisms for pre-synaptic potentiation and post-synaptic depression at the homosynaptic (P_0) and heterosynaptic (P_1) sites are illustrated. Also shown are the putative sites of action of some of the drugs used and their effects.

Next the effects of modifications to GABAergic transmission on synaptic plasticity evoked by 8Hz PF stimulation were examined. In the absence of the GABA receptor channel blocker picrotoxin RFS of PFs resulted in heterosynaptic potentiation of synaptic transmission. These results are in accordance with an earlier study in which exogenous application of GABA, a process that is likely to reduce post-synaptic calcium levels, led to induction of cerebellar LTP (Shibuki & Okada, 1992). Basket cells, which provide the strongest inhibitory inputs to PCs, like PCs, receive excitatory inputs from PFs, the axons of granule cells and contact the cell bodies of distant PCs. It

is therefore possible that under physiological conditions granule cells, which fire at high frequencies, activate PFs which in turn activate basket cells thereby inducing a form of "surrounded inhibition" such that LTD is formed in PCs which are innervated by the active PF beam while off beam PCs in which, due to the activity of basket cells, post-synaptic calcium levels are suppressed could undergo LTP if they are simultaneously activated by a separate beam of PFs.

Two distinct forms of potentiation have been demonstrated at synapses between inhibitory interneurones and PCs. Rebound potentiation, which is induced by CF activation is a post-synaptic phenomenon (Kano *et al.*, 1992; Kano *et al.*, 1996). Another form of cerebellar potentiation at inhibitory synapses can be induced by stimulation of the molecular layer in conjunction with applications of serotonin or noradrenaline (Mitoma & Konishi, 1996; Mitoma & Konishi, 1999). Although this latter process is mediated via an entirely presynaptic mechanism, both forms of plasticity require PKA. It seemed sensible therefore, to investigate if the heterosynaptic potentiation induced by 8Hz PF stimulation in the absence of picrotoxin, also spread to inhibitory synapses to the same cell.

Under these conditions potentiation of PF responses was found to be restricted to excitatory synapses. Since LTP was capable of spreading to excitatory synapses up to 170μm distant from the site of stimulation, it is unlikely that the lack of plasticity observed at inhibitory synapses was due to the distance between excitatory and inhibitory synapses since the electrode separations were in all cases much less than 170μm. However, this possibility cannot be entirely ruled out because the precise position of the inhibitory synapses with respect to the excitatory synapses is not known.

The data presented in chapter 3 suggest that the spread of cerebellar LTP involved the diffusible messenger NO. It is conceivable therefore, that NO does not play a role in the mechanism underlying synaptic plasticity at synapses between inhibitory interneurones and PCs.

The findings presented in this study indicate that the direction of cerebellar synaptic plasticity is negatively correlated to post-synaptic calcium activity. Furthermore, NO was found to have a dual role in modification of synaptic transmission at the PF-PC synapse. If calcium levels rise during RFS then post-synaptic depression is induced via the actions of NO and post-synaptic cGMP. If calcium levels do not increase, then presynaptic potentiation, which is also mediated via NO but requires unlike LTD the activation of PKA will take place.

The theory of motor learning, which was first proposed by Marr (1969) and later modified by Albus (1971), critically depends on the associative nature of conventional LTD. The CF normally fires irregularly at a relatively low rate of approximately one spike per second and in no particular relation to movement (for a review see Thach *et al.*, 1992). The hypothesis of cerebellar learning suggests that if a movement needs to be learned the CF fires once immediately after an error occurred. This then selectively reduces the strength of those PF synapses active at the same time. In this way, error signals, conveyed to Purkinje cells by CFs, reorganise the internal circuitry of the cerebellar cortex towards an overall reduction of subsequent errors. In this way, CFs act as "teachers" in the process of motor learning. However, the role of the CF in cerebellar processing is controversial. If CF activity essentially determines the strength of transmission at PF-PC synapses, then one would expect the behaviour of PCs to be a

mirror image of CF input. This has been reported to be the case in some (Thompson, 1986; Shidara *et al.*, 1993) but not all studies (Simpson *et al.*, 1996). Furthermore Llinas and co-workers suggested that since each granule cell fires spontaneously, the likelihood of conjunctive granule cell activation and spontaneous CF firing is high and consequently they predicted that depression should occur in all PF synapses on a PC within a short time (Llinas & Welsh, 1993).

The LTP and LTD induced by raised frequency PF stimulation were both independent of CF activation. Not only is there some debate as to the physiological role of CFs in cerebellar processing, it is by no means universally accepted that synaptic plasticity mediates learning and memory. Much of the recent evidence lending support to this concept comes from the use of transgenic animals. The ability of tissues taken from transgenic animals to express various forms of plasticity has been compared with the abilities of the animals to learn in several behavioural learning paradigms. In the cerebellum, animals with knockouts to various receptors or second messengers essential for LTD induction also show impairment in certain forms of motor learning associated with the cerebellum. Examples include acquisition of the eye-blink reflex (Aiba *et al.*, 1994; Shibuki *et al.*, 1996), the VOR or the nictitating membrane response (NMR) (DeZeeuw *et al.*, 1998) a defensive reflex elicited by tactile stimulation of areas of the face around the eye. However, unless the genetic modifications are targeted to specific cell types and brain regions and uncovered at defined stages of development (see Chapter 1 for further discussion), it is difficult to attribute the deficit in learning solely to the deficit in plasticity. Future experiments using this transgenic approach should ideally target key elements identified as potential mediators of LTP and LTD both developmentally and spatially.

An alternative approach to finding a causal relationship between plasticity and memory would be to use functional MRI studies. Monitoring brain activity during the acquisition of motor tasks has recently provided evidence that neuronal activity in the cerebellum increases during the motor learning (Imamizu *et al.*, 2000). Whereas these studies provide information about the areas of the brain involved in various motor tasks, the nature of the imaging method infers an increase in synaptic activity. LTD on the other hand might be expected to reduce synaptic activity and consequently blood flow. The situation is further complicated by the fact that NO, one of the principle mediators of LTD and LTP is a potent vasodilator.

At present, there is no conclusive evidence that CFs provide the predicted reinforcing input via conjunctive, LTD mechanisms in motor learning models. Use-dependent LTP and LTD of PF-PC synapses have now both been demonstrated here and by several other groups *in vitro* and neither require a reinforcing CF input. Either or both of these processes might contribute to behavioural learning. Notwithstanding this possibility, PF-induced LTD produced either by combined raised frequency and intensity stimulation (Hartell, 1996a) or by the 8Hz stimulation used in the present work could underpin a neuroprotective mechanism, particularly given the heterosynaptic nature of this plasticity.

The most obvious explanation for the existence of the cerebellar LTP described here and in previous studies (Salin *et al.*, 1996; Storm *et al.*, 1998) is to simply to provide a mechanism for reversing LTD to prevent its saturation. However, LTP does not reverse LTD, it appears to be a completely separate, CF-independent mechanism that is expressed presynaptically rather than post-synaptically. Although the net effect of both

mechanisms may be to maintain synaptic signalling, both forms could rapidly saturate producing a very energetically inefficient and "noisy" synapse.

The findings presented in this and other studies (Hartell, 1996a; Reynolds & Hartell, 2000; Hartell, 2000; Wang *et al.*, 2000) challenge the Marr-Albus theory of motor learning since LTD was found to be neither associative nor input-specific. It is therefore crucial to clarify the role of the CF input in cerebellar learning models such as the eye-blink reflex, VOR and NMR to establish whether it serves as a reinforcing input. One possible way of doing would be to repeat the experiments in animals that have undergone a chemical lesion to the inferior olive to destroy the CF input. Although the maintained existence of cerebellar plasticity and behavioural learning in these animals might suggest that CFs are not necessary for all forms of motor learning, such lesions dramatically alter the normal input output relationship of the cerebellar cortex and so proper interpretation of the results is difficult.

Little is know about the physiological role of cerebellar LTP. It has been suggested that LTP may be required for the conditioning of the eyeblink reflex (Perrett & Mauk, 1995). Indeed knock out mice that lack AC1, lack cerebellar LTP and show a deficit in motor performance when challenged with the roto-rod test (Storm *et al.*, 1998). These findings suggest that cerebellar LTP might be required for certain forms of motor learning and motor coordination. The fact that both heterosynaptic LTD (Hartell, 1996a; Reynolds & Hartell, 2000; Reynolds & Hartell, 2000; Wang *et al.*, 2000) and LTP have now been demonstrated following raised frequencies of PF activation also suggests that the generally accepted view that cerebellar plasticity is input specific at the cellular level requires re-evaluation.

REFERENCES

Abel, T., Nguyen, P. V., Barad, M., Deuel, T. S., & Kandel, E. R. (1997). Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. *Cell* **88**, 615-626.

Ahn, S., Ginty, D. D., & Linden, D. J. (1999). A late phase of cerebellar long-term depression requires activation of CaMKIV and CREB. *Neuron* **23**, 559-568.

Aiba, A., Chen, C., Herrup, K., Rosenmund, C., Stevens, C. F., & Tonegawa, S. (1994a). Reduced hippocampal long-term potentiation and context-specific deficit in associative learning in mGluR1 mutant mice. *Cell* **79**, 365-375.

Aiba, A., Kano, M., Chen, C., Stanton, M. E., Fox, G. D., Herrup, K., Zwingman, T. A., & Tonegawa, S. (1994b). Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. *Cell* **79**, 377-388.

Ajima, A. & Ito, M. (1995). A unique role of protein phosphatase in cerebellar long-term depression. *NeuroReport* **6**, 297-300.

Albus, J. S. (1971). A theory of cerebellar function. *Mathematical Bioscience* **28**, 167-171.

Anderson, W. W. & Collingridge, G. L. (1999). A data acquisition program for on-line analysis of long-term potentiation and long-term depression. *Soc.Neurosci.Abstr* **23**, 665.

Aramori, I. & Nakanishi, S. (1992). Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron* **8**, 757-765.

Arancio, O., Kandel, E. R., & Hawkins, R. D. (1995). Activity-dependent long-term enhancement of transmitter release by presynaptic 3',5'-cyclic GMP in cultured hippocampal-neurons. *Nature* **376**, 74-80.

Arancio, O., Kiebler, M., Lee, C. J., LevRam, V., Tsien, R. Y., Kandel, E. R., & Hawkins, R. D. (1996). Nitric-oxide acts directly in the presynaptic neuron to produce long- term potentiation in cultured hippocampal-neurons. *Cell* **87**, 1025-1035.

Ariano, M. A., Lewicki, J. A., Brandwein, H. J., & Murad, F. (1982). Immunohistochemical localization of guanylate cyclase within neurons of rat brain. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **79**, 1316-1320.

Atluri, P. P. & Regehr, W. G. (1998). Delayed release of neurotransmitter from cerebellar granule cells. *Journal Of Neuroscience* **18**, 8214-8227.

Baranes, D., Lederfein, D., Huang, Y. Y., Chen, M., Bailey, C. H., & Kandel, E. R. (1998). Tissue plasminogen activator contributes to the late phase of LTP and to synaptic growth in the hippocampal mossy fiber pathway. *Neuron* **21**, 813-825.

Batchelor, A. M., Madge, D. J., & Garthwaite, J. (1994b). Synaptic activation of metabotropic glutamate receptors in the parallel fibre-Purkinje cell pathway in rat cerebellar slices. *Neuroscience* **63**, 911-915.

Batchelor, A. M., Madge, D. J., & Garthwaite, J. (1994a). Synaptic activation of metabotropic glutamate receptors in the parallel fibre-Purkinje cell pathway in rat cerebellar slices. *Neuroscience* **63**, 911-915.

Beavo, J. A. (1988). Multiple isozymes of cyclic nucleotide phosphodiesterase. *Advances in 2nd Messenger and Phosphoprotein Res., 22.Ed.Greengard P and Robinson GA.Raven Press, New York, 1988.*

Berridge, M. J. (1993). A tale of two messengers. *Nature* **365**, 388-389.

Betz, W. J. & Bewick, G. S. (1992). Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. *Science* **255**, 200-203.

Betz, W. J., Bewick, G. S., & Ridge, R. M. (1992a). Intracellular movements of fluorescently labeled synaptic vesicles in frog motor nerve terminals during nerve stimulation. *Neuron* **9**, 805-813.

Betz, W. J., Mao, F., & Bewick, G. S. (1992b). Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *Journal Of Neuroscience* **12**, 363-375.

Bliss, T. V. P. & Collingridge, G. L. (1993). A synaptic model of memory: long term potentiation in the hippocampus. *Nature* **361**, 31-39.

Blond, O., Daniel, H., Otani, S., Jaillard, D., & Crepel, F. (1997). Presynaptic and postsynaptic effects of nitric oxide donors at synapses between parallel fibres and Purkinje cells: Involvement in cerebellar long-term depression. *Neuroscience* **77**, 945-954.

Bohme, G. A., Bon, C., Stutzmann, J. M., Doble, A., & Blanchard, J. C. (1991). Possible involvement of nitric oxide in long-term potentiation. *European Journal Of Pharmacology* **199**, 379-381.

Boulton, C. L., Irving, A. J., Southam, E., Potier, B., Garthwaite, J., & Collingridge, G. L. (1994). The nitric oxide-cyclic GMP pathway and synaptic depression in rat hippocampal slices. *European Journal Of Neuroscience* **6**, 1528-1535.

Boulton, C. L., Southam, E., & Garthwaite, J. (1995). Nitric oxide-dependent long-term potentiation is blocked by a specific inhibitor of soluble guanylyl cyclase. *Neuroscience* **69**, 699-703.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., & Silva, A. J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell* **79**, 59-68.

Boxall, A. R. & Garthwaite, J. (1996). Long-term depression in rat cerebellum requires both NO synthase and NO-sensitive guanylyl cyclase. *European Journal Of Neuroscience* **8**, 2209-2212.

Boxall, A. R., Lancaster, B., & Garthwaite, J. (1996). Tyrosine kinase is required for long-term depression in the cerebellum. *Neuron* **16**, 805-813.

Bredt, D. S., Ferris, C. D., & Snyder, S. H. (1992). Nitric-oxide synthase regulatory sites - phosphorylation by cyclic amp-dependent protein-kinase, protein-kinase-c, and calcium calmodulin protein-kinase - identification of flavin and calmodulin binding-sites. *Journal Of Biological Chemistry* **267**, 10976-10981.

Bredt, D. S., Glatt, D. E., Hwang, P. M., Fotuhi, M., Dawson, T. M., & Snyder, S. H. (1991). Nitric oxide synthase protein and mrna are discretely located in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron* 7, 615-624.

Bredt, D. S., Hwang, P. M., & Snyder, S. H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* **347**, 3768-3770.

Brune, B. & Lapetina, E. G. (1991). Phosphorylation of nitric oxide synthase by protein kinase A. *Biochemical & Biophysical Research Communications* **181**, 921-926.

Casadio, A., Martin, K. C., Giustetto, M., Zhu, H. X., Chen, M., Bartsch, D., Bailey, C. H., & Kandel, E. R. (1999). A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. *Cell* **99**, 221-237.

Castillo, P. E., Janz, R., Sudhof, T. C., Tzounopoulos, T., Malenka, R. C., & Nicoll, R. A. (1997). Rab3A is essential for mossy fibre long-term potentiation in the hippocampus. *Nature* **388**, 590-593.

Castillo, P. E., Weisskopf, M. G., & Nicoll, R. A. (1994). The role of Ca^{2+} channels in hippocampal mossy fiber synaptic transmission and long-term potentiation. *Neuron* **12**, 261-269.

Chavis, P., Mollard, P., Bockaert, J., & Manzoni, O. (1998). Visualization of cyclic AMP-regulated presynaptic activity at cerebellar granule cells. *Neuron* **20**, 773-781.

Chen, C., Kano, M., Abeliovich, A., Chen, L., Bao, S. W., Kim, J. J., Hashimoto, K., Thompson, R. F., & Tonegawa, S. (1995). Impaired motor coordination correlates with persistent multiple climbing fiber innervation in PKCγ mutant mice. *Cell* **83**, 1233-1242.

Chen, C. & Thompson, R. F. (1995). Temporal specificity of long-term depression in parallel fiber-Purkinje synapses in rat cerebellar slice. *Learning and Memory* **2**, 185-198.

Chen, C. F. & Regehr, W. G. (1997). The mechanism of cAMP-mediated enhancement at a cerebellar synapse. *Journal Of Neuroscience* **17**, 8687-8694.

Conquet, F., Bashir, Z. I., Davies, C. H., Daniel, H., Ferraguti, F., Bordi, F., Franz-Bacon, K., Reggiani, A., Matarese, V., Conde, F., Collingridge, G. L., & Crepel, F. (1994). Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1. *Nature* **372**, 237-243.

Crepel, F., Audinat, E., Daniel, H., Hemart, N., Jaillard, D., Rossier, J., & Lambolez, B. (1994). Cellular locus of the nitric oxide-synthase involved in cerebellar long-term depression induced by high external potassium concentration. *Neuropharmacology* **33**, 1399-1405.

Crepel, F. & Jaillard, D. (1990). Protein kinases, nitric oxide and long-term depression of synapses in the cerebellum. *NeuroReport* **1**, 133-136.

Crepel, F. & Jaillard, D. (1991). Pairing of pre-and postsynaptic responses in cerebellar Purkinje cells induces long-term changes in synaptic efficacy *in vitro*. *Journal Of Physiology-London* **432**, 123-141.

Crepel, F. & Krupa, M. (1988). Activation of protein kinase C induces a long-term depression of glutamate sensitivity of cerebellar Purkinje cells. An *in vitro* study. *Brain Research* **458**, 397-401.

Daniel, H., Hemart, N., Jaillard, D., & Crepel, F. (1993). Long-term depression requires nitric oxide and guanosine 3' 5'cyclic monophosphate production in rat cerebellar Purkinje cells. *European Journal Of Neuroscience* **5**, 1079-1082.

Daniel, H., Levenes, C., & Crepel, F. (1998). Cellular mechanisms of cerebellar LTD. *Trends In Neurosciences* **21**, 401-407.

DeVente, J., Bol, J. G. J. M., Berkelmans, H. S., Schipper, J., & Steinbusch, H. M. W. (1990). Immunocytochemistry of cGMP in the cerebellum of the immature, adult, and aged rat; the involvement of nitric oxide. A micropharmacological study. *European Journal Of Neuroscience* **2**, 845-862.

DeZeeuw, C. I., Hansel, C., Bian, F., Koekkoek, S. E., VanAlphen, A. M., Linden, D. J., & Oberdick, J. (1998). Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar LTD and adaptation of the vestibulo-ocular reflex. *Neuron* **20**, 495-508.

Dinerman, J. L., Dawson, T. M., Schell, M. J., Snowman, A., & Snyder, S. (1994). Endothelial nitric oxide synthase localized t hippocampal pyramidal cell; implications for synaptic plasticity. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 4214-4218.

Dubey, R. K., Gillespie, D. G., & Jackson, E. K. (1998). Cyclic AMP-adenosine pathway induces nitric oxide synthesis in aortic smooth muscle cells. *Hypertension* **31**, 296-302.

Eccles, J. C., Ito, M., & Szentagothai, J. (1967). *The Cerebellum as a Neuronal Machine* Springer Verlag, New York.

Edwards, F. A., Konnerth, A., Sakmann, B., & Takahashi, T. (1989). A thin slice preparation for patch clamp recordings from neurons of the mammalian central nervous-system. *Pflugers Archiv-European Journal Of Physiology* **414**, 600-612.

Eilers, J., Augustine, G. J., & Konnerth, A. (1995). Subthreshold synaptic Ca^{2+} signalling in fine dendrites and spines of cerebellar Purkinje neurons. *Nature* **373**, 155-158.

Eilers, J., Takechi, H., Finch, E. A., Augustine, G. J., & Konnerth, A. (1997). Local dendritic Ca2+ signaling induces cerebellar long-term depression. *Learning & Memory* **4**, 159-168.

Ekerot, C. F. & Kano, M. (1985). Long-term depression of parallel fiber synapses following stimulation of climbing fibers. *Brain Research* **342**, 357-360.

Finch, E. A. & Augustine, G. J. (1998). Local calcium signalling by inositol-1,4, 5-trisphosphate in Purkinje cell dendrites. *Nature* **396**, 753-756.

Fitzsimonds, R. M., Song, H., & Poo, M. (1997). Propagation of activity-dependent synaptic depression in simple neural networks. *Nature* **388**, 439-448.

Frey, U., Huang, Y.-Y., & Kandel, E. R. (1993). Effects of camp stimulate a late stage of LTP in hippocampal CA1 neurons. *Science* **260**, 1661-1664.

Fykse, E. M., Li, C., & Sudhof, T. C. (1995). Phosphorylation of rabphilin-3A by Ca2+/calmodulin-and cAMP-dependent protein kinases *in vitro*. *Journal Of Neuroscience* **15**, 2385-2395.

Galione, A., White, A., Wilmott, N., Turner, M., Potter, B. L., & Watson, S. P. (1993). cGMP mobilizes intracellular Ca2+ in sea urchin eggs by stimulating cyclic ADP-ribose synthesis. *Nature* **365**, 456-459.

Garthwaite, J., Southam, E., Boulton, C. L., Nielson, E. B., Schmidt, K., & Mayer, B. (1995). Potent and selective-inhibition of nitric oxide-sensitive guanylyl cyclase by 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Molecular Pharmacology* **48**, 184-188.

Glaum, S. R., Traverse, S. N., Rossi, D. J., & Millar, R. J. (1992). Role of metabotropic glutamate (t-ACPD) receptors at the parallel fiber-Purkinje cell synapse. *Journal Of Neurophysiology* **64**, 1453-1462.

Greengard, P., Valtorta, F., Czernik, A. J., & Benfenati, F. (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **259**, 780-785.

Gribkoff, V. K. & Bauman, L. A. (1992). Endogenous adenosine contributes to hypoxic synaptic depression in hippocampus from young and aged rats. *Journal Of Neurophysiology* **68**, 620-628.

Haley, J. E., Wilcox, G. L., & Chapman, P. F. (1992). The role of nitric oxide in hippocampal long-term potentiation. *Neuron* 211-216.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B., & Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Archiv-European Journal Of Physiology* **391**, 85-100.

Harris, E. W. & Cotman, C. W. (1986). Long-term potentiation of guinea pig mossy fiber responses is not blocked by N-methyl D-aspartate antagonists. *Neuroscience Letters* **70**, 132-137.

Hartell, N. A. (1994). cGMP acts within cerebellar Purkinje cells to produce long term depression via mechanisms involving PKC and PKG. *NeuroReport* **5**, 833-836.

Hartell, N. A. (1996a). Inhibition of cGMP breakdown promotes the induction of cerebellar long-term depression. *Journal Of Neuroscience* **16**, 2881-2890.

Hartell, N. A. (1996b). Strong activation of parallel fibers produces localized calcium transients and a form of LTD that spreads to distant synapses. *Neuron* **16**, 601-610.

Hartell, N. A. (1996c). The intracellular mechanisms underlying parallel fiber-induced, heterosynaptic long-term depression in the cerebellum. *Brit.J.Pharmacol.* **119**, 68.

Hartell, N. A. (1996d). Two separate pathways for cerebellar LTD: NO-dependent and NO- independent. *Behavioral And Brain Sciences* **19**, 453.

Hartell, N. A. (2000). Receptors, second messengers and protein kinases required for heterosynaptic cerebellar long-term depression. *Neuropharmacology* **40,** 148-161.

Hartell, N. A. & Okada, D. (1998). Imaging cGMP in cerebellar Purkinje cells. *Journal Of Physiology-London* **509**, 60P.

Hawkins, R. D. (1996). NO honey, I don't remember. *Neuron* **16**, 465-467.

Hawkins, R. D., Kandel, E. R., & Siegelbaum, S. A. (1993). Learning to modulate transmitter release: themes and variations in synaptic plasticity. *Annual Review Of Neuroscience* **16**, 625-665.

Hemart, N., Daniel, H., Jaillard, D., & Crepel, F. (1994). Properties of glutamate receptors are modified during long-term depression in rat cerebellar Purkinje cells. *Neuroscience* **19**, 213-221.

Hemart, N., Daniel, H., Jaillard, D., & Crepel, F. (1995). Receptors and second messengers involved in long-term depression in rat cerebellar slices *in vitro*: a reappraisal. *European Journal Of Neuroscience* **7**, 45-53.

Hirano, T. (1990a). Depression and potentiation of the synaptic transmission between a granule cell and a purkinje-cell in rat cerebellar culture. *Neuroscience Letters* **119**, 141-144.

Hirano, T. (1990b). Synaptic transmission between rat inferior olivary neurones and cerebellar Purkinje cells in culture. *Journal Of Neurophysiology* **63**, 181-189.

Holscher, C. (1997). Nitric oxide, the enigmatic neuronal messenger: Its role in synaptic plasticity. *Trends In Neurosciences* **20**, 298-303.

Huang, Y.-Y. & Kandel, E. R. (1994). Recruitment of long-lasting and protein kinase A-dependent long-term potentiation in the CA1 region of hippocampus requires repeated tetanization. *Learning and Memory* **1**, 74-82.

Huang, Y. Y., Kandel, E. R., Varshavsky, L., Brandon, E. P., Qi, M., Idzerda, R. L., McKnight, G. S., & Bourtchouladze, R. (1995). A genetic test of the effects of mutations in PKA on mossy fiber LTP and its relation to spatial and contextual learning. *Cell* **83**, 1211-1222.

Huang, Y. Y., Li, X. C., & Kandel, E. R. (1994). cAMP contributes to mossy fiber LTP by initiating both a covalently mediated early phase and macromolecular synthesis-dependent late- phase. *Cell* **79**, 69-79.

Imamizu, H., Miyauchi, S., Tamada, T., Sasaki, Y., Takino, R., Putz, B., Yoshioka, T., & Kawato, M. (2000). Human cerebellar activity reflecting an acquired internal model of a new tool. *Nature* **403**, 192-195.

Impey, S., Mark, M., Villacres, E. C., Poser, S., Chavkin, C., & Storm, D. R. (1996). Induction of cre-mediated gene-expression by stimuli that generate long-lasting ltp in area ca1 of the hippocampus. *Neuron* **16**, 973-982.

Inada, H., Shindo, H., Tawata, M., & Onaya, T. (1998). cAMP regulates nitric oxide production and ouabain sensitive Na+, K+-ATPase activity in SH-SY5Y human neuroblastoma cells. *Diabetologia* **41**, 1451-1458.

Inada, H., Shindo, H., Tawata, M., & Onaya, T. (1999). Cilostazol, a cyclic AMP phosphodiesterase inhibitor, stimulates nitric oxide production and sodium potassium adenosine triphosphatase activity in SH-SY5Y human neuroblastoma cells. *Life Sciences* **65**, 1413-1422.

Ito, I. & Sugiyama, H. (1991). Roles of glutamate receptors in long-term potentiation at hippocampal mossy fiber synapses. *NeuroReport* **2**, 333-336.

Ito, M. (1982). Cerebellar control of the vestibulo-ocular reflex around the flocculus hypothesis. *Annual Review Of Neuroscience* **5**, 0-96.

Ito, M. (1989). Long-term depression. *Annual Review Of Neuroscience* **12**, 85-102.

Ito, M. & Karachot, L. (1990). Messengers mediating long-term desensitization in cerebellar Purkinje cells. *NeuroReport* **1**, 129-132.

Ito, M. & Karachot, L. (1992). Protein kinases and phosphatase inhibitors mediating long-term desensitization of glutamate receptors in cerebellar Purkinje cells. *Neuroscience* **14** , 27-38.

Ito, M., Sakurai, M., & Tongroach, P. (1982). Climbing fiber induced depression of both mossy fiber responsiveness and glutamate sensitivity of cerebellar Purkinje cells. *Journal Of Physiology-London* **324**, 113-134.

Jacoby, S. & Hartell, N. A. (1999). Raised frequency parallel fibre stimulation can produce both nitric oxide-dependent LTP and LTD in the rat cerebellar cortex. *Journal Of Physiology-London* **520**, 38P.

Jayaraman, T., Ondrias, K., Ondriasova, E., & Marks, A. R. (1996). Regulation of the inositol 1,4,5-trisphosphate receptor by tyrosine phosphorylation. *Science* **272**, 1492-1494.

Kano, M., Fukunaga, K., & Konnerth, A. (1996). Ca^{2+}-induced rebound potentiation of gamma-aminobutyric acid-mediated currents requires activation of ca2+/calmodulin-dependent kinase-II. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **93**, 13351-13356.

Kano, M., Rexhausen, U., Dreessen, J., & Konnerth, A. (1992). Synaptic excitation produces a long-lasting rebound potentiation of inhibitory synaptic signals in cerebellar Purkinje cells. *Nature* **356**, 601-604.

Karachot, L., Kado, R. T., & Ito, M. (1995). Stimulus parameters for induction of long-term depression in *in vitro* rat Purkinje cells. *Neuroscience Research* **21**, 161-168.

Kashiwabuchi, N., Ikeda, K., Araki, K., Hirano, T., Shibuki, K., Takayama, C., Inoue, Y., Kutsuwasa, T., Yagi, T., Kang, Y., Aizawa, S., & Mishina, M. (1995). Impairment of motor coordination, Purkinje cell synapse formation, and cerebellar long-term depression in GluRδ2 mutant mice. *Cell* **91**, 43831.

Kasono, K. & Hirano, T. (1994). Critical role of postsynaptic calcium in cerebellar long-term depression. *NeuroReport* **6**, 17-20.

Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E., & Graybiel, A. M. (1998). A family of cAMP-binding proteins that directly activate Rap1. *Science* **282**, 2275-2279.

Kimura, S., Uchiyama, S., Takahashi, H. E., & Shibuki, K. (1998). cAMP-dependent long-term potentiation of nitric oxide release from cerebellar parallel fibers in rats. *Journal Of Neuroscience* **18**, 8551-8558.

Konnerth, A., Dreessen, J., & Augustine, G. J. (1992). Brief dendritic calcium signals initiate long-lasting synaptic depression in cerebellar Purkinje cells. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **89**, 7051-7055.

Konnerth, A., Llano, I., & Armstrong, C. M. (1990). Synaptic currents in cerebellar Purkinje cells. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **87**, 2662-2665.

Lev Ram, V., Makings, L. R., Keitz, P. F., Kao, J. Y., & Tsien, R. Y. (1995). Long-term depression in cerebellar Purkinje neurons results from coincidence of nitric oxide and depolarization-induced Ca2+ transients. *Neuron* **15**, 407-415.

Linden, D. J. (1994). Input-specific induction of cerebellar Long-term depression does not require presynaptic alteration. *Learning and Memory* **1**, 121-128.

Linden, D. J. (1997). Long-term potentiation of glial synaptic currents in cerebellar culture. *Neuron* **18**, 983-994.

Linden, D. J. (1998). Synaptically evoked glutamate transport currents may be used to detect the expression of long-term potentiation in cerebellar culture. *Journal Of Neurophysiology* **79**, 3151-3156.

Linden, D. J. & Connor, J. A. (1991). Participation of postsynaptic PKC in cerebellar long-term depression in culture. *Science* **254**, 1656-1659.

Linden, D. J. & Connor, J. A. (1992). Long-term depression of glutamate currents in cultured cerebellar Purkinje neurons does not require nitric oxide signalling. *European Journal Of Neuroscience* **4**, 10-15.

Linden, D. J., Dawson, T. M., & Dawson, V. L. (1995). An evaluation of the nitric oxide/cGMP-dependent protein kinase cascade in the induction of cerebellar long-term depression in culture. *Journal Of Neuroscience* **15**, 5098-5105.

Linden, D. J., Dickenson, M. H., Smeyne, M., & Connor, J. A. (1991). A long-term depression of AMPA currents in cultured cerebellar Purkinje neurons. *Neuron* **7**, 81-89.

Linden, D. J., Smeyne, M., & Connor, J. A. (1993). Induction of cerebellar long-term depression in culture requires postsynaptic action of sodium ions. *Neuron* **11**, 1093-1110.

Llinas, R. & Welsh, J. P. (1993). On the cerebellum and motor learning. *Current Opinion In Neurobiology* **3**, 958-965.

Lohmann, S. M., Walter, U., Miller, P. E., Greengard, P., & Camilli, P. (1981). Immunohistochemical localization of cyclic-GMP-dependent protein kinase in the mammalian brain. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **78** , 653-657.

Lu, Y. F., Kandel, E. R., & Hawkins, R. D. (1999). Nitric oxide signaling contributes to late-phase LTP and CREB phosphorylation in the hippocampus. *Journal Of Neuroscience* **19**, 10250-10261.

Lynch, G., Larson, J., Kelso, S., Barrionuevo, G., & Schottler, F. (1983). Intracellular injections of EGTA block induction of hippocampal long- term potentiation. *Nature* **305**, 719-721.

Malenka, R. C., Kauer, J. A., Zucker, R. S., & Nicholl, R. A. (1988). Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. *Science* **242**, 81-84.

Malenka, R. C. & Nicoll, R. A. (1993). NMDA-receptor-dependent synaptic plasticimultiple forms and mechanisms. *Trends In Neurosciences* **16**, 521-527.

Malinow, R. & Miller, J. P. (1986). Postsynaptic hyperpolarization during conditioning reversibly blocks induction of long-term potentiation. *Nature* **320**, 529-530.

Manabe, T., Wyllie, D. A., Perkel, D. J., & Nicoll, R. A. (1993). Modulation of synaptic transmission and long-term potentiation - effects on paired-pulse facilitation and epsc variance in the CA1 region of the hippocampus. *Journal Of Neurophysiology* **70**, 1451-1459.

Maragos, C. M., Morley, D., Wink, D. A., Dunams, T. M., Saavedra, J. E., Hoffman, A., Bove, A. A., Isaac, L., Hrabie, J. A., & Keefer, L. K. (1991). Complexes of NO with nucleophiles as agents for the controlled biological release of nitric-oxide - vasorelaxant effects. *Journal Of Medicinal Chemistry* **34**, 3242-3247.

Marr, D. (1969). A theory of cerebellar cortex. *Journal Of Physiology-London* **202**, 437-470.

Matthies, H. & Reymann, K. G. (1993). Protein kinase-a inhibitors prevent the maintenance of hippocampal long-term potentiation. *NeuroReport* **4**, 712-714.

Mauk, M. D. & Donegan, N. H. (1997). A model of Pavlovian eyelid conditioning based on the synaptic organization of the cerebellum. *Learning and Memory.* **4**, 130-158.

Meffert, M. K., Calakos, N. C., Scheller, R. H., & Schulman, H. (1996). Nitric oxide modulates synaptic vesicle docking fusion reactions. *Neuron* **16**, 1229-1236.

Meffert, M. K., Premack, B. A., & Schulman, H. (1994). Nitric oxide stimulates Ca^{2+}-independent synaptic vesicle release. *Neuron* **12**, 1235-1244.

Mitoma, H. & Konishi, S. (1996). Long-lasting facilitation of inhibitory transmission by monoaminergic and cAMP-dependent mechanism in rat cerebellar GABAergic synapses. *Neuroscience Letters* **217**, 141-144.

Mitoma, H. & Konishi, S. (1999). Monoaminergic long-term facilitation of GABA-mediated inhibitory transmission at cerebellar synapses. *Neuroscience* **88**, 871-883.

Miyata, M., Okada, D., Hashimoto, K., Kano, M., & Ito, M. (1999). Corticotropin-releasing factor plays a permissive role in cerebellar long-term depression. *Neuron* **22**, 763-775.

Moore, P. K., al Swayeh, O. A., Chong, N. W., Evans, R. A., & Gibson, A. (1990). L-NG-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br.J.Pharmacol.* **99**, 408-412.

Moore, P. K., Wallace, P., Gaffen, Z., Hart, S. L., & Babbedge, R. C. (1993). Characterization of the novel nitric oxide synthase inhibitor 7-nitro indazole and related indazoles: antinociceptive and cardiovascular effects. *Br.J.Pharmacol.* **110**, 219-224.

Musleh, W. Y., Shahi, K., & Baudry, M. (1993). Further studies concerning the role of nitric oxide in LTP induction and maintenance. *Synapse* **13**, 370-375.

Nakanishi, S. (1989). K-252-derivatives - K252a, K252b, KT5720, KT5962, KT5823. *Seitaino-Kagaku* **40** , 364-365.

Nakanishi, S. (1994). Metabotropic glutamate receptors - synaptic transmission, modulation, and plasticity. *Neuron* **13**, 1031-1037.

Nakazawa, K., Mikawa, S., Hashikawa, T., & Ito, M. (1995). Transient and persistent phosphorylation of AMPA-type glutamate receptor subunits in cerebellar Purkinje cells. *Neuron* **15**, 697-709.

Neher, E. (1992). Ion channels for communication between and within cells (Nobel lecture). *Angewandte Chemie-International Edition In English* **31**, 824-829.

Neher, E. & Sakmann, B. (1992). The patch clamp technique. *Sci.Am.* **266**, 28-35.

Nicoll, R. A. & Malenka, R. C. (1995). Contrasting properties of two forms of long-term potentiation in the hippocampus. *Nature* **377**, 115-118.

Nowicky, A. V. & Bindman, L. J. (1993). The nitric oxide synthase inhibitor, N-monomethyl-L-arginine blocks induction of a long-term potentiation-like phenomenon in rat medial frontal cortical neurons in vitro. *Journal Of Neurophysiology* **70**, 1255-1259.

Odell, T. J., Hawkins, R. D., Kandel, E. R., & Arancio, O. (1991). Tests of the roles of 2 diffusible substances in long-term potentiation - evidence for nitric-oxide as a possible early retrograde messenger. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **88**, 11285-11289.

Okada, D. (1992). Two pathways of cyclic GMP production through glutamate receptor mediated nitric oxide synthesis. *Journal Of Neurochemistry* **59**, 1203-1209.

Perrett, S. P. & Mauk, M. D. (1995). Extinction of conditioned eyelid responses requires the anterior lobe of cerebellar cortex. *Journal Of Neuroscience* **15**, 2074-2080.

Polte, T. & Schroder, H. (1998). Cyclic AMP mediates endothelial protection by nitric oxide. *Biochemical & Biophysical Research Communications* **251**, 460-465.

Pyle, J. L., Kavalali, E. T., Choi, S., & Tsien, R. W. (1999). Visualization of synaptic activity in hippocampal slices with FM1-43 enabled by fluorescence quenching. *Neuron* **24**, 803-808.

Qian, H. & Dowling, J. E. (1994). Pharmacology of novel GABA receptors found on rod horizontal cells of the white perch retina. *Journal Of Neuroscience* **14**, 4299-4307.

Raymond, J. L. (1998). Learning in the oculomotor system: from molecules to behavior. *Current Opinion In Neurobiology* **8**, 770-776.

Reynolds, T. & Hartell, N. A. (2000). An evaluation of the synapse-specificity of long-term depression induced in rat cerebellar slices. *Journal Of Physiology-London* **527**, 563-577.

Ross, C. A., Bredt, D. S., & Snyder, S. H. (1990). Messenger Molecules in the cerebellum. *Trends In Neurosciences* **13**, 216-222.

Ross, W. N. & Werman, R. (1987). Mapping calcium transients in the dendrites of Purkinje-cells from the guinea-pig cerebellum *in vitro*. *Journal Of Physiology-London* **389**, 319-336.

Sakmann, B. (1992). Elementary steps in synaptic transmission revealed by currents through single ion channels. *Biosci.Rep.* **12**, 237-262.

Sakmann, B. & Neher, E. (1984). Patch clamp techniques for studying ionic channels in excitable membranes. *Annu.Rev.Physiol* **46**, 455-472.

Sakurai, M. (1990). Calcium is an intracellular mediator of the climbing fibre induction of cerebellar long-term depression. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **87**, 3383-3385.

Salin, P. A., Malenka, R. C., & Nicoll, R. A. (1996). Cyclic-AMP mediates a presynaptic form of LTP at cerebellar parallel fiber synapses. *Neuron* **16**, 797-803.

Schoepp, D. D. & Conn, P. J. (1993). Metabotropic glutamate receptors in brain function and pathology. *Trends In Pharmacological Sciences* **14**, 13-20.

Schreurs, B. G. & Alkon, D. L. (1993). Rabbit cerebellar slice analysis of long-term depression and its role in classical conditioning. *Brain Research* **631**, 23-240.

Schreurs, B. G., Oh, M., & Alkon, D. L. (1996). Pairing-specific long-term depression of Purkinje cell excitatory postsynaptic potentials results from a classical conditioning proceedure in the rabbit cerebellar slice. *Journal Of Neurophysiology* **753**, 1051-1060.

Schuman, E. M. & Madison, D. V. (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* **254**, 1503-1506.

Schuman, E. M. & Madison, D. V. (1994a). Locally distributed synaptic potentiation in the hippocampus. *Science* **263**, 532-536.

Schuman, E. M. & Madison, D. V. (1994b). Nitric oxide and synaptic function. *Annual Review Of Neuroscience* **17**, 153-183.

Seamon, K. B. & Daly, J. W. (1986). Forskolin: its biological and chemical properties. *Adv.Cyclic.Nucleotide.Protein Phosphorylation.Res.* **20**, 1-150.

Shibuki, K., Gomi, H., Chen, L., Bao, S. W., Kim, J. K., Wakatsuki, H., Fujisaki, T., Fujimoto, J., Katoh, A., Ikeda, T., Chen, C., Thompson, R. F., & Itohara, S. (1996). Deficient cerebellar long-term depression, impaired eyeblink conditioning, and normal motor coordination in GFAP mutant mice. *Neuron* **16**, 587-599.

Shibuki, K. & Kimura, S. (1997). Dynamic properties of nitric oxide release from parallel fibres in rat cerebellar slices. *Journal Of Physiology-London* **498**, 443-452.

Shibuki, K. & Okada, D. (1991). Endogenous nitric oxide release required for long-tern synaptic depression in the cerebellum. *Nature* **349**, 326-328.

Shibuki, K. & Okada, D. (1992). Cerebellar long-term potentiation under surpressed postsynaptic Ca2+ activity. *NeuroReport* **3**, 231-234.

Shigemoto, R., Abe, T., Nomura, S., Nakanishi, S., & Hirano, T. (1994). Antibodies inactivating mgGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells. *Neuron* **12**, 1245-1255.

Shidara, M., Kawano, K., Gomi, H., & Kawato, M. (1993). Inverse-dynamics model eye movement control by Purkinje cells in the cerebellum. *Nature* **365**, 50-52.

Simpson, J. I., Wylie, D. R., & DeZeeuw, C. I. (1996). On climbing fiber signals and their consequence(s). *Behavioral And Brain Sciences* **19**, 384.

Son, H., Hawkins, R. D., Martin, K., Kiebler, M., Huang, P. L., Fishman, M. C., & Kandel, E. R. (1996). Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. *Cell* **87**, 1015-1023.

Southam, E., Morris, R., & Garthwaite, J. (1992). Sources and targets of nitric oxide in rat cerebellum. *Neuroscience* **137**, 241-244.

Spillane, D. M., Rosahl, T. W., Sudhof, T. C., & Malenka, R. C. (1995). Long-term potentiation in mice lacking synapsins. *Neuropharmacology* **34**, 1573-1579.

Storm, D. R., Hansel, C., Hacker, B., Parent, A., & Linden, D. J. (1998). Impaired cerebellar long-term potentiation in type I adenylyl cyclase mutant mice. *Neuron* **20**, 1199-1210.

Sutton, N. G. (1971). *Anatomy of the Brain and Spinal Medulla: a manual for students* Butterworth & Co. Publishers Ltd..

Takechi, H., Eilers, J., & Konnerth, A. (1998). A new class of synaptic response involving calcium release in dendritic spines. *Nature* **396**, 757-760.

Thach, W. T., Goodkin, H. P., & Keating, J. G. (1992). The cerebellum and the adaptive coordination of movement. *Annual Review Of Neuroscience* **15**, 403-442.

Thach, W. T. (1998). A Role for the Cerebellum in Learning Movement Coordination. *Neurobiol.Learn.Mem.* **70**, 177-188.

Thompson, R. F. (1986). The neurobiology of learning and memory. *Science* **233**, 941-947.

Thompson, R. F. & Krupa, D. J. (1994). Organization of memory traces in the mammalian brain. *Annual Review Of Neuroscience* **17**, 519-549.

Tong, G., Malenka, R. C., & Nicoll, R. A. (1996). Long-term potentiation in cultures of single hippocampal granule cells: A presynaptic form of plasticity. *Neuron* **16**, 1147-1157.

Trudeau, L. E., Emery, D. G., & Haydon, P. G. (1996). Direct modulation of the secretory machinery underlies PKA-dependent synaptic facilitation in hippocampal neurons. *Neuron* **17** , 789-797.

Villacres, E. C., Wong, S. T., Chavkin, C., & Storm, D. R. (1998). Type I adenylyl cyclase mutant mice have impaired mossy fiber long- term potentiation. *Journal Of Neuroscience* **18**, 3186-3194.

Vincent, P. & Marty, A. (1993). Neighboring cerebellar Purkinje cells communicate via retrograde inhibition of common presynaptic interneurons. *Neuron* **11**, 885-893.

Voogd, J. (1992). The morphology of the cerebellum; the last 25 years. *Eur.J.Morphology* **30**, 81-96.

Voogd, J. & Glickstein, M. (1998). The anatomy of the cerebellum. *Trends In Neurosciences* **21**, 370-375.

Wang, S. S., Khiroug, L., & Augustine, G. J. (2000). Quantification of spread of cerebellar long-term depression with chemical two-photon uncaging of glutamate. *Proc.Natl.Acad.Sci.U.S.A* **97**, 8635-8640.

Wang, Y. T. & Linden, D. J. (2000). Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. *Neuron* **25**, 635-647.

Weisskopf, M. G., Castillo, P. E., Zalutsky, R. A., & Nicoll, R. A. (1994). Mediation of hippocampal mossy fiber long-term potentiation by cyclic-amp. *Science* **265**, 1878-1882.

Weisskopf, M. G. & Nicoll, R. A. (1995). Presynaptic changes during mossy fiber ltp revealed by NMDA receptor- mediated synaptic responses. *Nature* **376**, 256-259.

Williams, J. H., Li, Y.-G., Nayak, A., Errigton, M. L., Murphy, K. J., & Bliss, T. V. P. (1993). The suppression of Long-term potentiation in rat hippocampus by inhibitors of nitric oxide synthase is temperature and age dependent. *Neuron* **11**, 877-884.

Wong, S. T., Athos, J., Figueroa, X. A., Pineda, V. V., Schaefer, M. L., Chavkin, C. C., Muglia, L. J., & Storm, D. R. (1999). Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. *Neuron* **23**, 787-798.

Xia, Z. G., Refsdal, C. D., Merchant, K. M., Dorsa, D. M., & Storm, D. R. (1991). Distribution of mRNA for the calmodulin-sensitive adenylate cyclase in rat brain: expression in areas associated with learning and memory. *Neuron* **6**, 431-443.

Xiang, Z., Greenwood, A. C., Kairiss, E. W., & Brown, T. H. (1994). Quantal mechanism of long-term potentiation in hippocampal mossy-fiber synapses. *Journal Of Neurophysiology* **71** , 2552-2556.

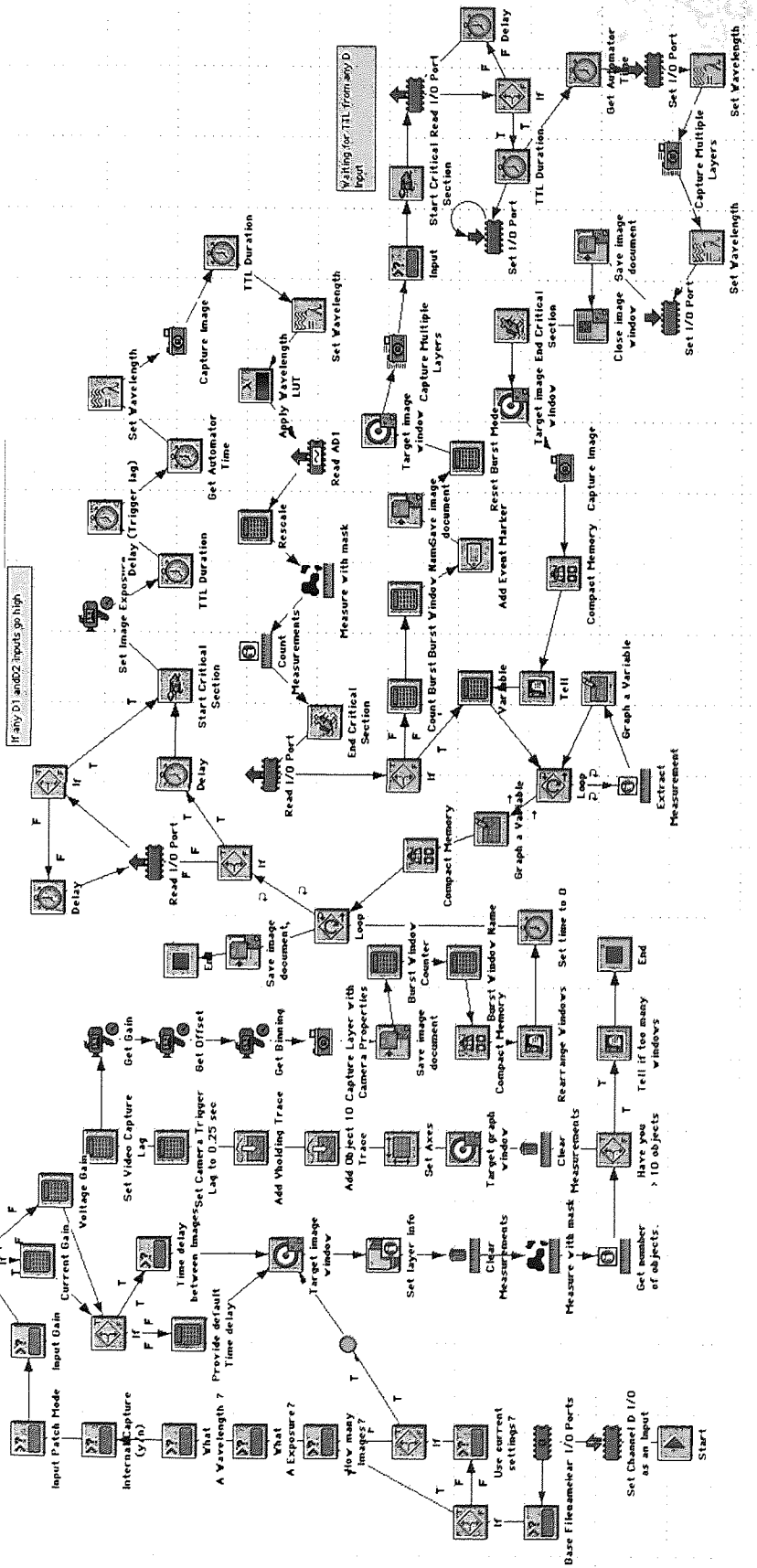
Yeo, C. H. & Hesslow, G. (1998). Cerebellum and conditioned reflexes. *Trends In Cognitive Sciences* **2**, 322-330.

Zalutsky, R. A. & Nicoll, R. A. (1990). Comparison of two forms of long-term potentiation in single hippocampal neurons. *Science* **248**, 1619-1624.

Zucker, R. S. (1973). Changes in the statistics of transmitter release during facilitation. *Journal Of Physiology-London* **229**, 787-810.

Zucker, R. S. (1989). Short-term synaptic plasticity. *Annual Review Of Neuroscience* **12**, 13-31.

APPENDIX 1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Input Patch Mode
Internal Capture (y/n)
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What λ Exposure?
How many images?
Use current settings?
Base Filename
Set Channel D I/O as an input
Start
Input Gain
Current Gain
Provide default Time delay
Time delay between Images
Voltage Gain
Target image window
Set layer info
Clear Measurements
Measure with mask
Get number of objects
Have you > 10 objects
Tell if too many windows
Rearrange Windows
Clear Measurements
Target graph window
Set Axes
Add Object ID Trace
Add Wholding Trace
Set Camera Trigger Lag to 0.25 sec
Set Video Capture Lag
Get Binning
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Get Gain
Capture Layer with Camera Properties
Save image document
Burst Window Counter
Compact Memory
Burst Window Name
Set time to 0
End
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Compact Memory
Graph a Variable
Delay
Read I/O Port
Delay
Start Critical Section
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Delay (Trigger lag)
TTL Duration
Get Automator Time
Set Wavelength
Capture Image
TTL Duration
Set Wavelength
Apply Wavelength LUT
Read AD1
Rescale
Measure with mask
Count Measurements
End Critical Section
Read I/O Port
Count Burst Window Name
Save image document
Add Event Marker
Reset Burst Mode
Target image window
Capture Multiple Layers
Start Critical Read I/O Port Section
Set I/O Port
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