An investigation of NMDA receptor subunit pharmacology

Andrew Peter Mallon

Institute of Biomedical and Life Sciences,

Division of Neuroscience and Biomedical Systems,

University of Glasgow.

April 2004

Thesis submitted in part fulfilment of the requirement for admission to the degree of Doctor of Philosophy of the University of Glasgow.

Abstract

N-Methyl-D-aspartate (NMDA) receptors are critically involved in synaptic transmission, neural development and various forms of neuronal plasticity including long-term potentiation (LTP) and long-term depression (LTD). They are also involved in the production of neuronal damage following excessive activation by glutamate released as a result of hypoxia or ischaemia. Each heteromeric receptor includes one or two NR1 subunits, at least two of the four NR2A-D subunits and less usually the NR3A/B subunits. This study demonstrates that the putative NR2B subunit-containing NMDA receptor antagonist Ro 25-6981 potentiates the effects of NMDA on rat hippocampal slices. The NR2A subunit antagonist PEAQX blocks the effects of NMDA alone and the potentiated response following Ro 25-6981 application. Furthermore, Ro 25-6981 was not neuroprotective as reported previously but unexpectedly precipitated excitotoxicity. The potentiating effect of Ro 25-6981 required around 20 minutes to become apparent, took a further 30 minutes to reach its maximum effect and was irreversible. It was not prevented by staurosporine (a broad-spectrum protein kinase inhibitor), okadaic acid (a potent inhibitor of the serine/threonine protein phosphatases types 1 and 2A) or anisomycin (a protein synthesis inhibitor). However, the potentiation was prevented by cyclosporin A (an inhibitor of Ca^{2+}/calmodulin-dependent phosphatase 2B [calcineurin]). The results

indicate that in an intact neuronal network, NR2B subunits tonically gate NR2A subunit-containing receptor function by a negative coupling mechanism involving calcineurin activation.

NMDA receptor-dependent LTP induced by high frequency stimulation was prevented by PEAQX, an NR2A antagonist. Ro 25-6981 was unable to prevent LTP induction but was associated with a marginal reduction in the magnitude of LTP induced.

There is evidence for the binding of homoquinolinic acid to an NMDA-insensitive novel binding site in the brain. This study investigated the pharmacology of homoquinolinate on the evoked field excitatory synaptic potential (fEPSP) recorded from the CA1 area of rat hippocampal slices. Two NMDA receptor agonists, quinolinic acid 150μM and homoquinolinic acid 2.5μM, caused an approximately 50% inhibition of fEPSP slope. Paired-pulse studies suggested there might be a presynaptic component to this action that is independent of presynaptic adenosine A_1 receptor activation. The broad-spectrum EAA antagonist kynurenic acid and the NMDA receptor blockers 2-amino-5-phosphonopentanoic acid and dizocilpine could prevent the inhibition of fEPSP slope. None of these antagonists revealed any other NMDA-insensitive activity of homoquinolinic acid. The use of 2-carboxy-3-carboxymethylquinoline (CCMQ) to displace the reported NMDA-insensitive binding had no effect on either baseline fEPSP slope or the depression caused by homoquinolinic

acid. It was also apparent that responses to homoquinolinic acid were blocked completely by the NR2A subunit-selective antagonist PEAQX, but not by the NR2B subunit-selective blocker Ro 25-6981. It was concluded that the novel binding site for homoquinolinic acid does not affect synaptic potentials in the hippocampus and that homoquinolinic acid appears to be a selective agonist at NMDA receptors that include the NR2A subunit. Although the NR2B agonist site may be maximally activated under normal conditions and therefore it is not possible to observe any additional effects upon fEPSP slope.

This study next investigated the negative coupling between NR2B and NR2A subunit-containing receptors, combining the NR2A/B subunit selective agonist HQA with the NR2B and NR2A selective antagonists Ro 25-6981 and PEAQX. The negative coupling observed previously with applications of NMDA was also seen using HQA and QA. The potentiation of responses to HQA by Ro 25-6981 application was also associated with an enhancement of paired-pulse interactions. The subsequent application of PEAQX was able to block both the depression of fEPSP slope and the associated enhancement of paired-pulse interactions. The presence of a presynaptic element during applications of HQA alone and potentiated responses alike and the blockade of these effects by PEAQX suggests the NR2A subunit-containing NMDA receptor is responsible for the presynaptic effects acting either directly at presynaptic

sites or indirectly at postsynaptic sites leading to the raising of a retrograde signal. The NR2B subunit in both its activated and antagonised state was associated with enhancements in paired-pulse interactions which suggest that it is not able to modulate directly the presynaptic element. However, whilst paired-pulse interactions are generally accepted to be presynaptic phenomena, it does not follow that postsynaptic effects cannot influence the appearance of changes in these interactions in field recordings. The absence of any observable difference between HQA, QA and NMDA results suggests that the NR2D subunit is not obviously involved in these processes.

Dedication

To my grandparents,

Peter and Agnes

Anthony and Agnes

Acknowledgements

I would like to extend my gratitude to Professor Trevor Stone and to Professor Hugh Nimmo for the opportunity to pursue research in their laboratories and for their help and expertise in realising my aims.

Special thanks go to the efforts made by the Bower Fire committee in their handling of this eponymous incident and the efforts made to prevent excessive ill effects to the course of my research.

I would also like to thank the many members of staff who provided me with help and advice.

I gratefully acknowledge Dr. Y. Auberson, Novartis Pharmaceuticals for the gift of PEAQX and Dr. Georg Jaeschke, F. Hoffman-LaRoche Pharmaceuticals for the gift of Ro 25-6981.

I would especially like to acknowledge the congenial company and assistance provided by the many members of the various laboratories I have researched in and frequented.

My fondest regards for my friends especially Ali, Annamieke, Chand, Chris, John, Kara, Leanne, Melissa, Sevil, Torfi and my girlfriend Julia.

In particular, I am indebted to my family, both immediate and extended, for their dependable love and support, especially Ben.

Contents

List of figures

14

List of tables

Abbreviations

θ_m	modification threshold
ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
2-AP5	D-2-amino-5-phosphono-pentanoic acid
BCM	Bienenstock-Cooper-Munro
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CCMQ	2-carboxy-3-carboxymethylquinoline
CNS	central nervous system
CREB	cAMP response element binding protein
CyA	cyclosporin A
DMSO	dimethyl sulfoxide
DPCPX	1,3-dipropyl-8-cyclopentyl-xanthine
EC_{50}	50% excitatory concentration
fEPSP	field excitatory postsynaptic potential
GABA	γ-aminobutyric acid

GlnBP	glutamine binding protein
HEK	human embryonic kidney
HFS	high frequency stimulation
HQA	homoquinolinic acid
IC_{50}	50% inhibitory concentration
IPSP	inhibitory postsynaptic potential
KA	kainate
KYA	kynurenic acid
LFS	low frequency stimulation
LIVBP	leucine/isoleucine/valine binding protein
LTD	long-term depression
LTP	long-term potentiation
mRNA	messenger ribonucleic acid
nNOS	neuronal NO synthase
NO	nitric oxide
n.s.	not significant
NMDA	N-methyl-D-aspartic acid

NRxy	NMDA receptor, x=subunit family, y=splice variant or family member
OA	okadaic acid
PEAQX	(R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077)
PKA	protein kinase A
PKC	protein kinase C
PP	protein phosphatase
PPD	paired-pulse depression
PPF	paired-pulse facilitation
PSD	postsynaptic density
QA	quinolinic acid
Ro 25-6981	(R*,S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanolol.
SAP	synapse-associated protein
s.e.m.	standard error of the mean
STA	staurosporine

Declaration

I declare that I myself carried out all the work in this thesis except where referenced and that it has not been submitted for any previous higher degree.

Publications

Papers

Mallon A.P., Auberson, Y.P., Stone T.W. (2005) Selective subunit antagonists suggest an inhibitory relationship between NR2B and NR2A-subunit-containing N-methyl-D-aspartate receptors in hippocampal slices. Experimental Brain Research, 162, 374-383.

Mallon A.P., MacGregor D.G., O'Kane M., Ross F., Nimmo H.G. & Stone T.W. (2004) The purification of an unknown protein from a crude source which induces long-term Depression (LTD) in hippocampal CA1 neurones. (*Publication Pending*)

Mallon A.P. & Stone T.W. (2004) Actions and NR2A subunit selectivity of homoquinolinic acid in the rat hippocampus. (*Publication Pending*)

Mallon A.P. & Stone T.W. (2004) NR2A and NR2B subunit-containing NMDA receptor pharmacology: excitotoxicity, hypoxia and epilepsy. (*In preparation*)

Mallon A.P., Morris B. & Stone T.W. (2004) Changes in phosphorylation levels of NMDA subunits following the disinhibition of the NR2B subunits. (*In preparation*)

Abstracts

A.P. Mallon, A.K. Shahraki, T.W. Stone. (2003) Effects of homoquinolinic acid in the rat hippocampal slice. Program No. 153.19. *2003 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience, 2003. Online.

A.P. Mallon, Stone T.W. The negative coupling of NR2B to NR2A subunits. (2004) Program No. 957.11. *2004 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience, 2004. Online.

A.P. Mallon, Stone T.W. NMDA subunit pharmacology: excitotoxicity, hypoxia and epilepsy. (2004) Program No. 957.11. *2004 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience, 2005. *(Publication Pending)*

Chapter 1. Introduction

NMDA receptor: an historical perspective

The excitatory amino acid transmitter field has its historical origins in the finding of the convulsive effects of L-glutamate and L-aspartate upon mammalian brain tissue (Hayashi, 1952; 1954) and their depolarising and excitatory effects on central neurones (Curtis, 1959; 1960). However the concept that such an amino acid, found in relatively high concentrations in the central nervous system (CNS), may be responsible for the majority of neurotransmission only became accepted with the accumulation of later evidence from electrophysiological, pharmacological and molecular biological studies. L-glutamate is now considered the principal excitatory neurotransmitter in the mammalian CNS acting upon a variety of receptor types. The existence of glutamate receptor subtypes was suggested by the observations that glutamate agonist analogues showed different potencies on different subsets of neurones (Curtis & Johnston, 1974; McLennan, 1983). Furthermore, the development of several antagonists in the early 1970's that had differential effects upon agonist responses resulted in an initial classification of NMDA, quisqualate and kainate receptors (Evans *et al.*, 1978; Davies & Watkins, 1979; McLennan & Lodge, 1979; Ault *et al.*, 1980). Since quisqualate was later shown to activate metabotropic glutamate receptors (Sladeczek *et al.*, 1985; Nicoletti *et al.*, 1986) and a

new agonist, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Krogsgaard Larsen *et al.*, 1980), was demonstrated to be selective for the quisqualate-activated non-NMDA receptors the ionotropic receptor family was reclassified as NMDA, AMPA and kainate (Collingridge & Lester, 1989). This has since been confirmed with the cloning and functional expression of various NMDA, AMPA and kainate receptor subunits (Hollmann & Heinemann, 1994).

NMDA receptor physiology

The NMDA receptor is a heteromeric ligand-gated ion channel that interacts with multiple intracellular proteins by way of different subunits (McBain & Mayer, 1994; Husi *et al.*, 2000). NMDA receptors are involved in a variety of neural processes, including long-term potentiation (LTP) (Collingridge & Singer, 1990; Bliss & Collingridge, 1993), long-term depression (LTD) (Dudek & Bear, 1992; Mulkey & Malenka, 1992), learning, memory (Olney, 1990; Nakanishi *et al.*, 1992), brain development, excitotoxicity (Choi & Rothman, 1990), neuropathologies such as epilepsy, psychosis and neurodegenerative diseases (Choi, 1988; Greenamyre & Young, 1989; Meldrum & Garthwaite, 1990; Doble, 1995). The NMDA receptor is characterised by a number of unique and important properties that distinguishes it from other ligand gated ion-channels. Firstly, extracellular Mg^{2+} at resting membrane potentials blocks the

NMDA receptor channel and it is only opened with simultaneous depolarisation and agonist binding (Mayer et al., 1984). Secondly, NMDA receptor channels are highly permeable to both monovalent cations and Ca^{2+} ions. NMDA receptor activation results in an influx of Ca^{2+} as well as the influx of Na^+ and efflux of K^+ that is characteristic of all excitatory amino acid (EAA) ionotropic receptors (MacDermott et al., 1986; Mayer & Westbrook, 1987; Schneggenburger et al., 1993). This characteristic Ca^{2+} entry is the key trigger for many important physiological phenomenon including LTP (Lynch et al., 1983; Bliss & Collingridge, 1993) and LTD (Dudek & Bear, 1992; Selig et al., 1995) where the relative magnitude of the rise in intracellular Ca^{2+} concentration and its temporal and spatial character determines which plasticity is induced (Lisman, 1989; Artola & Singer, 1993; Neveu & Zucker, 1996). Furthermore, prolonged activation of NMDA receptors results in excessive Ca^{2+} influx producing neuronal cell death in hypoxia, ischaemia and neurodegenerative disorders (Choi, 1988). Thirdly, the coincident binding of glutamate and the co-agonist glycine is necessary for receptor activation (e.g. two molecules of glycine and two molecules of glutamate in a tetrameric, heterodimeric, NR1/NR1/NR2/NR2 receptor) (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988). Glycine is present in cerebrospinal fluid at a concentration of about 10µM (Curtis & Johnston, 1974) and could be expected to attain a similar concentration in the extracellular fluid. At these concentrations the NMDA receptor could be fully saturated with

glycine, however it is conceivable that glycine is also released from glia or from the presynaptic terminal as a co-transmitter (Davanger *et al.*, 1994). Furthermore, the widespread expression of mRNA for glycine transporter-1 throughout the brain, with a distribution colocalized to that of the NR1 subunit suggests that the concentration of glycine is closely regulated (Smith *et al.*, 1992). It has been reported by some groups that the application of glycine potentiates NMDA receptor-mediated synaptic transmission (Danysz *et al.*, 1989; Wilcox *et al.*, 1996; Lim *et al.*, 2004) but not by others (Fletcher & Lodge, 1988; Obrenovitch *et al.*, 1997).

NMDA receptor molecular biology

Molecular studies have reported that native rat NMDA receptors may be either tetrameric or pentameric membrane proteins comprising the NMDA receptor 1 (NR1) subunit family, of which there are eight splice variants (NR1a-h based upon the presence or absence of 3 exons) encoded by a single gene, and the NR2 subunit family, of which there are four members (NR2A-D) (Laube *et al.*, 1998), each encoded by four distinct genes (Seeburg *et al.*, 1994; Dingledine *et al.*, 1999). A third family of NMDA receptor subunits, NR3, has also been described (Ciabarra *et al.*, 1995; Sucher *et al.*, 1995); of which there are two types NR3A and NR3B that less commonly contribute to NMDA receptors (Nishi *et al.*, 2001; Chatterton *et al.*, 2002; Eriksson *et al.*, 2002). The NR3A subunit has been

shown to function during early development and to modulate negatively ion channel activity (Das *et al.*, 1998). It has been reported that mutations in the NR2 subunit alters the NMDA receptor's response to glutamate whilst mutations in the NR1 subunit do not (Laube *et al.*, 1997; Anson *et al.*, 1998; Anson *et al.*, 2000). This is consistent with the pronounced glutamatergic heterogeneity of the native NMDA receptor being dependant upon the NR2A-D subunit complement and the slight heterogeneity seen with NR1 splice variants (Buller & Monaghan, 1997). In particular, the presence of different NR2A-D subunits within a heteromeric receptor confers different degrees of affinity for glutamate and dictates the functional properties of the receptor (table2). Thus, the NR2 subunit contains the glutamate binding site (Laube et al., 1997) whilst the co-agonist glycine's binding site is located on the NR1 and the NR3 subunit (Kuryatov et al., 1994). Native heteromeric receptor complexes are thought to be combinations of one or two NR1 and/or NR3 subunits forming the glycine-binding site and two or three NR2 subunits providing the glutamate-binding site. The exact subunit composition influencing the NMDA receptors' pharmacology and function (Kohr & Seeburg, 1996; Krupp *et al.*, 1996; Monaghan & Larsen, 1997; Vicini *et al.*, 1998; Pizzi *et al.*, 1999). The subunit composition of the NMDA receptor is dynamic, changing during synaptic development (Kew *et al.*, 1998a; Tovar & Westbrook, 1999), synaptic plasticity (Kiyama *et al.*, 1998; Manabe *et al.*, 2000) and other physiological and pathophysiological processes. The

activity-dependent trafficking of NMDA receptors is achieved both by receptors recycling through exocytosis-endocytosis from intracellular sites and lateral diffusion of receptors between synaptic and extrasynaptic sites (Tovar & Westbrook, 1999; Grosshans *et al.*, 2002; Groc *et al.*, 2004; Lavezzari *et al.*, 2004).

NMDA receptor distribution

Glutamate receptors are predominantly expressed in the CNS, but have also been reported in pancreatic islet cells, osteoclasts, osteoblasts, nerve terminals in the skin, mast cells, taste buds, cardiac ganglia and even in plant cells. The first evidence for differential NMDA receptor distribution came from electrophysiological studies into the endogenous agonist quinolinic acid (QA), which demonstrated variable potency in different brain regions (Stone & Burton, 1988). In the brain NMDA receptors are now known to be heterogeneously distributed according to their subunit composition, in particular to that of the NR2 family (Monaghan & Buller, 1994) (see table 1). The NR1 subunit is expressed throughout the brain with little differential distribution of splice variants (Nakanishi *et al.*, 1992; Laurie & Seeburg, 1994) The NR2A-D subunits are expressed in a very distinct pattern (Watanabe *et al.*, 1993; Buller *et al.*, 1994; Laurie & Seeburg, 1994). In the adult brain the NR1 and NR2A subunits are ubiquitous, the NR2B subunit is expressed mainly in the forebrain, NR2C

is found particularly in the cerebellum and various select nuclei and NR2D expression is limited to the diencephalon and the mid-brain (McBain & Mayer, 1994). Hippocampal CA1 pyramidal cells express mRNA for NR1, NR2A, NR2B and NR2D in the adult human (Scherzer et al., 1997) and the juvenile rat (Kirson et al., 1999).

NMDA receptors have been amply demonstrated on the postsynaptic membrane (Petralia *et al.*, 1994; Racca *et al.*, 2000), and there is accumulating evidence suggesting a presynaptic locus. AMPA, mGlu and kainate receptors have all been established as presynaptic autoreceptors in the hippocampus (Vazquez *et al.*, 1994; Chittajallu *et al.*, 1996; Alici *et al.*, 1997), modulating excitatory transmission by altering glutamate release probability. Immunohistochemical studies have suggested the existence of presynaptic NMDA autoreceptors (Liu *et al.*, 1994; Paquet & Smith, 2000). Also, functional studies have reported evidence for presynaptic NMDA receptors in the cerebellum (Glitsch & Marty, 1999), visual cortex (Sjostrom *et al.*, 2003), spinal cord (Liu et al., 1997), hippocampus (Breukel *et al.*, 1998), suprachiasmatic nucleus (Hamada et al., 1998) and entorhinal cortex (Berretta & Jones, 1996). Presynaptic NMDA receptors have been reported to be NR2B subunit-containing and tonically activated, causing an increase in glutamate release probability, dependent upon Ca^{2+} influx, in the entorhinal cortex (Woodhall et al., 2001).

NMDA receptor development

Developmentally only NR2B and NR2D subunits are present and widespread in the prenatal brain but shortly after birth, NR2A and NR2C subunits quickly predominate while the NR2B and NR2D subunits decline to adult levels and a limited distribution. NR2B subunits are expressed from late embryonic stages up to adulthood, while expression of NR2A subunits only develops during early postnatal stages and rises to mature levels by post-natal day 21 (Williams *et al.*, 1993; Mori & Mishina, 1995; Wenzel *et al.*, 1995; Portera Cailliau *et al.*, 1996). This transformation is particularly significant for the age-dependence of synaptic plasticity (Kemp et al., 2000) and glutamate toxicity (Liu et al., 1996). NR1 subunits are present and widespread both pre- and post-natally (Lynch & Guttmann, 2001)(see tables 1, 2 and 3).

Subunit	Localisation in adult animals	Developmental course
NR1	Ubiquitous with splice variant distribution limited to specific cells	Prenatal and postnatal
NR2A	Widespread throughout the forebrain. Less prominent in the basal ganglia	Develops post-natally
NR2B	Largely limited to forebrain	Widespread with later decline to adult levels and distribution
NR2C	Largely limited to cerebellum	Appears postnatally
NR2D	Isolated cells of thalamus, sub thalamus and nNOS containing cells of cortex	Widespread prenatally

Table 1. Summary of the distribution and developmental course of NMDA receptor subunits.

NMDA receptor property	Subunit heterogeneity
Glutamate and glycine affinity	2D > 2B > 2C
Channel open probability	2A (2-5 fold higher) > 2B
Peak current density	2A (4 times larger) > 2B
Channel conductance	2A, 2B >> 2C, 2D
Receptor kinetics	2A > 2B = 2C > 2D
Current decay time	2A > 2B > 2C > 2D
Ca^{2+} influx	2B > 2A
Sensitivity to Mg^{2+} block	2A, 2B >> 2C, 2D
Deactivation time	2D >> 2B, 2C > 2A
Desensitisation sensitivity	2A >> 2D >> 2B, 2C
Speed of recovery from desensitisation	2A > 2A/2B > 2B
Proton sensitivity	2A > 2B > 2D > 2C

Table 2. Summary of the functional heterogeneity of NMDA receptors depending on the presence of specific NR2 subunits.

NMDA	Widely distributed in mammalian CNS (especially enriched in hippocampus and cerebral cortex).
AMPA	Widespread in CNS; similar distribution to NMDA receptors.
Kainate	Concentrated in a few specific areas of CNS, corresponding to NMDA and AMPA receptor distribution.
Metabotropic	A class of receptors positively linked to inositol triphosphate or negatively to cyclic AMP formation.

Table 3. Summary of the general distribution of excitatory amino acid (EEA) receptors in the mammalian CNS.

NMDA receptor pharmacology

The complicated physiology of the NMDA receptor complex provides agonists and antagonists with several distinct binding sites: glutamate, strychnine-insensitive glycine, ion-channel, polyamine and other modulatory sites (Figure 1).

Glutamate, NMDA, aspartate, quinolinic acid, homoquinolinic acid and other agonists activate the glutamate site; the prototypic antagonist of this site is 2-AP5. The glycine site is activated by glycine (Johnson & Ascher, 1987) and several antagonists exist including ACBC, CGP 78608 and 7-chlorokynurenic acid (Fletcher & Lodge, 1988; Kemp et al., 1988). The NMDA receptor ion channel is blocked by Mg^{2+} ions at resting membrane potentials. Other modulators have also been discovered that block the ion channel in a non-competitive manner; notably, dizocilpine (MK 801), PCP and ketamine. Zinc, like other group IIB metal ions produces inhibition of the NMDA receptor and is selective at nanomolar concentrations to NR2A subunit-containing NMDA receptors (Paoletti et al., 1997). Polyamines such as spermine and spermidine can inhibit or potentiate the NMDA receptor, at high and low concentrations respectively. Polyamines promote channel opening at low micromolar concentrations by glycine-dependent and glycine-independent mechanisms (Rock & Macdonald, 1992; Williams, 1997). At higher concentrations, they block the channel in a

voltage-dependent manner. Antagonists at this site, inhibiting the NMDA receptor, include ifenprodil and haloperidol (Reynolds & Miller, 1989; Ilyin *et al.*, 1996; Lynch & Gallagher, 1996). Modulation also occurs at the Ro 25-6981 site, the redox site and with ethanol and the volatile anaesthetics.

NR1

NR2

Polyamine
Mg^{2+}

Modulatory
LIVBP-like
Domain

Agonist
binding
GlnBP-like
Domain

?

Zn^{2+} (NR2A)
Ifenprodil (NR2B)

S-S

Glycine

Redox

Glutamate

Extracellular

MK801
Ketamine
PCP
Mg^{2+}

Cytoplasmic
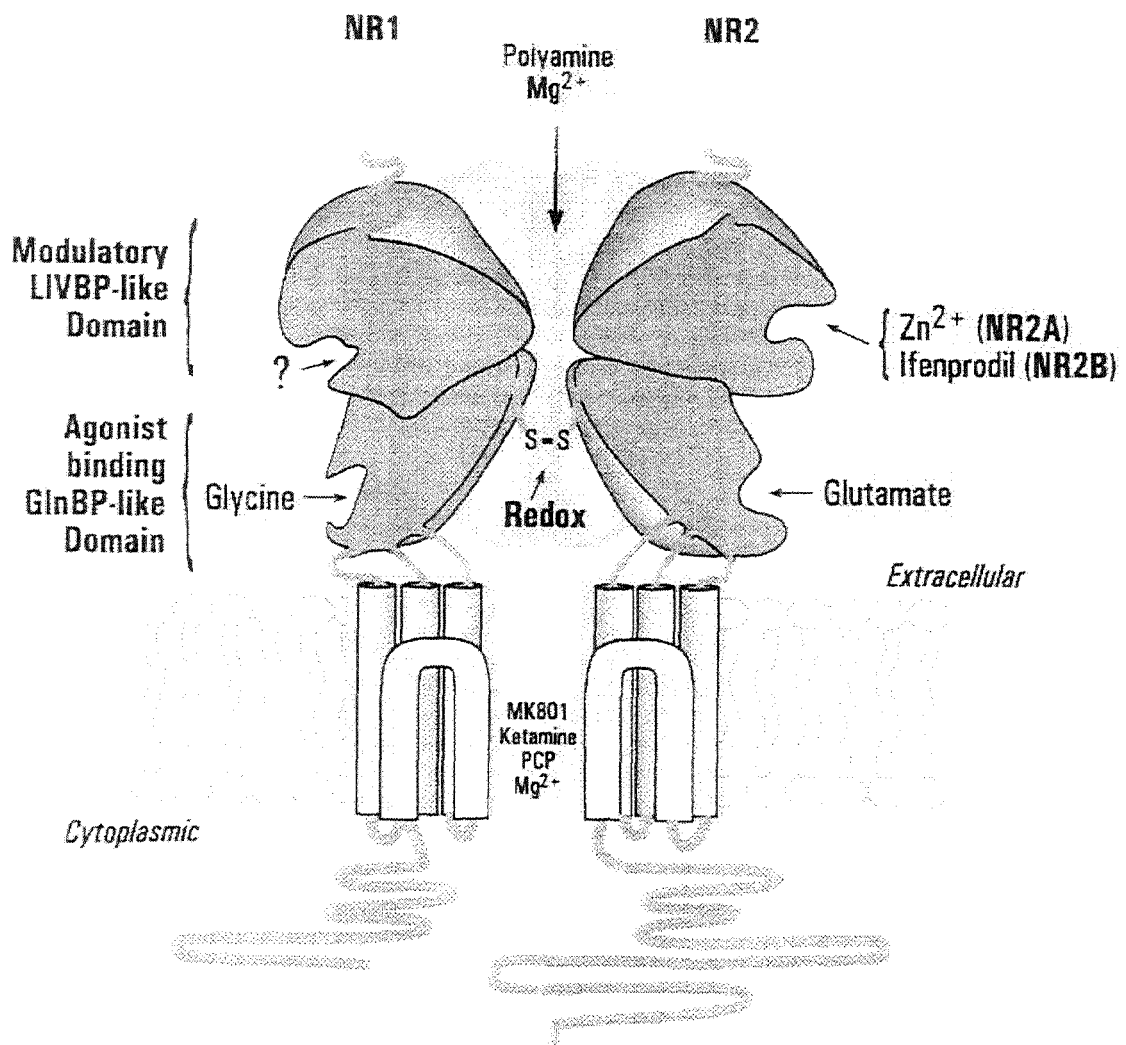
Figure 1. The NMDA receptor.

A model showing the agonist binding sites for glutamate and glycine and the competitive antagonists that can block these sites. Additionally, other modulators can block the ion-channel and modulate the receptor at discrete sites (Kemp & McKernan, 2002).

AMPA and kainate receptors

The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) receptors are the non-NMDA ionotropic receptor subtypes that activate Na^+ and K^+ conductances whilst the NMDA receptors activate Na^+, K^+ and Ca^+ conductances (Monaghan *et al.*, 1989; Barnard & Henley, 1990). AMPA receptors are hetero-oligomeric proteins consisting of the subunits GluR1 to GluR4, each receptor comprising four subunits (Hollmann & Heinemann, 1994; Rosenmund *et al.*, 1998). The AMPA and kainate receptors have at least three separate binding sites at which agonists and antagonists can act: glutamate binding, desensitisation and intra-ion channel binding sites. Kainate receptors consist of GluR5-7 and KA1-2 subunits, and have approximately 40% sequence homology to AMPA receptor GluR1-4 subunits. High affinity binding of kainate is observed to the KA1 and KA2 subunits compared to low affinity binding to GluR5-7. The same agonists and antagonists can modulate both AMPA and kainate receptors with little differentiation. However, certain desensitisation inhibitors, which act allosterically to modulate positively sites on the ion channel, can distinguish between AMPA and kainate receptors (Table 3).

Inhibitory GABA-ergic transmission

As well as excitatory transmission mediated by glutamate there is also the presence of GABA-ergic interneurones that mediate feedforward and feedback inhibition of excitatory transmission. Activation of these inhibitory interneurones by glutamate results in a dual component inhibitory postsynaptic potential (IPSP)(Alger & Nicoll, 1982; Benardo, 1994). This consists of a $GABA_A$-mediated fast component which results in the activation of $GABA_A$ ionotropic receptors releasing hyperpolarising Cl^- ions into the neurone. The other component is a slow $GABA_B$-mediated component which results in the activation of the $GABA_B$ metabotropic receptor (Alger, 1984) leading to the hyperpolarising influx of K^+ ions and the negative modulation of the influx of Ca^{2+} ions. IPSPs can inhibit excitatory synaptic transmission by moving the membrane potential to values that are more negative and increasing the amount of depolarisation required by EPSPs to reach the threshold for action potential generation. $GABA_B$ mediated slow IPSPs are possibly better poised to counter longer-lasting $EPSP_{NMDA}$ which because of their long slow kinetics have been reported to escape fast $GABA_A$ inhibition (Benardo, 1995) although one report suggested that when stimulation evokes only a small EPSP (2-5mV) the $GABA_B$ component is negligible (Otmakhova & Lisman, 2004). In addition to postsynaptic GABA receptors there is also a presynaptic $GABA_B$ autoreceptor in the Schaffer collateral feedforward

interneurones (but not in the perforant pathway). When the interneurone is repeatedly activated, GABA is released in sufficient quantity to feedback on these $GABA_B$ receptors, which inhibits the release of further GABA, and synaptic inhibition is depressed (Davies *et al.*, 1990).

Excitotoxicity

The term excitotoxicity was first coined by John Olney who observed that a number of excitatory amino acids (EAAs) killed neurones in the hypothalamus with a potency that correlated with their efficacy in depolarising cells. Excitotoxicity is a critical mechanism contributing to neurodegeneration during ischaemia and hypoxia. Both the loss of calcium homeostasis and the activation of EAA receptors are postulated to be responsible for excitotoxic neuronal damage (Olney, 1969; 1986; Seisjo, 1988; Choi, 1991; Sattler et al., 1999). With the activation of NMDA, receptors both of these hypotheses are fulfilled. The over-activation of NMDA receptors leads to an influx of Ca^{2+} resulting in necrosis or apoptosis by several proposed mechanisms. The subsequent activation of Ca^{2+}-sensitive enzymes (nNOS, endonucleases and proteases), the production of highly reactive free radical species, mitochondrial impairment and the activation of immediate-early response sense genes all contributing to neuronal death. Blockade of this influx, by NMDA antagonists and calcium antagonists, is neuroprotective in animal models of stroke and seizure (Lee et al., 1999). The clinical use of non-selective NMDA receptors as neuroprotective drugs has been hindered by their narrow therapeutic window, which produces adverse drug reactions including sedation, muscular weakness, ataxia, confusion and tinnitus. Subunit selective antagonists such as ifenprodil have been associated with a

decrease in side effects allowing an effective therapeutic dose to be titrated (Gotti et al., 1988). Nevertheless, therapeutic NMDA receptor antagonists have yet to be developed with clinical success (De Keyser *et al.*, 1999). Several studies have specifically implicated both the NR2A- and NR2B-containing NMDA receptors in excitotoxicity. Excitotoxicity in cultured embryonic forebrain neurones coincides with a dramatic increase in NR2B mRNA levels during the first 10 days, thereafter remaining stable (Cheng et al., 1999). However, decreased expression of NR2A and NR2B mRNA has also been reported in CA1 and other hippocampal areas, in response to a severe ischaemic insult, altering the function of the NMDA receptors (Zhang et al., 1997). In addition to changes in subunit expression, ischaemia has also been shown to increase the tyrosine phosphorylation of both the NR2A and NR2B subunits thereby altering their function (Kim *et al.*, 1998; Cheung *et al.*, 2000).

Anatomy of the hippocampus

The electrophysiological experiments described in this study were carried out in the hippocampal slice preparation of the 7-8 week old Wistar rat. The hippocampus along with the amygdala, adjacent regions of the limbic cortex and the septal area constitute the main structures of the limbic system. These structures form major interconnections with portions of the thalamus and the cerebral cortex (cingulated gyrus).

For three reasons the hippocampus is one of the most studied areas of central nervous system. Firstly, it has an easily identifiable structure at the gross and histological levels. Secondly, it has been recognized that the hippocampus plays a fundamental role in learning and memory (patients who have had bilateral hippocampalectomy have suffered from a permanent loss of storing new information into their long-term memory). Thirdly, the hippocampus is especially susceptible to seizure disorders and is particularly vulnerable to the effects of ischaemia and hypoxia (Johnston & Amaral, 1998).

In the brains of mammals, the hippocampus is a bilateral structure that appears as a ridge extending into the lateral ventricle. The outer surface of the hippocampus is composed of myelinated fibres, appears white and is called the alveus. The hippocampal formation can be divided into Ammon's horn, the subiculum, the dentate gyrus and the entorhinal cortex.

Based on the Golgi method of staining, the anatomist Lorente de Nó (1934) (see Johnstone & Amaral, 1998) divided Ammon's horn into four cornu Ammonis subfields: CA1 to CA4. The designation CA4 is no longer used because it referred to the region occupied by the polymorphic layer of the dentate gyrus. CA1 is equivalent to the regio superior and CA2 and CA3 fields are equivalent to the regio inferior. A narrow transitional zone, CA2, separates CA1 and CA3 (Figure 2).

The dentate gyrus contains round, tightly packed neurons called granule cells and consists of three layers: the granule layer, which is the principal layer, the molecular layer above the granule cell layer and a polymorphic layer below the granule cell layer (Isaccson, 1987).

In all CA fields below the alveus is the stratum oriens, which contains the basal dendrites of the pyramidal cells. The cell bodies are clearly visible under a microscope as a dark band. Below this is the stratum radiatum consisting of the apical dendrites of the pyramidal cells and the Schaffer collaterals, which are collateral branches from axons of pyramidal cells in the CA3 region (Cajal, 1968).

Neuronal circuits in the hippocampus

The excitatory neuronal circuitry of the hippocampal formation is illustrated in figure 2. The functional organisation of the hippocampus has been described in terms of a 'trisynaptic' circuit. Information that flows from the neocortex into and out of the hippocampus travels in a unidirectional manner through this trisynaptic pathway. The entorhinal cortex is considered the starting point of the circuit. Neurones located in layer II of the entorhinal cortex give rise to the perforant pathway that projects through the subiculum and terminates both in the dentate gyrus and in the CA3 region. Neurones forming the medial entorhinal cortex produce axons that terminate in the middle layer of the molecular layer of the dentate gyrus; the outer third of the molecular layer receive axons from the lateral entorhinal cortex. These two components of the perforant pathway also terminate in the stratum lacunosum-molculare of the CA2 and CA3 regions. Neurones located in layer III of the entorhinal cortex project to the CA1 and the subiculum.

The granule cells also synapse onto neurones of the polymorphic layer, which supplies associational connections to other levels of the dentate gyrus. The CA3 pyramidal cells project heavily within the CA3 and as the Schaffer collateral projection to the CA1 stratum radiatum and stratum oriens. CA1 neurones project to both the subiculum and the deep layers of

the entorhinal cortex, which in turn produce projections back into many of the cortical areas that originally terminated in the entorhinal cortex. Therefore information sent from cortical neurones to the entorhinal cortex can then negotiate the complete hippocampal circuit and be returned back to its cortical origin (Witter, 1989).

As well as the main excitatory circuitry, there are also inhibitory pathways throughout the hippocampus. Three types of interneurones control this circuit in the pyramidal cell layers: axo-axonic cells, basket cells and bistratified cells. The majority of interneurones in the hippocampus use the inhibitory transmitter γ-aminobutyric acid (GABA). The axo-axonic cells synapse upon the initial segment of pyramidal neurones and have a strong influence over the generation of action potentials. Basket cells synapse onto the somata and bistratified cell synapse at apical and basal dendrites of the pyramidal neurones. The dendrites of these three classes of interneurone project into the stratum radiatum and stratum oriens where they can encounter excitatory inputs from the Schaffer collaterals, commissural-association fibres and feedback synapses from local pyramidal neurones (Isaccson, 1987; Johnston, 1998).
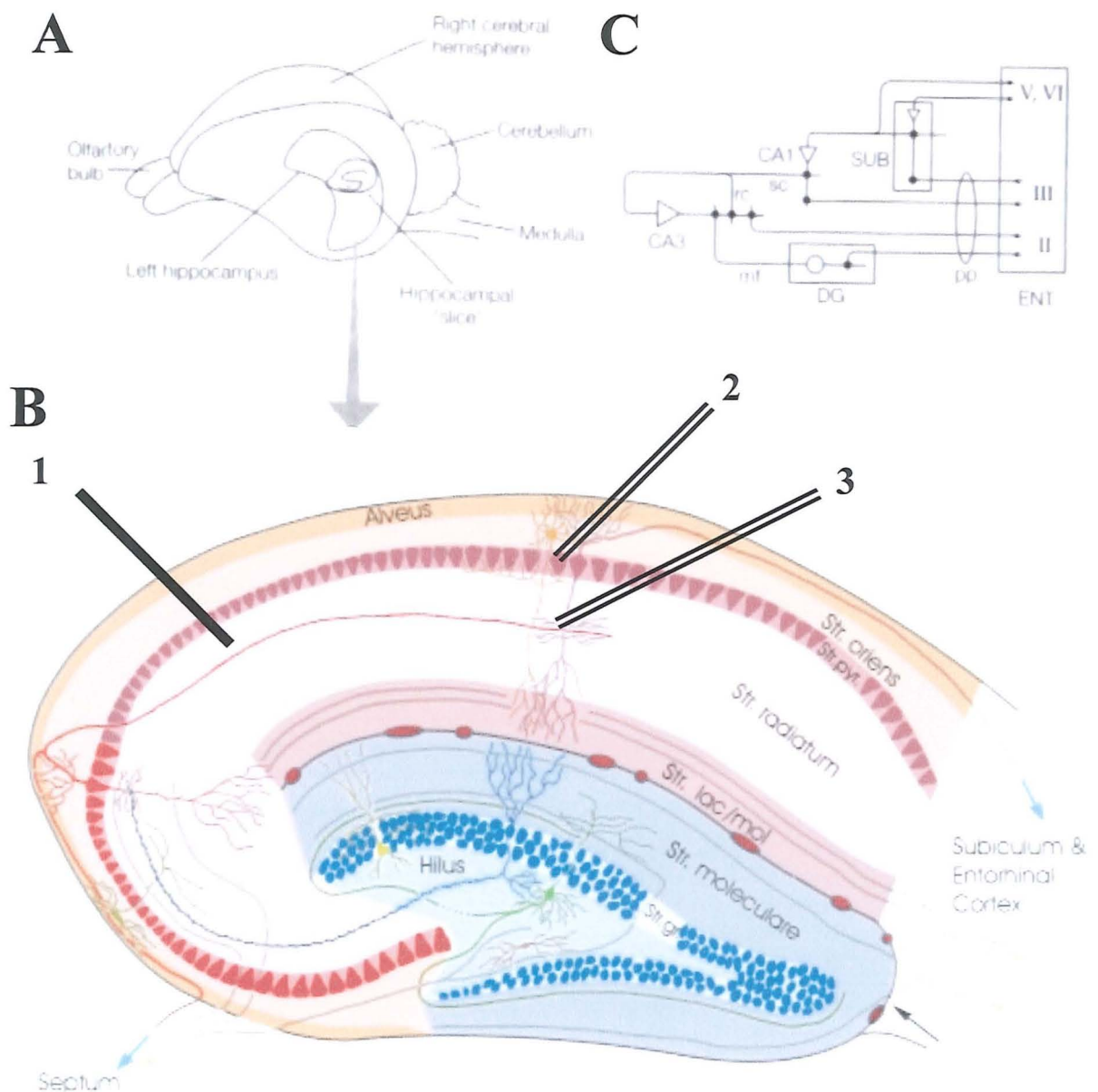
Figure 2. Diagram showing the anatomy of the rat hippocampal formation and the placement of the stimulating and recording electrodes.

A, location of the left hippocampus in rat brain. B, structure of a hippocampal slice showing the principal excitatory neurons. The stimulating electrode (1) was positioned in the stratum radiatum at the CA1/CA2 junction. Orthodromic population spikes and excitatory postsynaptic potentials were recorded from the stratum pyramidale and stratum radiatum respectively using electrodes (2) and (3) placed in the CA1 region. C, hippocampal circuitry. DG, dentate gyrus; ENT, entorhinal cortex; mf, mossy fibre; pp, perforant pathway; rc, recurrent collateral; sc, Schaffer collateral; SUB, subiculum. (Revest & Longstaff, 1998).

The hippocampal slice preparation

Ever since cortical slices were demonstrated to have comparable electrical activity with the intact animal (Yamamoto & McIlwain, 1966) the slice preparation has been used extensively in the study of synaptic transmission and electrophysiological experiments in the mammalian CNS. The studies leading to our present understanding of how NMDA, AMPA, and metabotropic glutamate receptors contribute to synaptic transmission; and investigations of LTD and LTP have come almost exclusively from brain slice studies. Hippocampal slices have also shown good conditions to allow intracellular recording (Yamamoto, 1972; Kerkut, 1981). The hippocampus is an essentially laminar structure, each transverse slice having the same 'trisynaptic' circuitry. The neuronal function and anatomy of the slice preparation, in the plane in which it is cut, is calculated to be similar to the intact brain producing representative electrophysiological and pharmacological responses. The slices should be well enough prepared to allow recording at physiological temperatures (36-37^{0}C for rodent slices) however, this is not usually achievable and a sub-physiological temperature of 28-30°C is normally used.

Advantages of the in vitro slice preparation over in vivo techniques include:

1. Technical simplicity: it is relatively easy to record via extracellular, intracellular and whole-cell patch-clamp compared with in vivo experiments.

2. Control over the conditions of the preparation. If one can reduce the number of contributory variables, greater significance can be drawn upon results from a constant environment (e.g. temperature, movement, O_2/CO_2 saturation, absence of the influence of an anaesthetic maintenance dose).

3. Improved visualization of tissue. The tissue is easily identified under low magnification facilitating the positioning of stimulating and recording electrodes.

4. Control of the extracellular environment and the field potential to study neurophysiology and pharmacology. This is helpful when applying drugs and doses that are impractical in whole animal studies.

5. Humane treatment of animals, because slice preparation does not require restraint or the maintenance of physiological functions it is a relatively more humane method.

6. The ability to use technically demanding methods that are difficult or impossible in intact preparations. For example, the ability to isolate specific pathways and record from specific postsynaptic cells without contamination of other synaptic inputs from other brain regions which have been dissected (Grover & Teyler, 1990), or the ability to use optical techniques.

These advantages of the slice preparation have led to increasing numbers of neuroscientists to use this technique. However, there are also some disadvantages with brain slice preparations:

1. The dissection and slicing process severs the hippocampal slices' normal sensory input and motor output. Long feedback loops, some excitatory pathways and some inhibitory pathways are also lost. One obvious symptom of this is the markedly lower levels of spontaneous activity found in the slice preparation compared to the intact animal. This brings into question the similarity and relevance of the in vitro slice preparation compared to the in vivo environment. Furthermore, it has been demonstrated that phenomena such as LTD induction are more readily induced in vitro than in vivo (Bear & Abraham, 1996)

2.   The crude method of slicing causes damage along the faces of the slice, this results in cell damage and the release of substances that may affect the slice.

3.   The tissue is subject to an anoxic period during preparation (Lipton & Whittingham, 1979).

4.   The ionic environment does not mimic exactly the normal extracellular conditions in vivo. This is of particular relevance in the induction and maintenance of LTD and LTP that are dependent upon relative Ca^{2+} and Mg^{2+} ion concentrations. The continuous perfusion of artificial cerebrospinal fluid (ACSF) can also wash out any modulatory compounds endogenous to the slice (such as growth factors released following stimulation).

5.   The addition of drugs to the artificial cerebrospinal fluid (ACSF) results in the exposure of the whole slice to pharmacological modulation. This confounds the significance of results seen with the subset of cells that are being recorded.

6.   Finally, the diversity of methods used in each brain slice laboratory makes the comparison of findings difficult (Lipton et al., 1995). The most obvious differences include: (A) the maintenance of brain slices at the interface of the ACSF and oxygenated atmosphere

versus those maintained entirely submerged in ACSF, (B) cutting brain slices on a manual tissue chopper versus a vibratome or a vibraslice, (C) differing ACSF recipes and (D) temperature differences.

Extracellular responses

Single electrical stimulations of the Schaffer collaterals result in a distinctive sequence of excitation followed by inhibition in the target CA1 pyramidal neurones. Field extracellular recordings represent the summed responses from a population of neurones close to the recording electrode. The field excitatory postsynaptic potential (fEPSP) recorded in the stratum radiatum consists of an initial fibre volley of action potentials in the presynaptic fibres producing a negative-going transient; this is followed by a slower negative-going potential with a time-period similar to that of the underlying synaptic currents. The current flowing into these dendrites during the fEPSP will exit the neurons near the cell body layer in the stratum pyramidale where a recording electrode will record a concurrent positive going potential. If the strength of the synaptic input is adequate to evoke action potentials in the neurons, then a negative-going potential (population spike) can also be recorded in the stratum pyramidale resulting from the inward current during the postsynaptic action potentials.

Paired-pulse interactions

The magnitude of synaptic transmission in the mammalian brain can be modulated in different ways. Two examples of such modulation are paired-pulse facilitation (PPF) and paired-pulse depression (PPD).

Paired-pulse facilitation

Paired-pulse facilitation (PPF) is a form of short-term plasticity in which the synaptic response to the test pulse (second stimulus) given after the conditioning pulse (first stimulus) is enhanced in comparison to the conditioning pulse when interstimulus intervals are relatively long (≥ 20ms). The most widely accepted hypothesis to explain the mechanism of PPF is based on the accumulation of presynaptic Ca^{2+} after the first stimulation. The first pulse induces a Ca^{2+} influx that lingers in the presynaptic terminal for several hundred milliseconds. This residual Ca^{2+} combined with fresh Ca^{2+} entering during the second pulse leads to the increased transmitter release (Katz & Miledi, 1968; Thomson, 2000; Zucker & Regehr, 2002). The Ca^{2+} concentration in the presynaptic nerve terminal affects directly the release probability of neurotransmitter. Furthermore, reports at the neuromuscular junction have suggested that the second of the two action potentials induces release with higher probability than the first one because of a small but persistent increase of the intracellular Ca^{2+} concentration in the axon terminal. This residual Ca^{2+}

52

can enhance the release probability by increasing the fusion of synaptic vesicles with the presynaptic membrane and enhancing the number of vesicles released by the action potential (Katz & Miledi, 1968; Mennerick & Zorumski, 1995; Debanne *et al.*, 1996). Neuromodulators and physiological processes that alter transmitter release probability also affect the paired-pulse ratio. Increasing the external Mg^{2+}/Ca^{2+} ratio (Davies & Collingridge, 1993; Lambert & Wilson, 1994; Wilcox & Dichter, 1994) and applying adenosine (Lupica *et al.*, 1992; Higgins & Stone, 1995) decreases the probability of release of neurotransmitter during the first stimulus and increases the probability of release by the second stimulus producing enhanced PPF. In the hippocampus, PPF of excitatory synaptic potentials in the CA1 and CA3 is observed when large numbers of axon are concurrently stimulated (Creager *et al.*, 1980; Manabe *et al.*, 1993) whereas, during conditions in which the release probability is enhanced by decreasing the Mg^{2+}/Ca^{2+} ratio then PPF is reduced (Nathan *et al.*, 1990; Kahle & Cotman, 1993).

Paired-pulse depression

When two evoked potentials are elicited when interstimulus intervals are small ($\leq 10ms$), the size of the second response is smaller than the first, a phenomenon called paired-pulse depression (PPD). The mechanism underlying PPD of synaptic transmission is unclear. One hypothesis is the activation of $GABA_B$ autoreceptors (Deisz & Prince, 1989), but studies

53

have shown that $GABA_B$ receptor antagonists did not attenuate PPD of unitary inhibitory post synaptic potentials (IPSPs) in pairs of cultured hippocampal neurons (Wilcox & Dichter, 1994). An alternative presynaptic mechanism for PPD is a transient decrease in the quantal content caused by depletion of the readily releasable vesicle pool by the first stimulus (Mennerick & Zorumski, 1995; Stevens & Wang, 1995; Debanne *et al.*, 1996). Postsynaptic mechanisms such as the desensitisation of $GABA_A$ receptors (Alger, 1991) and a reduced driving force due to intracellular accumulation of chlorine ions and/or extracellular accumulation of potassium ions may also explain PPD (McCarren & Alger, 1985). Since paired-pulse depression is observed most clearly and reliably when the interstimulus interval is around 15–100 ms, which corresponds to the time course of the $GABA_A$ mediated fast inhibitory potential recorded intracellularly in pyramidal cells (Davies *et al.*, 1990), it is thought to be largely caused by the activation of $GABA_A$ receptors (Wilcox & Dichter, 1994). It is difficult to test this proposition using bath application of $GABA_A$ receptor antagonists such as bicuculline which increase the excitability of the pyramidal cells by blocking the effects of spontaneously released GABA.

Whilst changes in paired-pulse interactions are widely considered to be due to presynaptic alterations in release probability; when studied on fEPSPs and especially where there are depolarisation-induced changes in the size of the postsynaptic potential in response to the test agents many factors affect them which can confound the interpretation. These include changes in presynaptic release probability (Andreasen & Hablitz, 1994), activity of presynaptic autoreceptors on inhibitory or excitatory terminals (Davies *et al.*, 1990; Stanford *et al.*, 1995), modulation of postsynaptic conductances (e.g. voltage-dependent NMDA receptor) and the presence of GABA-ergic inhibition following the fEPSP and the depression cause by NMDA agonist action (Duguid & Smart, 2004). In particular, it has been reported that there is a non-linear inverse relationship between feedforward inhibition and the NMDA dependent PPF component of the fEPSP (Papatheodoropoulos & Kostopoulos, 1998) although, this NMDA-component of PPF is manifested by an increase in the duration of the fEPSP and not the amplitude of the slope.

In hippocampal slices paired-pulse inhibition has been reported at interstimulus intervals less than 40 ms and in the present experiments at less than 20 ms while PPF is observed at longer time intervals (20 and 50 ms) (Lynch *et al.*, 1983; Higgins & Stone, 1995; Nikbakht & Stone, 2000).

Aims

1. To investigate NMDA receptor subunit pharmacology in the intact, adult hippocampal slice preparation using the newly developed NR2A and NR2B subunit-selective NMDA receptor antagonists Ro 25-6981 and PEAQX.

2. To address the contribution of NR2A and NR2B subunit-containing NMDA receptors in the induction of NMDA receptor dependent synaptic plasticity in the Schaffer collateral-CA1 pyramidal synapse.

3. A report had suggested that the NR2A/2B subunit selective NMDA receptor agonist homoquinolinic acid was binding to a novel NMDA-insensitive site in the brain. An investigation of NMDA insensitive effects on the fEPSP was undertaken to reveal any residual effects.

   Furthermore, the selectivity of homoquinolinic acid allowed the investigation of the NR2A and NR2B components of the previous study (1) and to find evidence for the involvement of the NR2D subunit. In addition, this study investigated changes of paired-pulse interactions using these compounds.

Chapter 2. Material and methods

Preparation of hippocampal slices

Male Wistar rats (130-180g, 7-8 weeks) were anaesthetised by an intraperitoneal injection of urethane solution (1.5 g/Kg); urethane has been shown not to interfere with synaptic plasticity, a problem encountered when using phenobarbitone (unpublished observations). The animals were then killed by cervical dislocation, decapitated using a guillotine, and the brain gently and rapidly removed to ice-cold and oxygenated artificial cerebrospinal fluid (ACSF). The intact brain was kept cold and moist by copious amounts of ACSF whilst it was transferred to a petri dish lined with filter paper to prevent sliding. The cerebellum was removed and the two cerebral hemispheres were separated with a scalpel blade. Each hippocampus was dissected free from the surrounding tissue using small spatulas and cut transversely using a McIlwain tissue chopper into slices 450μm thick, perpendicular to their longitudinal axis. The slices were gently separated from each other using blunt glass microelectrodes to ensure that they receive adequate amounts of oxygen and nutrients and to allow individual slices to be transferred easily. The slices were incubated at room temperature (21-23^{0}C) on a fresh filter paper lined petri dish containing a small amount of freshly gassed ACSF to cover the slices. The petri dish was kept in an incubation chamber saturated in an atmosphere of

95% O_2 and 5% CO_2 for at least 1 hour prior to individual slices being transferred to the recording chamber.

Composition of ACSF

The composition of the ACSF was (in mM): KH_2PO_4 2.2, KCl 2, $NaHCO_3$ 25, NaCl 115, $CaCl_2$ 2.5, $MgSO_4$ 1.2, and glucose 10. It was gassed with a mixture of 95% O_2 and 5% CO_2 yielding a pH ~7.4.

Bath superfusion and application of drugs

Following incubation, individual slices were transferred to a 1 ml submerged recording chamber using a wide tipped plastic Pasteur pipette. A seeker wire, the end of which was modified in such a way as to form a ⎍ shape was used to gently hold the slice in place. The slice was left to stabilise whilst being continuously superfused with ACSF by a gravity-fed silicone tube at a rate of 4 ml/min. The ACSF was kept saturated with 95% O_2 and 5% CO_2 and heated using a thermostatically controlled water bath to 28-30°C. Drugs were added to the ACSF and fed via a three-way tap through the silicon tubing to the recording chamber. Due to the length of tubing, it took 1 minute for fluid to reach the recording chamber. To prevent the build-up of organic matter the silicon tubing and recording chamber were flushed with distilled water before and after use. Additionally, the system was more aggressively cleaned using diluted

bleach once a month and the tubing replaced every six months and as required.

Stimulation

Stimuli were AC amplified square wave constant-current pluses of 300μs duration. Paired stimuli, where used, were delivered through the same electrode. The slices were stimulated using a concentric bipolar electrode (Clark Electromedical Instruments Ltd, Harvard Apparatus) positioned in the stratum radiatum near the commissural border of CA1/CA2 for orthodromic activation of pyramidal cells. The slice was briefly and occasionally stimulated at 1 Hertz (Hz) until a population of neurones was identified; once this was achieved, a stimulus frequency of 0.05Hz was used. The population of neurones was then allowed to stabilise. Population spikes were recorded at 70% of maximum amplitude whilst fEPSP slopes were recorded at 50% of their maximum amplitudes. Only field responses that produced clear fEPSP and population spikes with maximum amplitudes greater than 2mV were used in experiments. Furthermore, responses that showed greater than 5% drift over the 20-minute baseline-recording period were excluded (Figure 3).

A

B

1mV

5ms

Population
spike

Presynaptic
fibre volley

Example points of maximum
slope measurement

1mV

5ms

fEPSP

Stimulation
artefacts
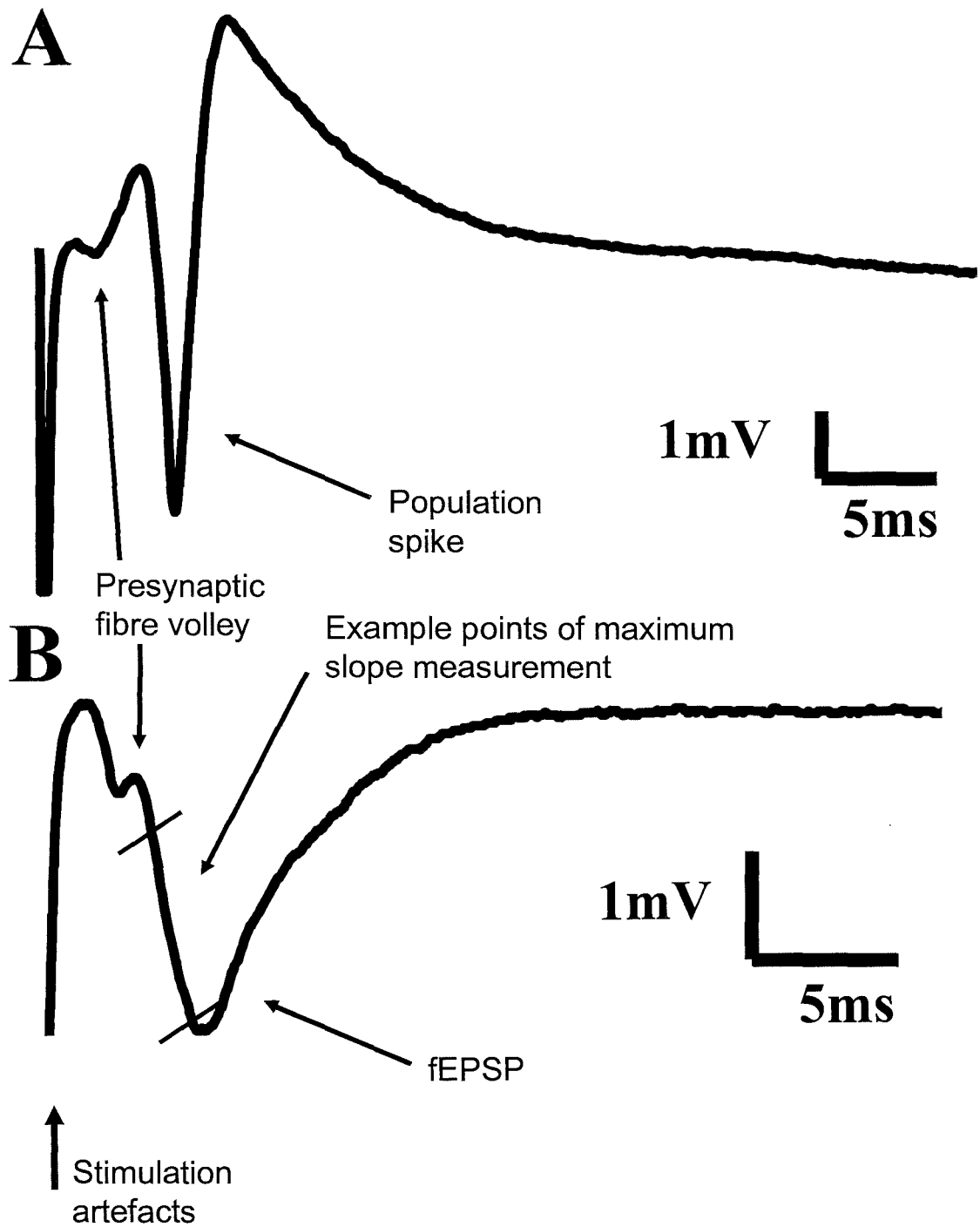
Figure 3. Example responses recorded from the CA1 region of the rat hippocampal slice.

Trace (A) shows a population spike recorded from the stratum pyramidale and trace (B) shows a fEPSP, recorded from the stratum radiatum.

Recording

Extracellular population spike potentials and field excitatory postsynaptic potentials (fEPSPs) were recorded extracellularly from the stratum pyramidale and the stratum radiatum, respectively, using borosilicate glass microelectrodes, which were produced on a Kopf vertical puller. The electrode tips were broken back to produce an opening 2-4µm in diameter under low magnification using a glass probe, resistances approximately 2–5MΩ. The electrodes were filled with sodium chloride 0.9% solution using a fine 36-gauge needle.

Evoked responses were amplified through a Neurolog system, displayed on a digital oscilloscope and recorded onto a personal computer via a Cambridge Electronic Device (CED) micro 1401 interface and Signal analysis software (Cambridge Electronic Design, version 1). Responses were filtered between DC and 5kHz; line frequency interference was removed by digitally filtering (Humbug, Digitimer Ltd).

Data analysis

Responses were quantified as the amplitude of the population spike in mV (measured as the difference between peak negativity and the averaged values of the two peaks of the positive-going synaptic potential) and the fEPSP was measured as the maximum slope of the initial negative going gradient with two static cursors using Signal software; manually selecting and recording the slope in response to changes in the size of the fEPSP response and electrical interference (figure 3). Individual responses were measured, normalised and compared to the 20-minute initial baseline recordings. Every time point was pooled and graphed using GraphPad Prism software (GraphPad Prism Software, San Diego, CA, version 3) showing mean ± standard error of the mean (s.e.m.). Rarely, single responses had to be excluded due to anomalous electrical interference disrupting the recording of evoked responses.

When paired-pulse interactions were investigated, inhibition or facilitation was expressed as the ratio of the second response of a pair compared with the first response.

Statistical analysis

The statistical significance of a difference was assessed on raw data by a paired Student's t-test or, if the difference was between three or more

means, significance was calculated with a one-way or repeated measures analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-test using GraphPad Prism software; p values less than 0.05 were considered statistically significant. IC_{50} and EC_{50} values were calculated using GraphPad Prism software by generating non-linear regression, sigmoidal concentration-response curves. In the figures statistically differences between data points are sometimes indicated by asterisks: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Chemical agents and drugs

Constituents of ACSF were of analar grade and were dissolved in distilled water to prepare fresh every day. N-Methyl-D-aspartic acid (NMDA), D-(-)-2-amino-5-phosphonopentanoic acid (2-AP5), dizocilpine (MK 801), zinc acetate (Zn^{2+}), (RS)-AMPA hydrobromide (AMPA) and kainate (KA) were dissolved in distilled water to obtain stock solutions. Quinolinic acid (QA) and homoquinolinic acid (HQA) were dissolved in 1 molar equivalencies of NaOH to give stock solutions. Dimethyl sulphoxide (DMSO) was used to dissolve kynurenic acid (KYA), 2-carboxy-3-carboxymethylquinoline (CCMQ), okadaic acid (OA), anisomycin, (R:(*), S:(*))-alpha-(4-hydroxyphenyl)-beta-methyl-4-(phenylmethyl)-1-piperidinepropanol (Ro 25-6981), staurosporine (STA) and 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX) to produce stock solutions. The final

concentration of DMSO never exceeded 0.1% when applying these agents to slices; furthermore, control applications of DMSO 0.1% alone had no effect compared to baseline field recordings. Cyclosporin A (CyA) and (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (PEAQX) were dissolved in ethanol.

The majority of drugs were obtained from Tocris Cookson Ltd, analar grade compounds were obtained from BDH chemicals Ltd. UK. NMDA was obtained from Sigma chemical Co. Ltd. Ro 25-6981 was a gift from Dr Georg Jaeschke of F. Hoffmann-LaRoche AG Roche pharmaceuticals and PEAQX was a gift from Dr Yves Auberson of Novartis Pharmaceuticals (table 4).

Compound	Supplier	Stock solution
NMDA	Sigma chemical Co. Ltd	H_2O
2-AP5	Tocris Cookson Ltd	H_2O
MK 801	Tocris Cookson Ltd	H_2O
AMPA	Tocris Cookson Ltd	H_2O
kainate	Tocris Cookson Ltd	H_2O
quinolinic acid	Tocris Cookson Ltd	NaOH 1M equivalencies
homoquinolinic acid	Tocris Cookson Ltd	NaOH 1M equivalencies
CCMQ	Tocris Cookson Ltd	DMSO 0.1%
kynurenic acid	Tocris Cookson Ltd	DMSO 0.1%
okadaic acid	Tocris Cookson Ltd	DMSO 0.1%
anisomycin	Tocris Cookson Ltd	DMSO 0.1%
Ro 25-6981	F. Hoffmann-LaRoche	DMSO 0.1%
staurosporine	Tocris Cookson Ltd	DMSO 0.1%
DPCPX	Tocris Cookson Ltd	DMSO 0.1%
cyclosporin A	Tocris Cookson Ltd	Ethanol
PEAQX	Novartis Pharmaceuticals	Ethanol

Table 4. List of pharmacological agents, suppliers and solvents used to produce stock solution.

Chapter 3. The negative coupling of NR2B to NR2A subunit-containing receptors.

Introduction

There has been a considerable amount of research over the last 10 years that has identified the NR2B subunit as being especially important in determining the function of the NMDA receptor. For example, in forming the glutamate binding site (Laube et al., 1997), controlling the Mg^{2+} block (Williams *et al.*, 1998) and in a variety of phenomena including learning and memory formation, synaptic plasticity (Hrabetova et al., 2000), pain (Chizh et al., 2001), schizophrenia (Grimwood *et al.*, 1999; Gao *et al.*, 2000), Parkinson's disease (Nash *et al.*, 1999; Oh *et al.*, 1999; Dunah *et al.*, 2000; Steece Collier *et al.*, 2000), Huntington's disease (Chen *et al.*, 1999; Zeron *et al.*, 2001) and excitotoxicity (Zhang *et al.*, 1997; Cheng *et al.*, 1999). The NR2B subunit consists of 1456 amino acids with a molecular weight of approximately 170-180 kDa. All of the NR2 subunits have the same basic structure as the NR1 subunit (Hollmann & Heinemann, 1994) but varying in that they have an especially large intracellular C-terminal domain. This C-terminal domain, it has been suggested, plays a role as the target for modulatory or accessory proteins in promoting receptor assembly, sorting or targeting and playing a part in different channel conformations to modulate receptor function (Kohr *et al.*, 2003; Loftis &

Janowsky, 2003). The NR2B subunit is modulated by its co-assembly with other subunits, alterations in the interaction with associated proteins such as PSD-95 and SAP102, proteases, growth factors, hormones and post-translational modifications such as phosphorylation. The phosphorylation of tyrosine residues can contribute to enhanced receptor responses and signal transduction into the neurone (Gurd, 1985; Gurd & Bissoon, 1997; Hisatsune *et al.*, 1999; Manabe *et al.*, 2000; Nakazawa *et al.*, 2001). Receptor function is also modulated by the phosphorylation of serine residues by protein kinase A (PKA), protein kinase C (PKC), and calmodulin-dependent protein kinase II (CaMKII) (Smart, 1997).

Ro 25-6981 is an ifenprodil derivative that has high affinity and selectivity for the NR2B subunit-containing NMDA receptor but without the affinity for α_1-adrenergic and serotonergic receptors found with ifenprodil itself (Chenard *et al.*, 1991; McCool & Lovinger, 1995). It is a high affinity, selective (5000-fold greater for the NR2B than the NR2A subunit), activity-dependent, voltage-independent and non-competitive NR2B subunit-containing NMDA receptor antagonist (Fischer *et al.*, 1997; Mutel *et al.*, 1998; Lynch *et al.*, 2001).

F. Hoffman-La Roche Pharmaceuticals first described Ro 25-6981 in 1997. They used cloned NMDA receptors expressing NR1c/NR2A, NR1c/NR2B and NR1f/NR2B subunits to confirm that Ro 25-6981 was selective for the NR2B subunit-containing receptors. They also studied the

66

electrophysiology of Ro 25-6981 in young cultured rat cortical neurones, which express NR2B as the dominant NMDA receptor subunit. It was observed that the effect of Ro 25-6981 was dependent upon the relative level of NMDA receptor activation. Ro 25-6981 potently blocked the current evoked by NMDA 100μM but it potentiated the slight responses to NMDA 1μM. Protons (H^+) exert a tonic inhibition of the NMDA receptor with an IC_{50} at physiological pH 7.4 by binding at a proton sensor site. The NR2A and NR2B subunit-containing NMDA receptors are the most sensitive to this effect. The proton sensor's binding site overlaps structurally and mechanistically with the binding site of Ro 25-6981, ifenprodil and the endogenous modulators spermine and spermidine (Mott *et al.*, 1998). The binding stabilises a protonated, agonist-bound state of the receptor that has a low open probability. This shifts the pKa of the proton sensor to values that are more alkaline and increases tonic inhibition. However, the potentiation of responses to NMDA by these compounds is reported to be due to an allosteric modulation with the glutamate and glycine binding sites. The binding of these compounds causes a decrease in NMDA receptor affinity for glycine and an increase in affinity for glutamate and NMDA leading to potentiation. However, while ifenprodil and Ro 25-6981 both bind to the N-terminal third of the NR2B receptor, Ro 25-6981 has a distinct binding site that has greater three-dimensional requirements. Furthermore, the binding of ifenprodil is

decreased and its IC_{50} is increased by rising concentrations of spermidine whilst under the same conditions the binding of Ro 25-6981 is unaffected and its IC_{50} decreases (Kew *et al.*, 1996; Kew *et al.*, 1998b; Grimwood *et al.*, 2000; Lynch *et al.*, 2001; Perin-Dureau *et al.*, 2002). In addition, Ro 25-6981 had no direct effect on the ion channel as seen with ifenprodil. This clearly demonstrates distinct properties of action by Ro 25-6981, which have not yet been fully investigated. This activity-dependent property is of great significance in therapeutic development as it predicts that NMDA receptors that are pathologically and continuously activated will be blocked whilst leaving inactive receptors relatively unaffected. In neuroprotective experiments, Ro 25-6981 was found to be very effective in protecting cultured cortical neurones from excitotoxic and ischaemic insults (Fischer *et al.*, 1997).

A further study followed, using autoradiography to investigate the binding of Ro 25-6981 to adult rat brain membranes and tissue sections. It was observed that Ro 25-6981 binding was similar to NR2B subunit distribution and dissimilar to NR2A subunit distribution (Mutel et al., 1998).

More recently, a study of Ro 25-6981 was carried out to characterise its NR2B selective pharmacology using human embryonic kidney (HEK) 293 cells transfected with heterodimers consisting of NR1a and either wildtype, chimeric or mutant NR2B subunits (Lynch *et al.*, 2001) which co-assemble

into tetrameric or pentameric receptors. It was observed that specific regions of the N-terminal leucine/isoleucine/valine-binding protein (LIVBP)-like domain of the NR2B subunit protein modulated the interaction between Ro 25-6981 and the NMDA receptor. Ro 25-6981 may be binding directly to the NR2B subunit or indirectly to the NR1 subunit at a site that is modulated by the NR2B subunit in a way that is analogous to glycine binding to the NR1 subunit where the NR2 subunit influences glycine affinity (Gallagher *et al.*, 1996; Masuko *et al.*, 1999; Perin-Dureau *et al.*, 2002). Whilst Ro 25-6981 is an ifenprodil analogue it has a binding site and pharmacology that is distinct from the polyamine site (Lynch *et al.*, 2001).

Although there are several NR2B antagonists available, Ro 25-6981 is the first compound to be developed that is a highly selective and full antagonist at NR2B subunit-containing NMDA receptors. Ifenprodil is a partial antagonist only inhibiting NR1/NR2B receptors by 80% on average and affecting NR1/NR2A/NR2B receptors with little (Luo *et al.*, 1997) or intermediate effect (Tovar & Westbrook, 1999). Also, ifenprodil is not very selective for the NR2B subunit (400-fold, recombinant NR1/NR2B compared to NR1/NR2A) and also blocks α_1-adrenergic, $5HT_{1A}$, $5HT_2$, $5HT_3$ and sigma receptors and N, P and Q type calcium channels (Chenard *et al.*, 1991; McCool & Lovinger, 1995). Interestingly, Pfizer has recently developed an NR2B subunit-selective antagonist CP-101, 606, which has a

very low affinity for the NR1/NR2A/NR2B synaptic receptors but a high affinity for the purely extrasynaptic NR1/NR2B population (Chenard *et al.*, 1995; Tovar & Westbrook, 1999). It would be of particular interest to repeat this study using CP-101, 606 to investigate further the contribution of synaptic versus extrasynaptic receptor populations (Hardingham *et al.*, 2002). There have been several recent reports demonstrating that the different subunit-containing NMDA receptors and the locus of activation has a great effect on determining the direction of synaptic plasticity (Liu *et al.*, 2004; Massey *et al.*, 2004; Rusakov *et al.*, 2004; Wong *et al.*, 2004; Mallon *et al.*, 2005) and the phosphorylation of CREB (cAMP response element binding protein) leading to BDNF expression and pro-survival pathways (Hardingham *et al.*, 2002; Riccio & Ginty, 2002).

Novartis Pharmaceuticals have recently developed an NR2A subunit-containing NMDA receptor antagonist (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (PEAQX, also known as NVP-AAM077, appendix 2). PEAQX is a water-soluble 5-methyl-quinoxalinedione with a high affinity for the glutamate-binding site of the NMDA receptor ([3H]-CGP39653 binding assay: IC_{50} = 11nM). At 10µM PEAQX showed no additional activity in a series of 68 radioligand binding assays for CNS receptors. PEAQX has 126-fold selectivity for the NMDA NR1A/NR2A over the NR1A/NR2B receptors expressed in Xenopus oocytes (IC50 = 14nM and

1800nM respectively). Experiments on embryonic cortical cells and on neocortical slices have confirmed these results with native receptors. Cultured embryonic cortical cells express NR2A subunits at a very low level, resulting in a low potency of PEAQX ($IC_{50} = 1\mu M$). In adult slices where NR2A subunits are predominantly expressed the effect of PEAQX is stronger ($IC50 = 68nM$) than in cultured embryonic cortical cells that express NR2A at very low levels (undetectable) ($IC_{50} = 1\mu M$). Furthermore, in vivo PEAQX was found to be a potent anticonvulsant following intravenous dosing with few of the side effects associated with non-selective or NR2B selective antagonists (Auberson et al., 2002).

Although these compounds have been examined on recombinant receptors and immature cultures, which are systems that over-express the NR2B subunit-containing NMDA receptors, this study has used these compounds to examine the role of NR2 subunits in an intact neuronal network in which adult heteromeric NMDA receptors are involved in synaptic transmission and plasticity. Of great importance in this study was the use of adult animals that are 7-8 weeks old because they are past the 3rd postnatal-week threshold for the maturation of NMDA receptor subunit expression (where NR2A subunits are the last to reach their mature levels) and the 4th postnatal-week threshold during which the basal levels of receptor tyrosine phosphorylation are attained (Cudmore & Gurd, 1991; Tovar & Westbrook, 1999).

Results

NMDA

We started this study using 10-minute applications of NMDA, which reversibly depressed the slope of the fEPSP. The depression of fEPSP slope being a reflection of changes in membrane conductance and depolarisation of the neurones. This leads to the depolarisation block of the CA1 neurones recorded from and the subsequent blockade of excitatory transmission. The concentration-response relationship was very steep, with $1\mu M$ having had no effect, $4\mu M$ producing a response that was discernible but not statistically significant, while $7.5\mu M$ produced a depression to $69.36\pm16.33\%$, $10\mu M$ produced a depression to $15.18\pm4.43\%$ (Figure 5) and $20\mu M$ producing a depression to $-1.05\pm1.61\%$ of fEPSP slope (Figure 4). Furthermore, investigation of changes to the paired-pulse paradigm during the depression elicited by NMDA $10\mu M$ found a significant decrease of paired-pulse inhibition, and increase of paired-pulse facilitation at all interstimulus intervals (10ms $p<0.001$, 20ms, $p<0.01$ and 50ms $p<0.01$, n=5) (Figure 6 and 7).
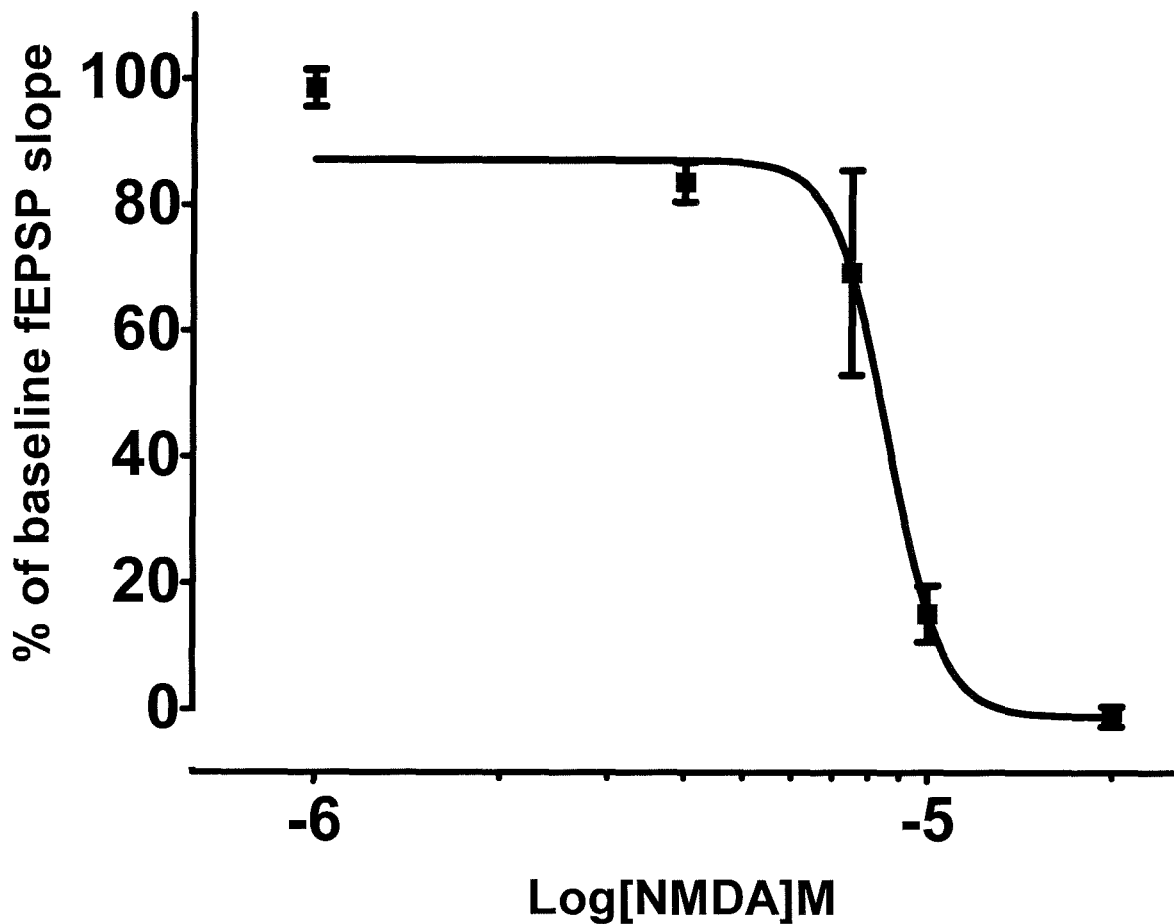
Figure 4. Concentration curve of the inhibitory effect of NMDA upon fEPSP slope.

Graph showing the non-cumulative concentration-response curve of depression of fEPSP slope in response to 10-minute applications of NMDA concentrations of 1µM, 4µM, 7.5µM, 10µM and 20µM, n=4, 13, 4, 8 and 4 respectively. A concentration of 8.6µM is predicted to cause a 50% depression of fEPSP slope. Each point shows the mean±s.e.m.
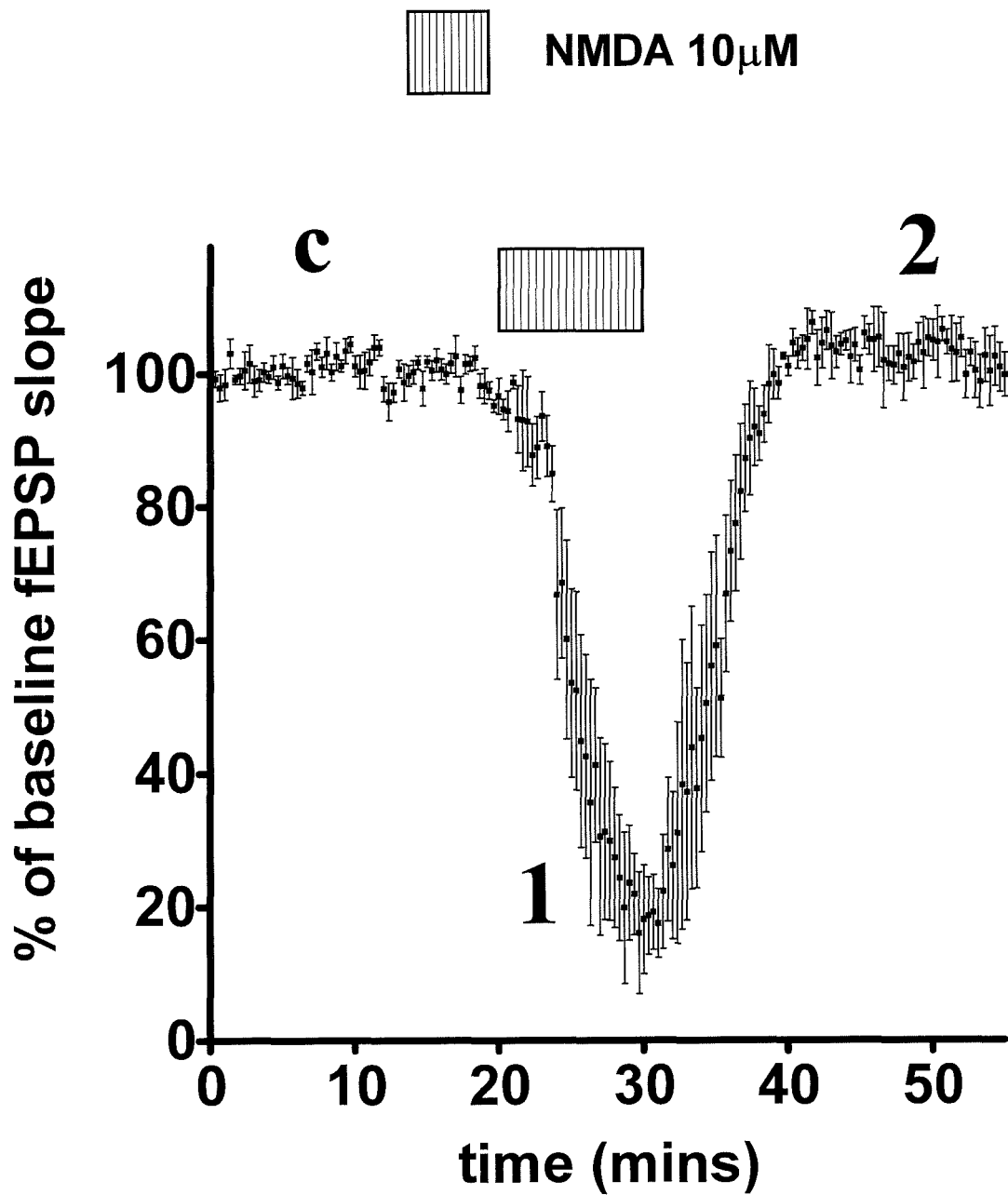
Figure 5. Effect of NMDA 10μM on fEPSP slope.

Plot showing the effect of a 10-minute application of 10μM NMDA, which elicits a depression in the slope to $15.18 \pm 4.43\%$ of fEPSP ($p<0.001$). Points (c), (1) and (2) indicate the timing of the paired-pulse tests summarised in Figure 6. Each point shows the mean±s.e.m. for $n=8$ experiments.
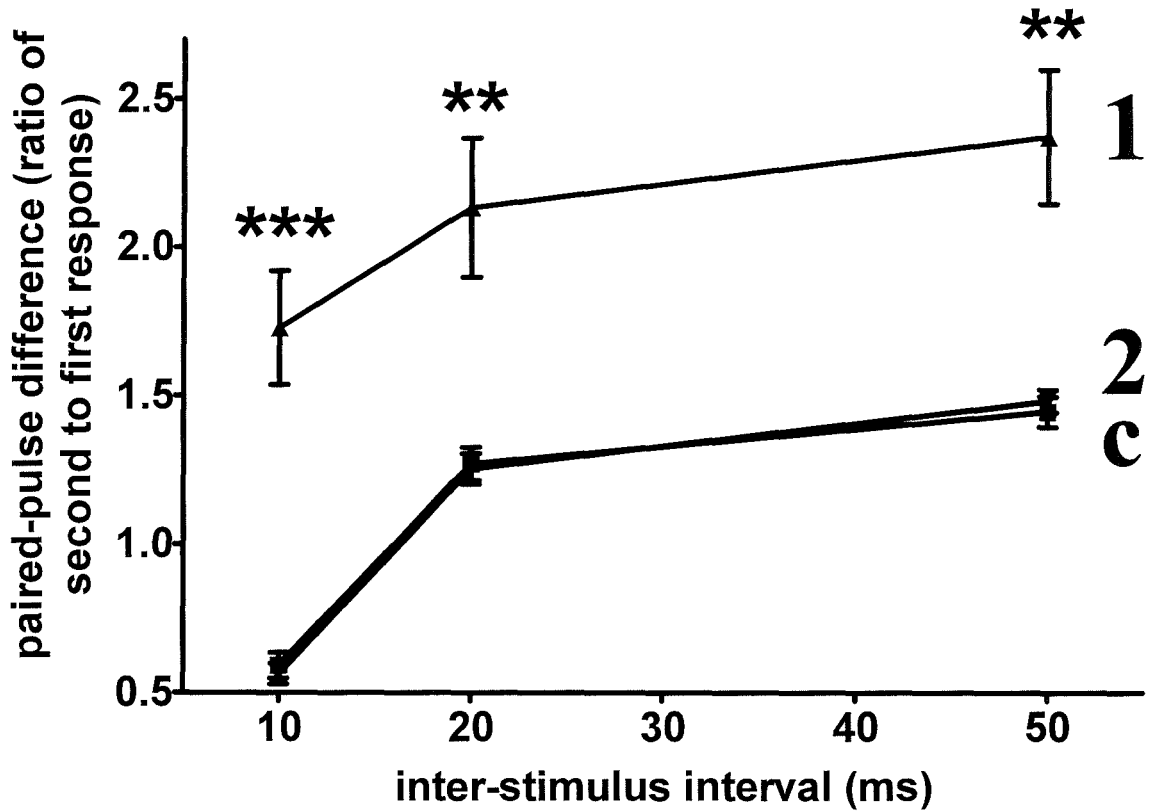
Figure 6. Effect of NMDA 10μM on paired-pulse interactions.

Graph showing the paired-pulse interactions of fEPSP slope at interstimulus intervals of 10ms, 20ms and 50ms during the baseline control responses (c), 10μM NMDA (1) and the washout period (2) (10ms p<0.001, 20ms p<0.01, 50ms p<0.01, n=5, 1 compared to 2 and c)(see plot in figure 5 for corresponding times points and example records in figure 7). Each point shows the mean±s.e.m. for n=5 experiments.
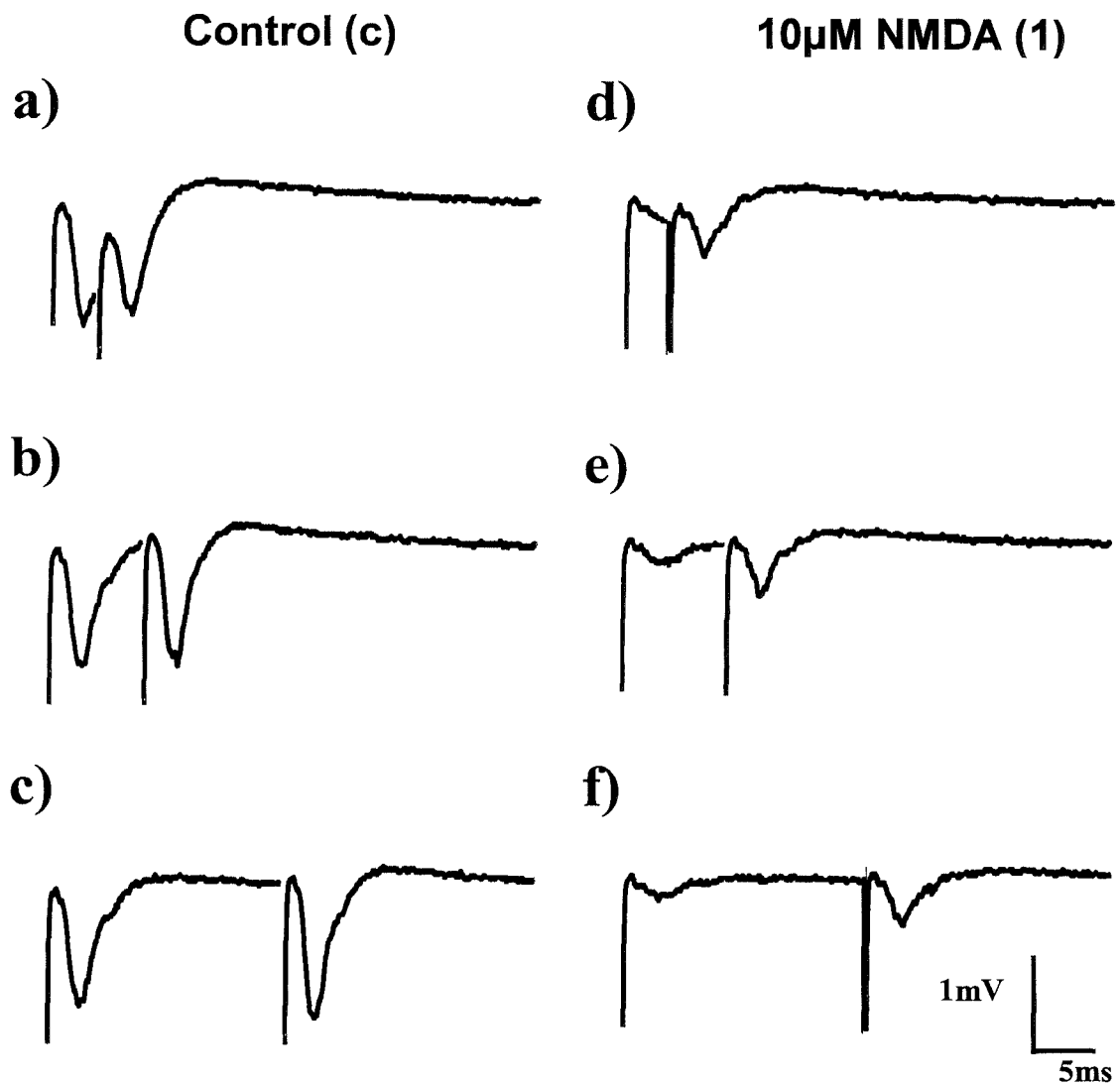
Figure 7. Example records of paired-pulse interactions during baseline recording and under the influence of 10μM NMDA.

Showing paired-pulse interactions recorded during baseline recording: (a) 10ms (b) 20ms and (c) 50ms and during the depression caused by NMDA 10μM: (d) 10ms (e) 20ms and (f) 50ms (points relate to same points in figures 5 and 6).

Ro 25-6981

The large depressions of fEPSP slope elicited by applications of NMDA were caused by the activation of native heteromeric NMDA receptors containing a combination of NR1, NR2A, NR2B and NR2D subunits. This study investigated the contribution of NR2B subunit-containing NMDA receptors using the NR2B antagonist Ro 25-6981. A concentration of $3\,\mu M$ Ro 25-6981 was chosen as it has previously been shown to block selectively the NR2B component of the NMDA excitatory postsynaptic current ($NMDA_{EPSC}$) in 3-4 week old rats and not the NR2A component (Liu *et al.*, 2004). Repeated applications of NMDA $10\,\mu M$ caused two similar reversible depressions of fEPSP slope to $16.06 \pm 9.16\%$ then to $12.85 \pm 7.85\%$ ($p < 0.001$, n=4). The co-application of $3\,\mu M$ Ro 25-6981 had no immediate effect upon this depression, which again reversibly depressed the fEPSP slope to $8.96 \pm 2.77\%$ ($p < 0.001$, n=4). However, the next 10-minute application of NMDA started 5 minutes after the end of the 20-minute application of Ro 25-6981 $3\,\mu M$ totally abolished the fEPSP, which then slowly and partially recovered to $37.56 \pm 7.26\%$ of the baseline responses and remained depressed for at least the 100 minutes recorded ($p < 0.001$, n=4) (figure 8 and 9). Attempts to recover the response by increasing afferent stimulation or by HFS stimulation, which normally induces LTP (responses not shown), were unsuccessful. This observation is consistent with a terminal excitotoxic insult to the neurones recorded

from and inconsistent with the induction of LTD as witnessed by the failure

to reverse it with an LTP inducing protocol or by increasing the stimulation

of the presynaptic afferents (Schurr et al., 1995).

Following the intensity of the response to $10\mu M$ NMDA, a concentration of

NMDA $4\mu M$ was used, a concentration that produced a smaller control

depression of baseline fEPSP slope (figure 10). This would be less likely

to result in an excitotoxic insult after the application of Ro 25-6981.

Applications of NMDA $4\mu M$ caused two small yet discernible depressions

of fEPSP slope that were not statistically significant (i and ii). Again the

co-application of Ro 25-6981 $3\mu M$ with a third application of NMDA (iii)

had no immediate effect but a fourth application, during the washout phase

of Ro 25-6981 (iv), showed a significant enhancement of the depression of

fEPSP slope. The fifth application of NMDA (v) caused an even greater

depression of fEPSP slope that was matched in size by the sixth and

seventh (vi and vii) applications with no washout of the effect being

observed up to 100 minutes after Ro 25-6981 had been removed from the

perfusate (significant depressions of fEPSP slope to (iv) $40.94\pm4.56\%$, (v)

$19.78\pm6.7\%$, (vi) $19.28\pm10.74\%$ and (vii) $13.23\pm4.18\%$ (all $p<0.001$

compared to the second NMDA $4\mu M$ induced depression, n=4)). Lastly,

the application of the NR2A subunit-selective antagonist PEAQX $0.1\mu M$

during the eighth and final application of NMDA $4\mu M$ (viii) completely

blocked any depression of fEPSP slope i.e. both the initial control NMDA 4μM portion and the enhanced portion induced by Ro 25-6981 (figure 10).

This study also investigated the effect of Ro 25-6981 upon responses to low concentrations of NMDA using intracellular recording techniques to investigate the effect solely on the excitability of the postsynaptic pyramidal cell (Appendix 1). A similar enhancement of responses to NMDA was induced over the same time course as seen with extracellular recordings. This suggests a predominantly postsynaptic site of action for these effects.
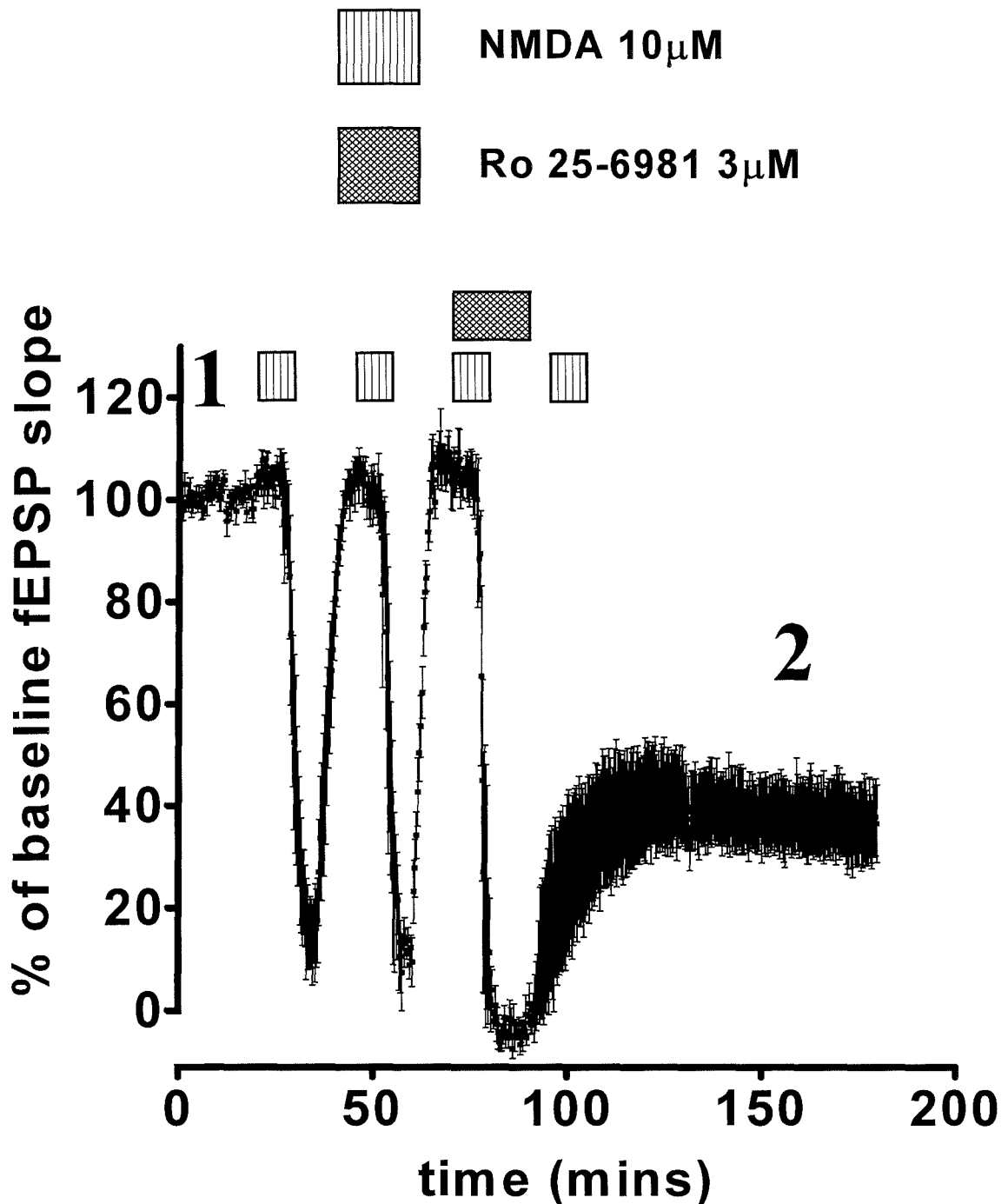
Figure 8. Repeated 10-minute applications of 10μM NMDA and the effect of 3μM Ro 25-6981.

Plot showing the co-application of 3μM Ro 25-6981 had no immediate effect upon the response to 10μM NMDA, whereas the next application of NMDA was potentiated to the extent that the fEPSP was initially abolished and then shows only partial recovery ($p<0.001$, $n=4$). Each point shows the mean±s.e.m. for $n=4$ experiments.

1

2

1mV

5 ms
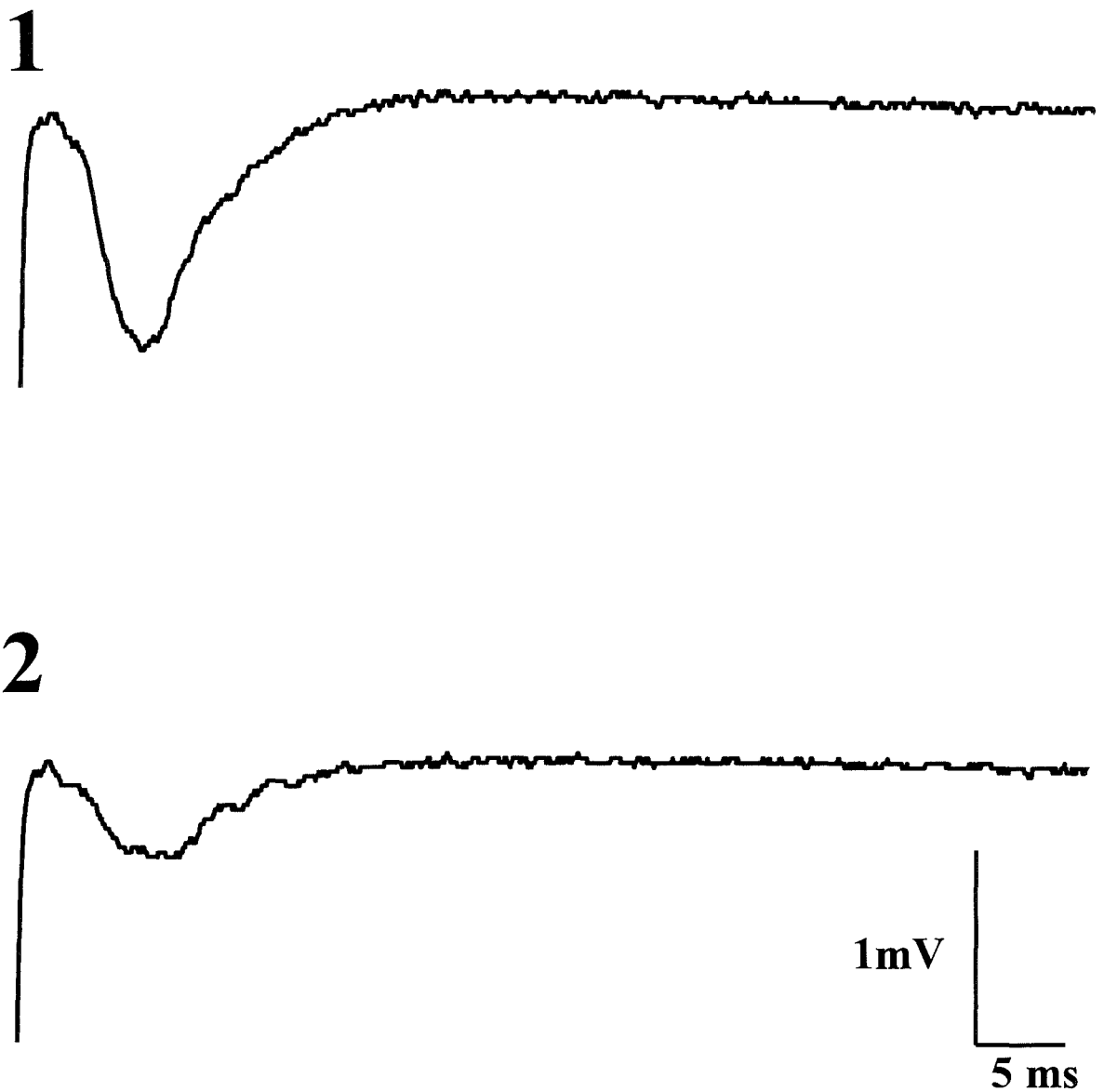
Figure 9. Representative fEPSPs showing excitotoxic damage.

Recordings of fEPSPs showing the irrecoverable depression of baseline responses caused by the excitotoxic insult of NMDA 10μM and Ro 25-6981 3μM (2) compared with baseline responses (1). Numbered traces correspond to the time course in figure 8.
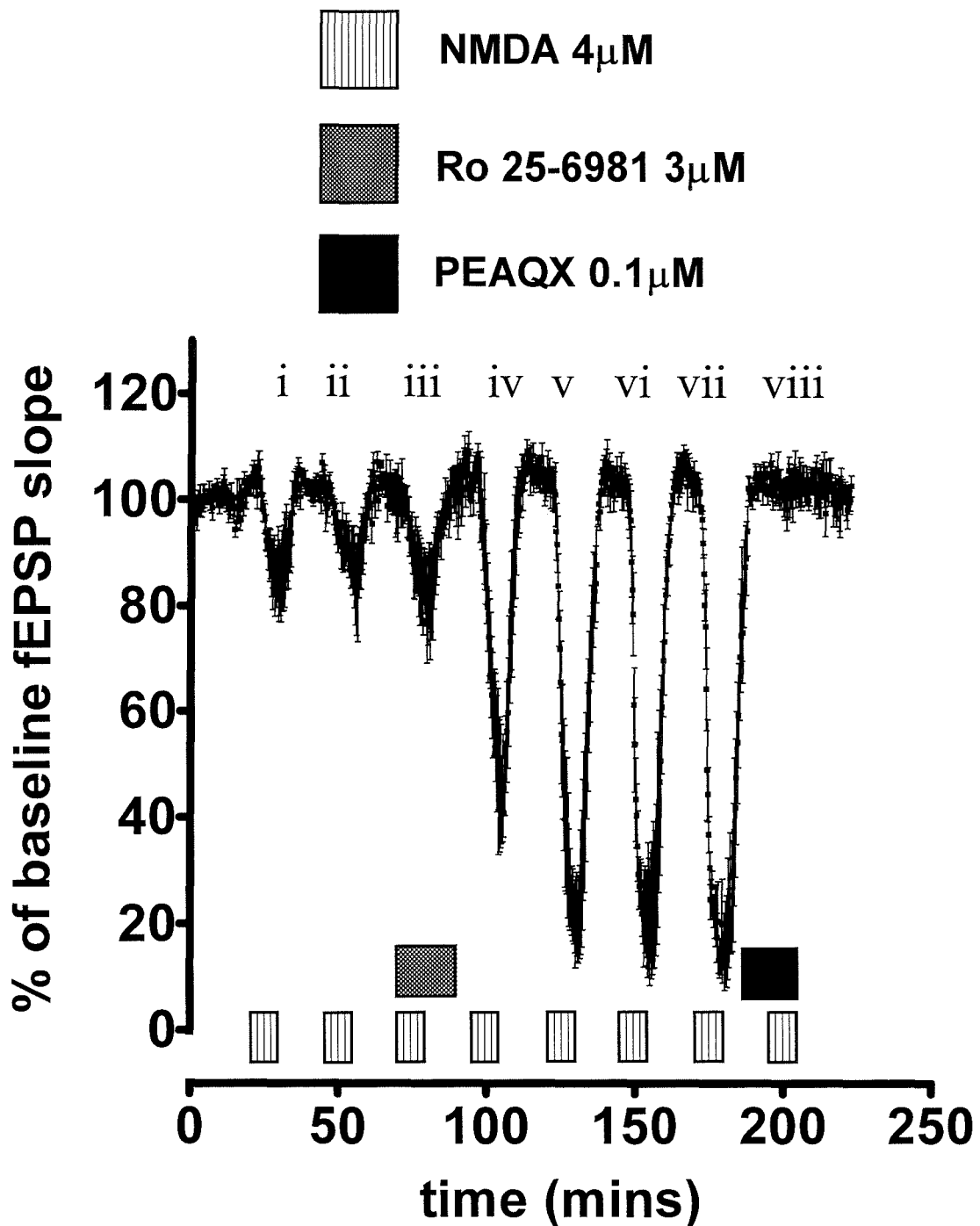
Figure 10. Effect of Ro 25-6981 and NMDA 4μM on fEPSP slope.

Plot showing the effect of repeated 10-minute applications of 4μM NMDA, which produced a small depression of the fEPSP slope. The application of 3μM Ro 25-6981 had no immediate effect upon this, but subsequent applications of 4μM NMDA show the development of potentiation in the fEPSP slope ($p<0.001$, $n=4$). The co-application of PEAQX at 0.1μM completely blocked the final potentiated response to NMDA. Each point shows the mean±s.e.m. for $n=4$ experiments

NMDA receptor antagonism

A surprising feature of these experiments was that the effect of Ro 25-6981 developed slowly, required at least 20 minutes to elicit a clearly significant decrease in response size and the effect gradually continued to develop during the washout phase. In addition this effect, once fully developed, was long lasting, with no attenuation of the depressed response size being observed for up to 100 minutes post-washout. The continuous bath-application of NMDA 4μM was employed in order to observe better how the effect of Ro 25-6981 developed. As shown in figure 11, NMDA 4μM initially produced a small, stable depression of baseline fEPSP slope, the co-perfusion of Ro 25-6981 3μM produced a slight recovery of the responses but near the end of the 20-minute perfusion the depression gradually develops. As Ro 25-6981 started to washout the depression had reached 70.42±12.18% of baseline fEPSP slope. In spite of the absence of perfusing Ro 25-6981 the depression continued to gradually develop. The depression stabilised 30 minutes after the washout of Ro 25-6981 at 33.84±0.32% ($p<0.001$; n=5) of baseline fEPSP slope. The application of the competitive, non-selective NMDA receptor antagonist 2-AP5 50μM reversed the depression to near-baseline responses. However, after the washout of 2-AP5 the depression reasserted itself within approximately 18 minutes ($p<0.001$; n=5). At the end of the experiment and with the washout of NMDA, the fEPSP slope returned to a level not significantly

different from the initial baseline responses (Figure 11). An examination of paired-pulse interactions during this experiment found that paired-pulse ratios were enhanced during the Ro 25-6981-mediated enhancement of NMDA 4µM (p<0.001, n=5 at all interstimulus intervals) but were returned to baseline responses during the reversal of this depression caused by 2-AP5 (figure 12).

Repeating the same protocol using a slightly higher level of NMDA at a concentration of 7.5µM (figure 13) produced a modest and stable depression of fEPSP slope to 64.6±10.4% (not significant, n=4). The co-application of Ro 25-6981 3µM caused an enhancement of this depression, which started during Ro 25-6981 co-perfusion and continued during washout until it reached a stable plateau of -0.16±0.24% (p<0.01; n=4) of baseline fEPSP slope within approximately 18 minutes of the depression beginning. The application of the competitive, non-selective NMDA receptor antagonist 2-AP5 50µM significantly reversed the depression of fEPSP slope back to the level previously seen with NMDA alone (p<0.01; n=4). Again, during the washout of 2-AP5 the depression reasserted itself within approximately 18 minutes to 0.80±0.29% (p<0.001; n=4). At the end of the experiment and with the washout of the NMDA, the fEPSP returned to a level not significantly different from the baseline responses although it was apparent that the level and duration of NMDA agonism had had a deleterious effect upon the response size. The rate of onset of effect

was quicker with NMDA $7.5\mu M$ (18 minutes) than with NMDA $4\mu M$ (30 minutes) perhaps reflecting the increased rate of binding with increased concentration of NMDA as previously reported with glutamate (Mutel *et al.*, 1998) and with ifenprodil (Grimwood *et al.*, 2000). Figure 14 shows a concentration-response curve of the effect of NMDA concentrations and the degree to which they are potentiated to under the influence of Ro 25-6981 $3\mu M$. This shows a shift to the left of the NMDA concentration-response curve after the application of Ro 25-6981. The concentration required to elicit a 50% depression of fEPSP slope changed from $1.7\mu M$ with NMDA alone to $8.6\mu M$ under the influence of Ro 25-6981.
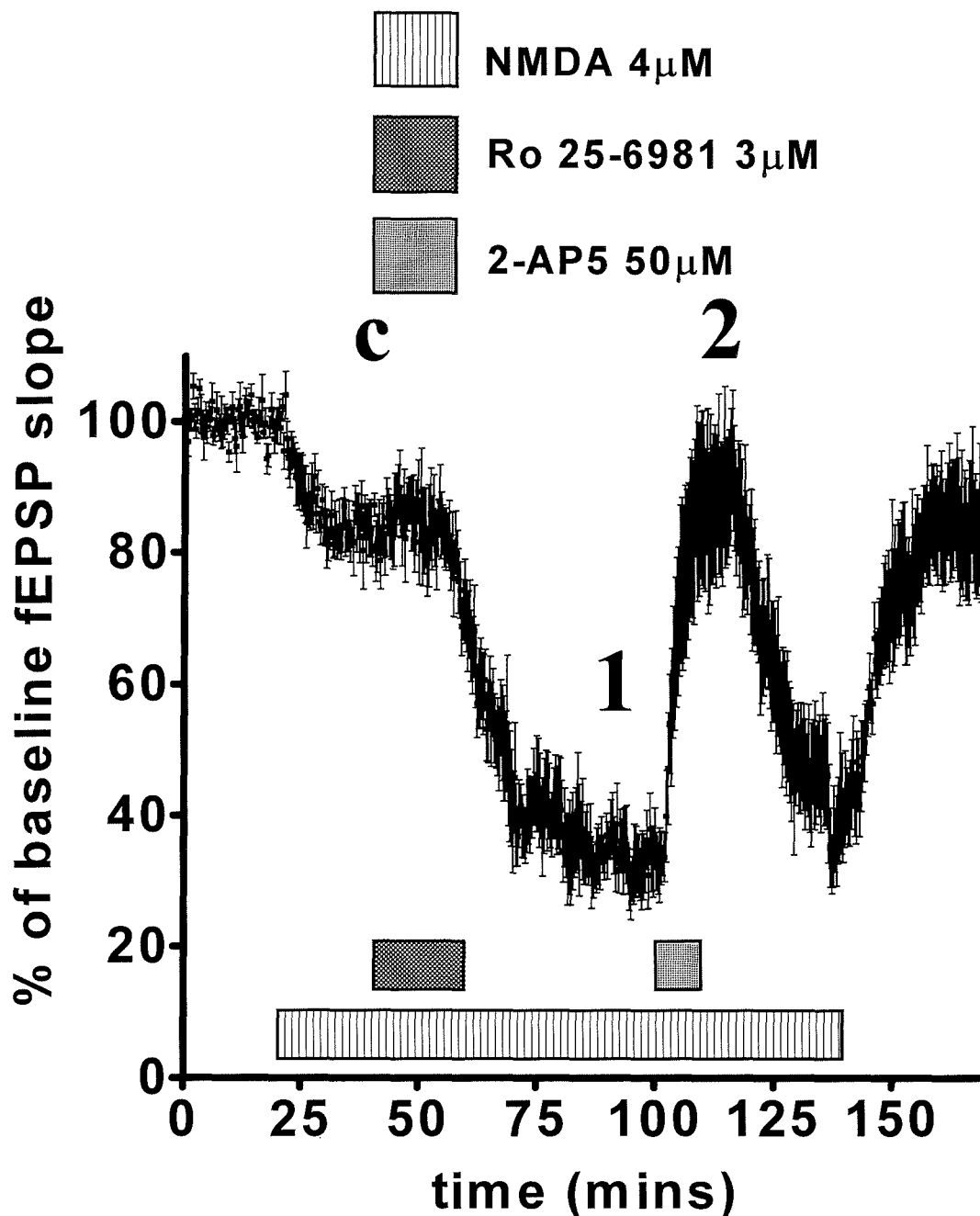
Figure 11. Effect of Ro 25-6981 on the continuous application of NMDA 4μM and its reversal by 2-AP5.

4μM NMDA caused a slight depression in the fEPSP slope (c). The co-application of 3μM Ro 25-6981 increased this response; the increase occurred slowly during Ro 25-6981 washout into a stable plateau, (1) fEPSP slope of 33.84±0.32% (p<0.001; n=5) compared with the baseline. The competitive NMDA receptor antagonist 2-AP5 (50μM) reversed the depression (2) (p<0.001; n=5). During the washout of 2-AP5, the potentiated response to NMDA re-asserted itself (p<0.001; n=5), but recovered to baseline values upon removing the NMDA. Each point shows the mean±s.e.m. for n=5 experiments.
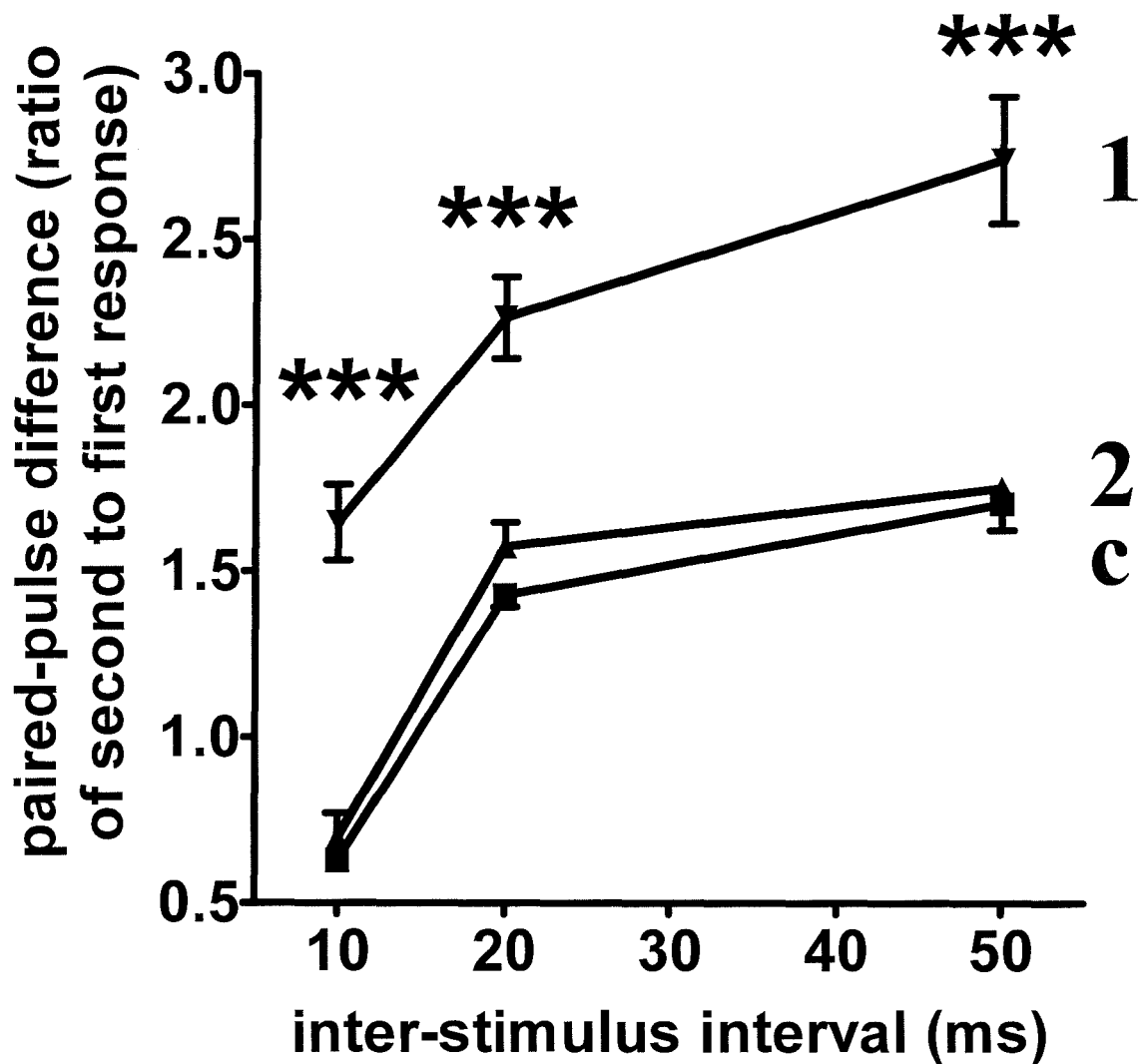
Figure 12. Effect of NMDA $4\mu M$ and Ro 25-6981 on paired-pulse interactions.

This graph summarises paired-pulse interactions at interstimulus intervals of 10 ms, 20 ms and 50 ms, corresponding to points (c), (1) and (2) in figure 11. The results demonstrate a significant enhancement of paired-pulse interactions during the trough of the depression caused by Ro 25-6981 and NMDA (1) (10 ms $p<0.001$, 20 ms $p<0.001$, 50 ms $p<0.001$, $n=5$) compared with baseline responses (c). These changes were reversed by 2-AP5 (2). Each point shows the mean±s.e.m. for $n=5$ experiments
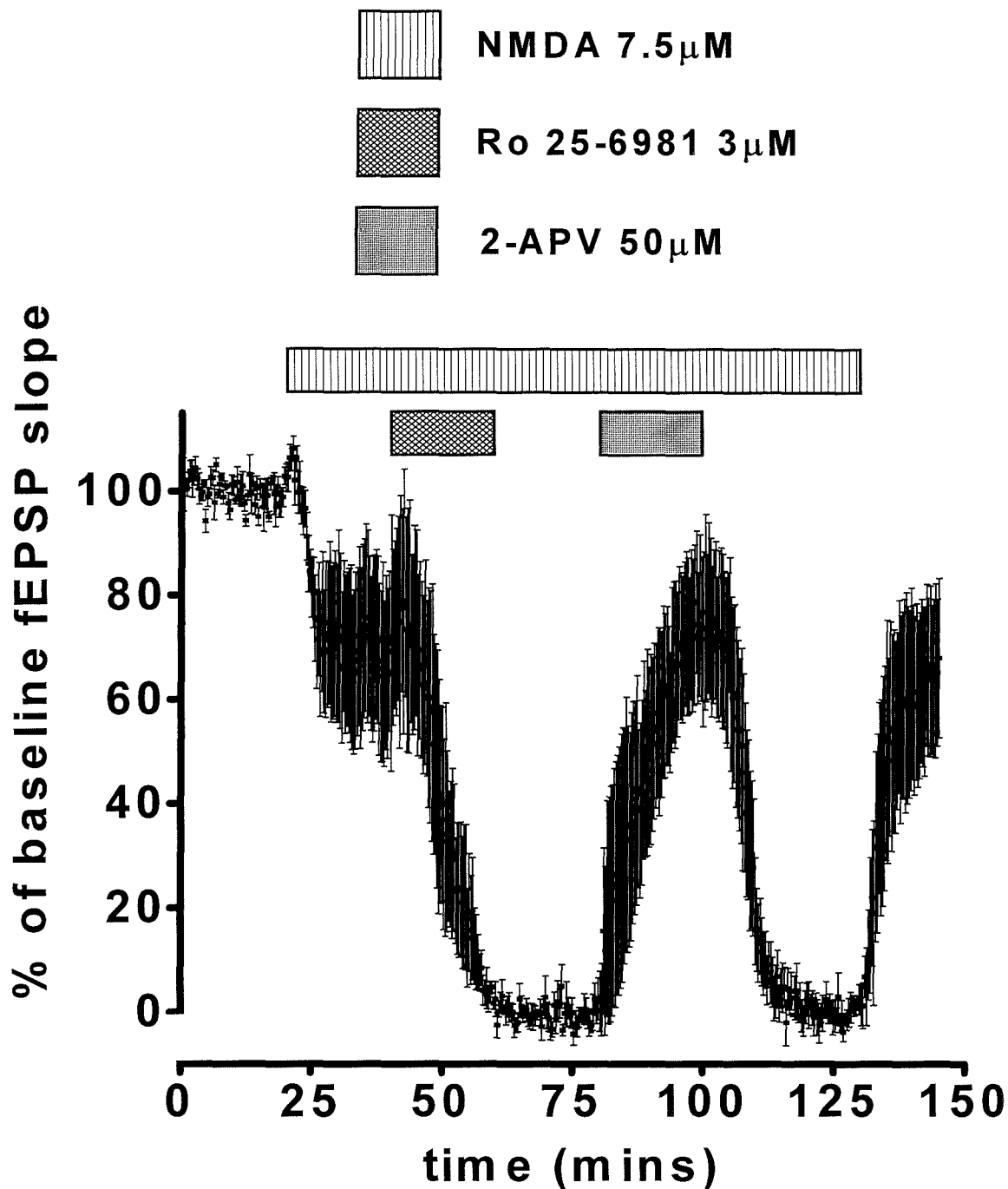
Figure 13. Effect of Ro 25-6981 on the continuous application of NMDA 7.5μM and its reversal by 2-AP5.

7.5μM NMDA caused a modest (64.6±10.4%; n.s. n=4) depression of the fEPSP slope. The co-application of 3μM Ro 25-6981 increased this response; the increase occurred slowly during Ro 25-6981 washout into a stable plateau fEPSP slope of -0.16±0.24% (p<0.01; n=4) compared with the baseline. The competitive NMDA receptor antagonist 2-AP5 (50μM) reversed the depression ($p<0.01$; $n=4$). During the washout of 2-AP5, the potentiated response to NMDA re-asserted itself ($p<0.001$; $n=4$), but recovered to baseline values upon removing the NMDA. Each point shows the mean±s.e.m. for $n=4$ experiments.
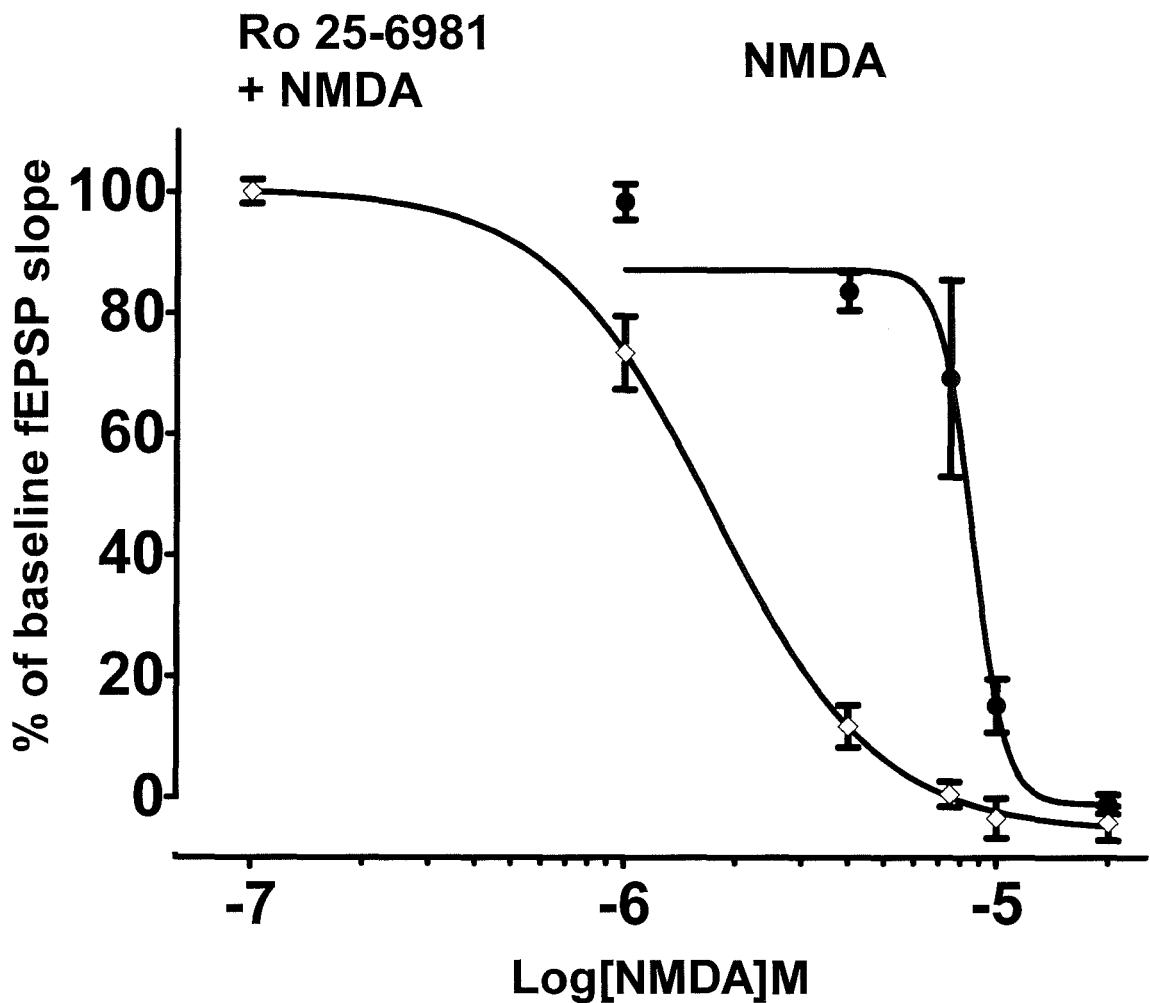
Figure 14. The concentration-response curve of NMDA alone and after Ro 25-6981.

Graph summarising the concentration-response curves for responses to NMDA in the presence and absence of 3μM Ro 25-6981, and showing the marked leftward shift produced by the combination. NMDA alone (1, 4, 7.5, 10 and 20μM) and Ro 25-6981 potentiated responses to NMDA (0.1, 1, 4, 7.5 and 10μM). The concentration eliciting a 50% depression of fEPSP slope changes from 8.6μM for NMDA alone to 1.7μM under the influence of Ro 25-6981. Each point shows the mean±s.e.m.

2-AP5 is a competitive antagonist at the NMDA receptor's glutamate binding site, in order to test that this was not a compounding factor in the blockade of the effect of Ro 25-6981 the non-competitive, open channel blocker dizocilpine (MK 801) was used to antagonise the NMDA receptor during the co-application of Ro 25-6981 and NMDA. The application of NMDA $4\mu M$ caused a small, stable depression of fEPSP slope that was reversed by the application of MK 801 $10\mu M$ to baseline response size. The perfusion of Ro 25-6981 $3\mu M$ had no effect upon the fEPSP slope and during the washout of MK 801 the response size remained at baseline levels (figure 15). However, the non-competitive antagonism provided by MK 801 was not washed out of the slice over the time-period recorded from and this is a likely reason for the lack of any depression observed.
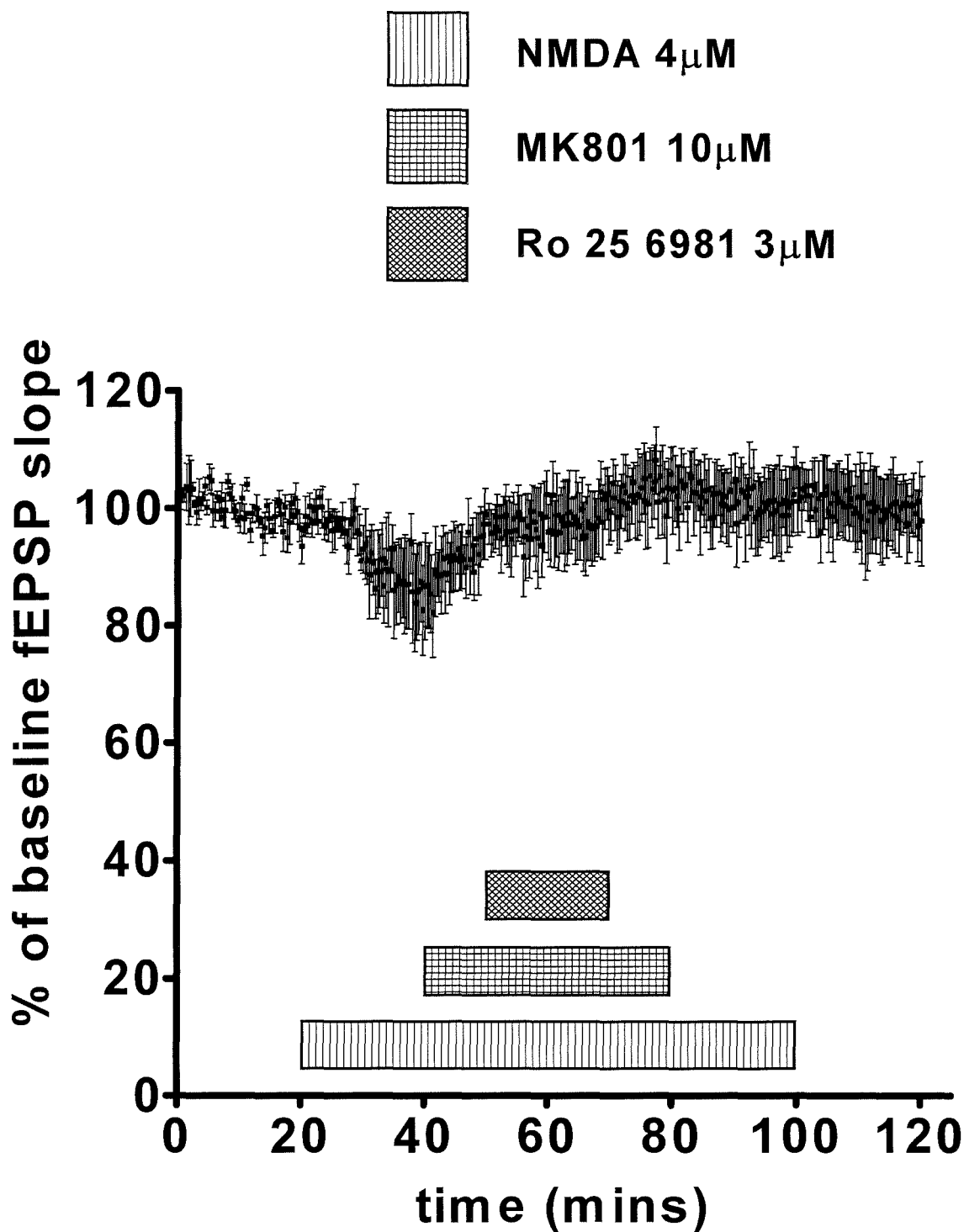
Figure 15. Effect of 4μM NMDA and Ro 25-6981 in the presence of MK 801 on fEPSP slope.

Plot showing the effect of 10μM MK 801 applied throughout the combined application of 4μM NMDA and 3μM Ro 25-6981. An initial, small depression (approximately 5%) produced by NMDA was blocked by 10μM MK 801. This prevents the development of a potentiated response to NMDA plus Ro 25-6981 even after the washout of MK 801. Each point is the mean±s.e.m. for n=3.

Having previously demonstrated that the Ro 25-6981-mediated potentiation of responses to NMDA was transiently reversible by applying 2-AP5 50μM this study investigated whether, by applying this competitive antagonist before, during and after Ro 25-6981, the induction of the depression of fEPSP slope could be prevented. As shown in figure 16 the application of NMDA 4μM produced a slight, stable depression of the fEPSP slope that was reversed to above the baseline levels by the subsequent application of 2-AP5. The co-application of Ro 25-6981 3μM had no further effect upon the fEPSP response, 2-AP5 preventing the depression normally elicited. However, as 2-AP5 was washed out the depression of fEPSP slope developed rapidly to 14.06±5.09% of baseline fEPSP slope (p=0.0178, n=4) within approximately 20 minutes of it starting to reduce. The gradient of the development of this depression was similar to that seen with 2-AP5 washout in figure 11 and 13; i.e. a depression that is linear and rate-dependent upon the washout of 2-AP5. The gradient was dissimilar to the Ro 25-6981/NMDA 4μM gradient of figure 11, which is rate-dependent on the effect initiated by Ro 25-6981, which is a slower developing effect (approximately 30 minutes). Therefore, the effect of Ro 25-6981 would seem to have already been realized during 2-AP5 co-perfusion suggesting that it does not require NMDA receptor activation. The difference in these gradients may be explained by the binding of Ro 25-6981 having already taken place during 2-AP5 perfusion; 2-AP5 was able to suppress the NR2A

activation-dependent expression of its effect but not the binding of Ro 25-6981 to its unique site on the NR2B subunit and the mechanism it puts into effect to produce the enhanced depression of fEPSP slope.
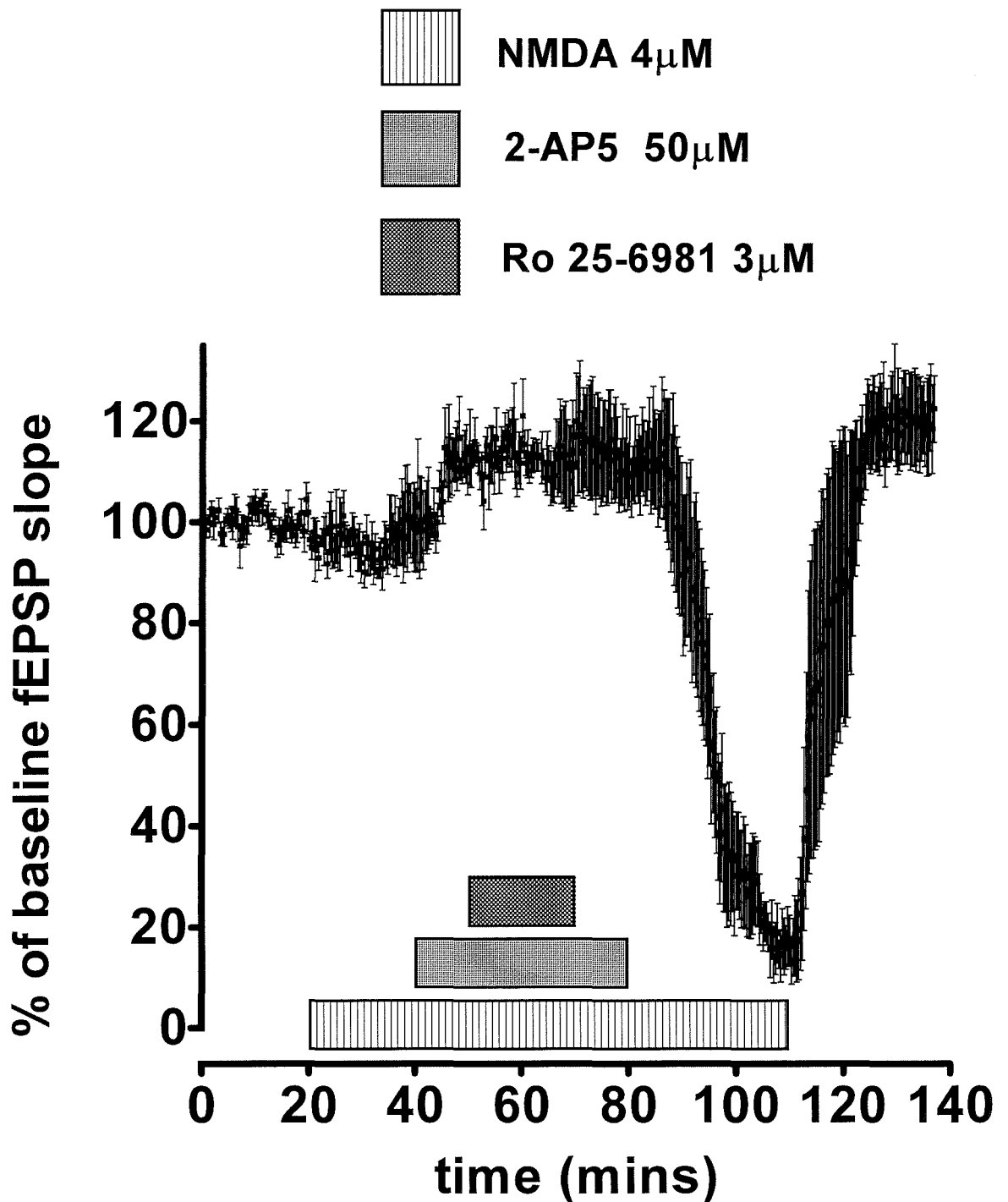
Figure 16. Effect of 2-AP5 continuously bath-applied during the application of 4μM NMDA and Ro 25-6981 on fEPSP slope.

A plot showing the effect of 50μM 2-AP5 applied throughout the combined application of 4μM NMDA and 3μM Ro 25-6981. An initial, small depression (approximately 5%) produced by NMDA was blocked by 50μM 2-AP5. Despite this, the application of 2-AP5 did not prevent the development of a potentiated response to NMDA plus Ro 25-6981, which appeared after washout of the 2-AP5 and persisted until the NMDA was removed. Each point is the mean±s.e.m. for n=4 experiments.

PEAQX

The bath application of NMDA in the adult rat hippocampus will activate NMDA receptors containing NR2A, NR2B and NR2D subunits. In view of the potentiating activity produced when the NR2B element to the action of NMDA was blocked by Ro 25-6981, the putative NR2A subunit antagonist PEAQX was used to investigate the residual response to NMDA. NMDA $4\mu M$ was continuously bath-applied producing a small, stable depression of fEPSP slope (figure 17). The co-perfusion of $3\mu M$ Ro 25-6981 transformed this into a large depression to $31.90\pm1.01\%$ of the baseline fEPSP slope ($p<0.05$, $n=3$) that developed over approximately 30 minutes. It was then reversed by the application of PEAQX $0.1\mu M$ back to baseline fEPSP responses. The rate of washout of PEAQX was initially rapid but after 8 minutes, it slowed to such an extent that it still did not completely washout for at least 2 hours. These experiments were therefore concluded before the depression might be observed to fully re-establish.
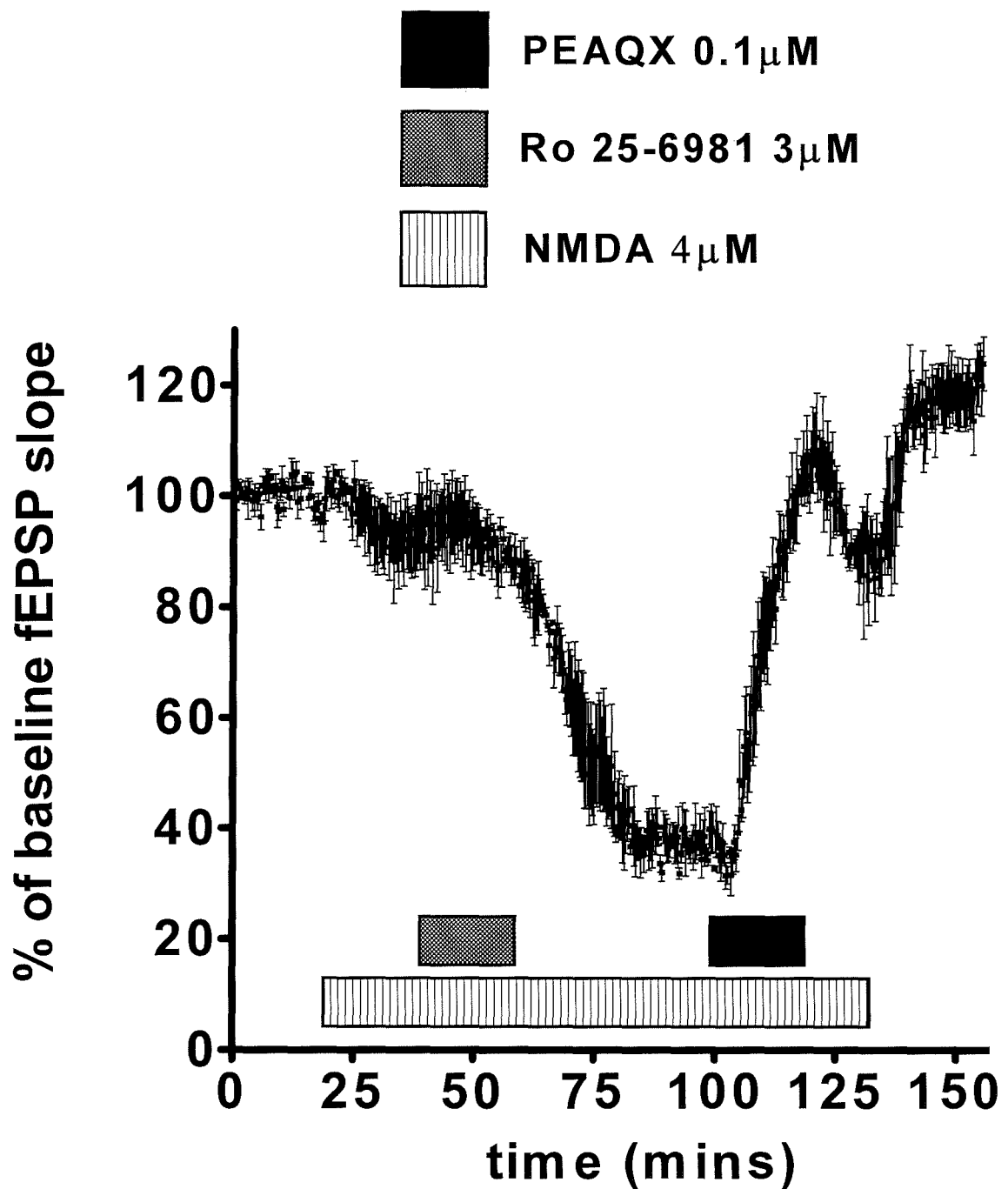
Figure 17. Effect of NMDA, Ro 25-6981 and PEAQX on fEPSP slope.

This plot shows the interaction of NMDA, Ro 25-6981 and PEAQX. 4µM NMDA produces a small, stable depression, but co-perfusion of 3µM Ro 25-6981 transforms this into a large depression that reaches $31.90 \pm 1.01\%$ of the baseline fEPSP slope ($p < 0.05$, $n=3$). This depression was reversed by 0.1 µM PEAQX. The partial washout of PEAQX stabilised after 8 minutes but does not completely wash out for at least two hours. The experiment was therefore terminated before the depression was fully re-established. Each point is the mean±s.e.m. for n=3 experiments.

Protein phosphorylation

The phosphorylation state of the NMDA receptor and many other cellular proteins determines their activity and function (Smart, 1997). This study next investigated the involvement of protein kinases and protein phosphatases on the Ro 25-6981 effect.

Staurosporine (STA) is a broad-spectrum protein kinase inhibitor, enzymes inhibited include: PKC (IC_{50}=3nM), PKA (IC_{50}=7nM), tyrosine protein kinase (IC_{50}=6nM) and CaMKII (IC_{50}=20nM) (Tamaoki *et al.*, 1986; Ruegg & Burgess, 1989; Yanagihara *et al.*, 1991). As shown in figure 18 the initial 10-minute application of NMDA 4μM caused a small depression of the fEPSP slope, staurosporine 100nM was continuously bath-applied and had no effect upon baseline fEPSP slope or the depression produced by a further 10-minute application of NMDA 4μM. The application of Ro 25-6981 3μM and the subsequent application of NMDA produced a significant depression of fEPSP slope to 17.66±10.83% ($p<0.01$, n=3) compared to baseline responses. After washout, a final application of NMDA 4μM produced another significant depression of fEPSP slope to 1.77±0.87% ($p<0.001$, n=3) compared to baseline responses (Figure 18). Therefore, staurosporine was unable to prevent the potentiation of responses to NMDA 4μM by Ro 25-6981.

The first phosphatase inhibitor investigated was okadaic acid (OA), a potent inhibitor of the serine/threonine protein phosphatases types 1 and 2A (PP1 and PP2A) (Bialojan & Takai, 1988). The continuous application of NMDA $4\mu M$ elicited a small, stable depression of fEPSP slope. The addition of okadaic acid before, during and after the co-application of Ro 25-6981 $3\mu M$ was unable to prevent the development of a significant depression of the fEPSP slope to $1.56 \pm 0.31\%$ ($p < 0.001$, $n = 4$). Interestingly, upon washout the fEPSP slope recovered to an average of $156.5 \pm 14.62\%$ ($p < 0.05$, $n = 4$) of baseline responses, a significant LTP of responses that was stable (figure 19). This LTP-inducing effect was not investigated further at this time.

Cyclosporin A (CyA), a widely used serine/threonine Ca^{2+}/calmodulin-dependent phosphatase 2B (calcineurin) inhibitor, was used next. The continuous bath-application of NMDA $4\mu M$ again produced a slight, stable depression of fEPSP slope. The application of $1\mu M$ cyclosporin A had no significant effect on the baseline fEPSP slope but it was able to prevent the anticipated enhancement of the NMDA $4\mu M$-mediated depression of fEPSP slope previously seen after the application of Ro 25-6981 $3\mu M$ (figure 20). Interestingly the baseline fEPSP returned to values that were significantly potentiated ($132 \pm 0.81\%$, $p < 0.05$, $n = 4$) compared to baseline responses. The application of cyclosporin A prevented the depression of fEPSP slope seen with both the control group and the experiments using

okadaic acid and staurosporine, which were unable to inhibit the development of the depression. Interestingly, the magnitude of the depression of fEPSP slope was significantly greater than the control group for both okadaic acid and staurosporine (figure 21).
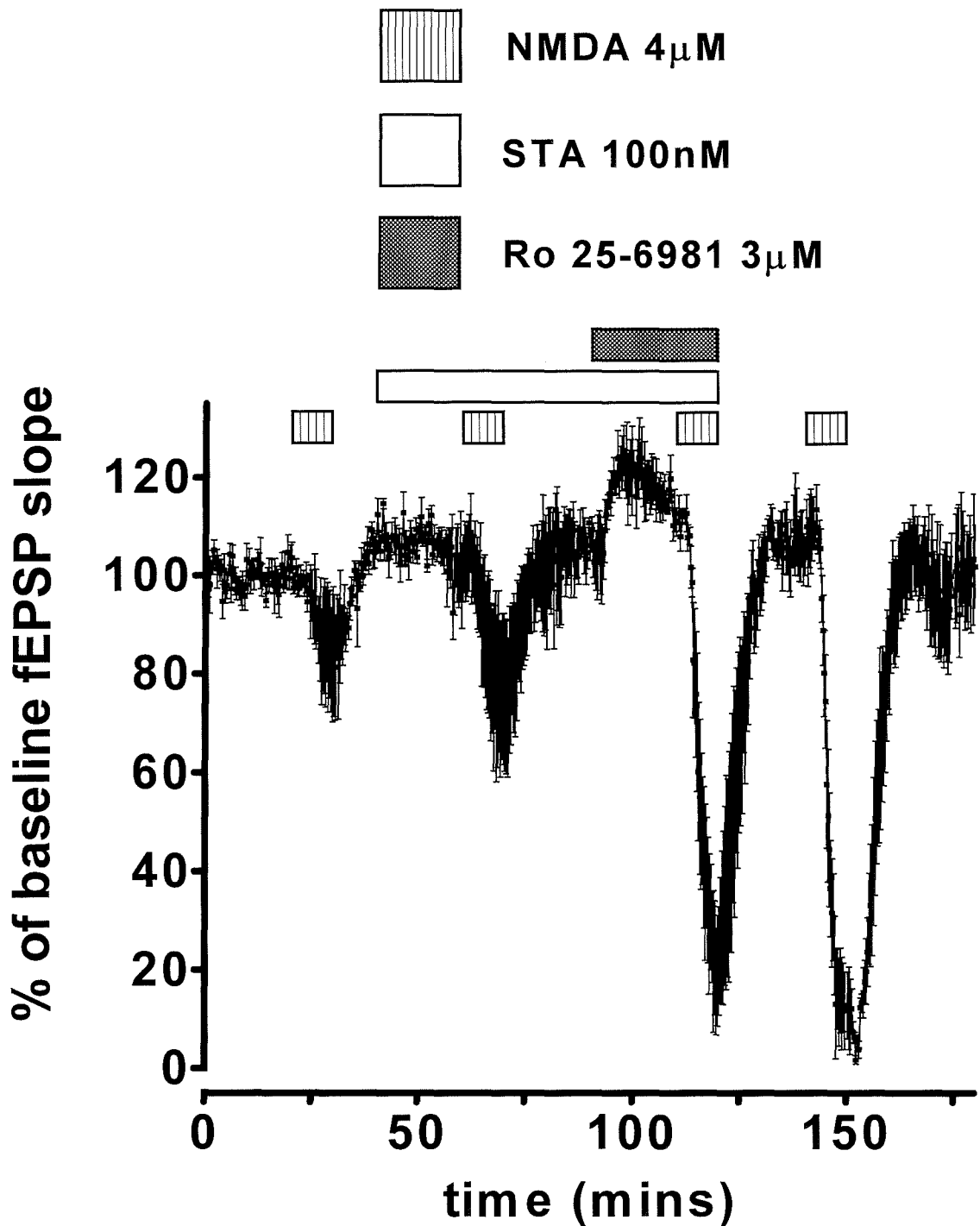
Figure 18. Action of staurosporine upon the effect of the co-application of 4µM NMDA and Ro 25-6981 on fEPSP slope.

Plot showing repeated applications of NMDA 4µM produced a small depression of fEPSP slope that was not prevented by the co-application of 100nM staurosporine. The co-application of staurosporine was also not able to prevent the enhancement of NMDA responses by Ro 25-6981 3µM which produced a significant depression of the fEPSP slope to $17.66 \pm 10.83\%$ ($p<0.01$). Each point shows the mean±s.e.m. for n=3 experiments.

100
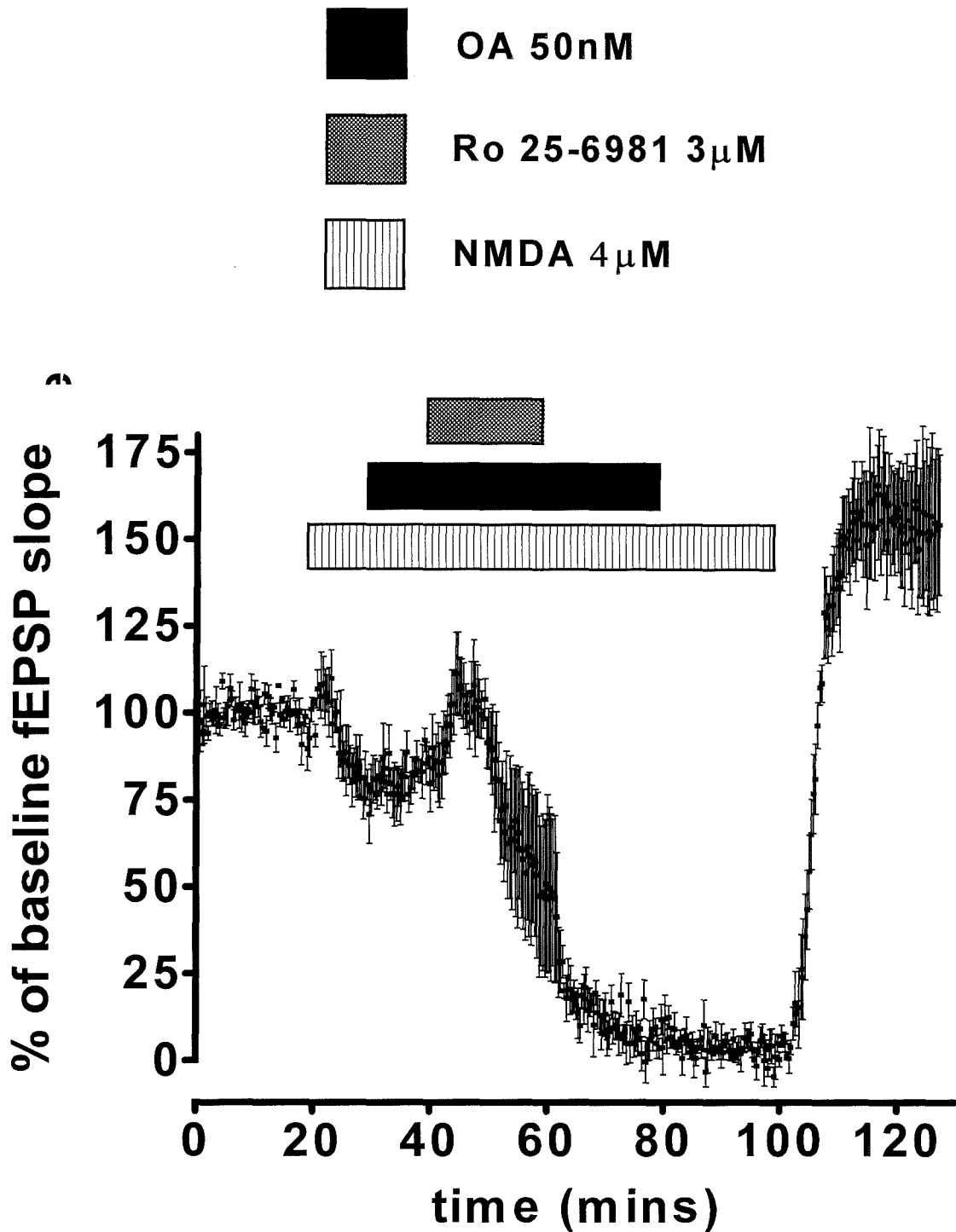
Figure 19. Action of okadaic acid upon the effect of the co-application of 4µM NMDA and Ro 25-6981 on fEPSP slope.

Plot showing that the continuous application of NMDA 4µM produced a small depression of fEPSP slope. The co-application of staurosporine before, during and after Ro 25-6981 was not able to prevent the enhancement of NMDA responses by Ro 25-6981 3µM which produced a significant depression of the fEPSP slope to $1.56 \pm 0.31\%$ ($p < 0.001$, $n=4$). Each point shows the mean±s.e.m. for $n=4$ experiments.
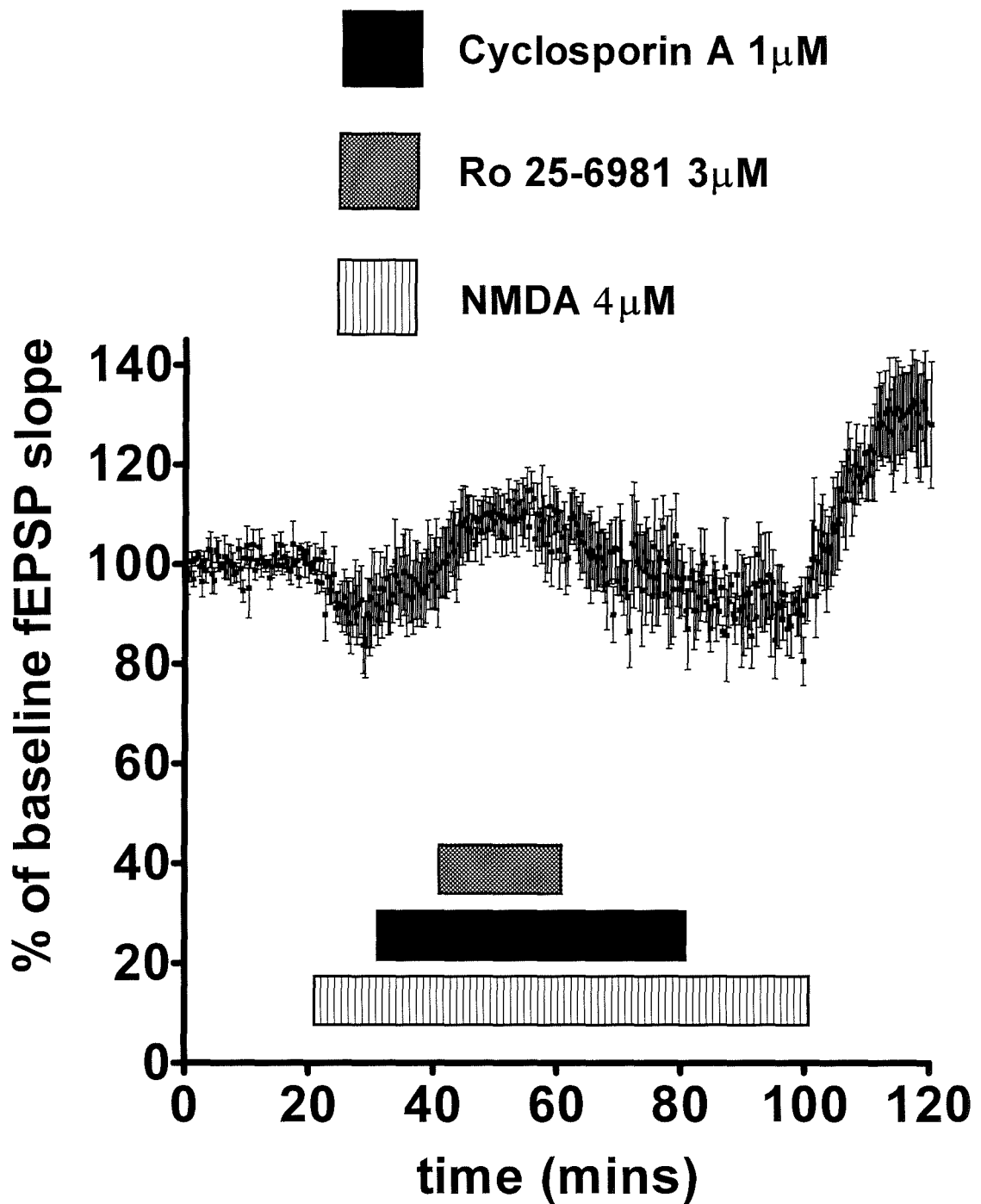
Figure 20. Prevention by cyclosporin A of the effect of the co-application of 4µM NMDA and Ro 25-6981 on fEPSP slope.

Plot showing the effect of 1µM cyclosporin A (CyA) upon the Ro 25-6981 potentiation of NMDA responses. 4µM NMDA produced a slight, non-significant depression of fEPSP slope; the application of CyA had no significant effect on this but prevented the enhancement of the NMDA response by 3µM Ro 25-6981. Each point shows the mean±s.e.m. for n=4 experiments
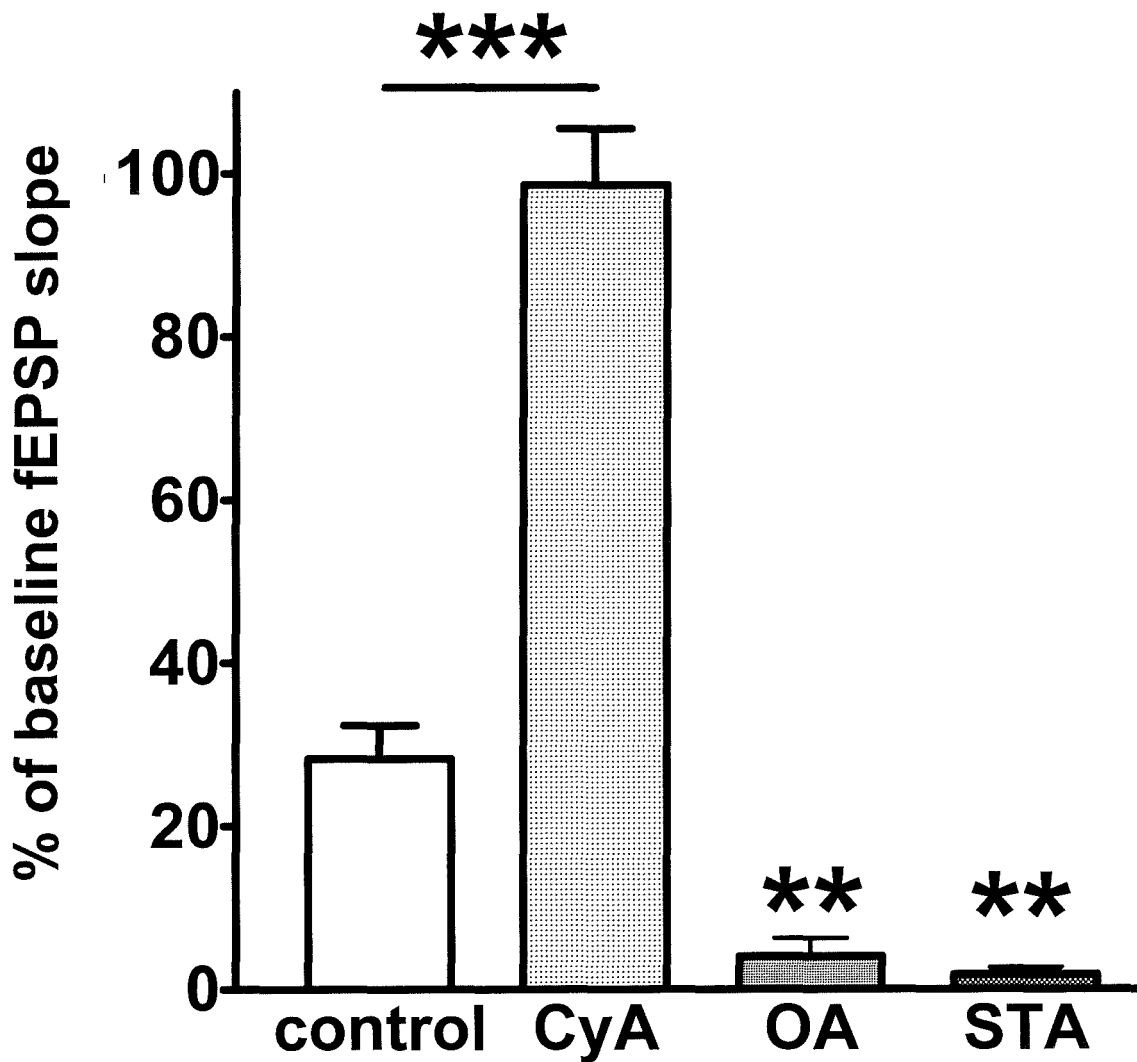
Figure 21. Column bar graph summary of phosphorylation modulators.

This histogram summarises the depression caused by the co-application of 4μM NMDA and 3μM Ro 25-6981 under control conditions (figure 11, n=4) and with the co-perfusion of 1μM cyclosporin A (CyA) (figure 20, n=4), 50nM okadaic acid (OA) (figure 19, n=4) and 100nM staurosporine (STA) (figure 18, n=3). Cyclosporin A prevented the depression of the fEPSP slope induced by NMDA in the control group (p<0.001). However, okadaic acid and staurosporine increased the magnitude of the depression (both p<0.01).

Protein synthesis

In view of the latency of onset, the long-lasting effect of Ro 25-6981, and the involvement of protein phosphorylation the question arose of whether any permanent change was required and induced of new protein synthesis. To investigate this possibility anisomycin, a widely used reversible translation inhibitor was used (Grollman, 1967). The application of NMDA 4μM produced a small, stable depression of fEPSP slope. The continuous application of anisomycin 30μM had no significant effect upon the fEPSP slope nor was it able to interfere with the subsequent NMDA and Ro 25-6981-mediated depression of fEPSP slope to $10.65\pm0.3628\%$ (p=0.0128, n=4)(figure 22).
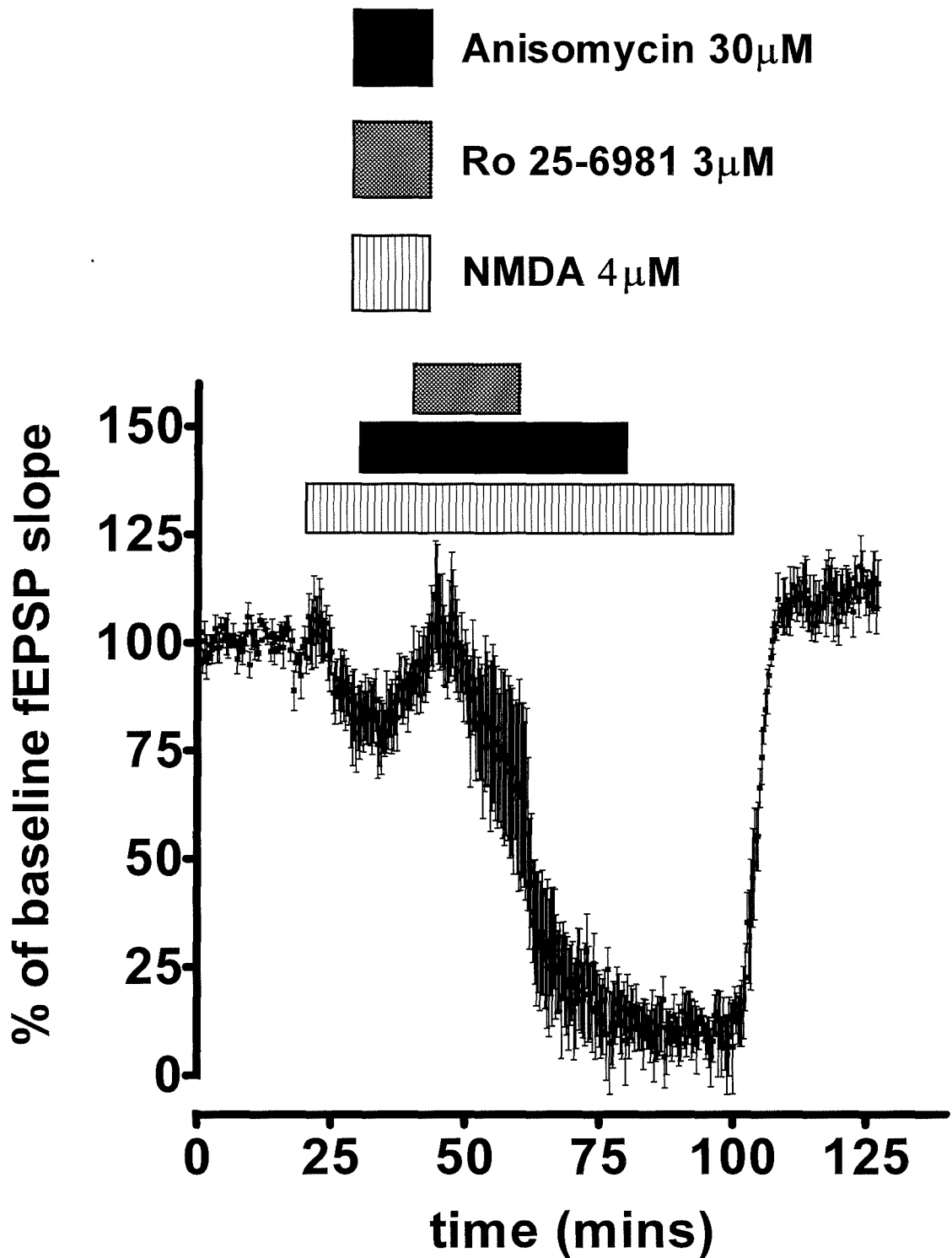
Figure 22. Effect of anisomycin on the Ro 25-6981/NMDA effect upon fEPSP slope.

Plot showing that 30μM anisomycin was unable to prevent the development of the Ro 25-6981-mediated enhancement of NMDA responses (p=0.0128, n=4). Each point shows the mean±s.e.m. for n=4 experiments.

Discussion

Applications of NMDA produced a concentration dependent depression of evoked fEPSP slope that was associated with an enhancement of paired-pulse ratios at all interstimulus intervals. The fEPSP under normal 0.05Hz stimulation is predominantly a reflection of the fast kinetics of the AMPA receptors which underlie this recording, with fast rise times (10-90% rise time of ~400μs) and fast decay time constant (~1.3ms). The NMDA component, in contrast, has slow kinetics with a slow rise time (~9ms) and a slow mean decay time constant (~52ms) (Gibb *et al.*, 1994). Schaffer collateral inputs onto CA1 neurones elicit fEPSPs where the NMDA receptors contribute only very a small component (~3%) (Arrigoni & Greene, 2004), principally as a result of the magnesium block of the channel at resting membrane potentials. At these negative membrane potentials, the magnesium ion blocks the ion channel in a voltage-dependent non-competitive manner. The depolarisation induced by a single fEPSP is not sufficient to overcome the threshold leading to NMDA receptor activation because the membrane potential does not reach a sufficient level to expel the magnesium ion. However, repetitive activation of the synapse such as that seen in HFS and LFS stimulation protocols to induce LTP and LTD produces a non-linear summating effect which overcomes this threshold and leads to the activation of the NMDA receptors. It is apparent from current-voltage plots for NMDA-induced

conductances that even at -90mV, although the NMDA current is very small it is never entirely prevented by magnesium block (Mayer *et al.*, 1984; Nowak *et al.*, 1984). The bath application of NMDA receptor agonists will lead to the activation of synaptic and extrasynaptic NMDA receptors which will result in large conductance through their 50pS channels over longer time periods than that seen with AMPA receptors, producing a regenerative feedback that leads to the unblocking of NMDA receptors and their activation. This conductance then leads to the depolarisation of the cells recorded from and the subsequent depolarisation block of excitatory synaptic transmission, manifested as a loss of the fEPSP slope. The washout of NMDA agonist allows the neurones to rebuild the ionic gradients that underlie the membrane potential and the fEPSP response recovers. The application of NMDA agonists which leads to this depolarisation will also lead to the activation of $GABA_A$ and $GABA_B$ receptors responsible for fast and slow components of feed-forward and feedback inhibitory transmission. Although this study did not address the contribution of these effects on the changes in the depression of fEPSP slope or on paired-pulse interactions previous investigations found that GABA, bath-applied to the hippocampal slice, elicited no inhibition of the fEPSP slope or altered the paired-pulse interactions in concentrations up to 1mM (unpublished observations). This suggested that the contribution of additional GABA release in this preparation is limited or already saturated by concentrations of GABA released in response to afferent stimulation.

Ro 25-6981 has been described as a selective antagonist at the NR2B subunit of the NMDA receptor. However, the previous work was carried out deliberately on immature tissue or cloned receptors overexpressing the NR2B subunit and not in adult tissues expressing mature NMDA receptors. When tested on the intrinsic neuronal circuitry of the adult hippocampal slice Ro 25-6981 unexpectedly potentiated NMDA receptor activation at all concentrations of NMDA tested and showed no inhibition of NMDA responses. The increased depression of fEPSP slope was generated through NR2A subunit-containing receptors, since the potentiated response could be blocked selectively by PEAQX. Furthermore, this enhancement appeared to have a presynaptic component, since the paired-pulse ratios were significantly enhanced during potentiated responses to NMDA at concentrations that previously showed no such change from baseline responses. It is widely recognised that a loss of paired-pulse inhibition and an increase of paired-pulse facilitation represents a presynaptic action of the test agents. Although it does not follow that postsynaptic effects cannot influence the appearance of PPD and PPF in field recordings (Seabrook *et al.*, 1999). Additionally, evidence from intracellular recordings of the enhancement of NMDA responses by Ro 25-6981 suggests that this phenomenon has a predominantly postsynaptic locus. The presynaptic element of responses to NMDA suggested in this study may be mediated via NR2A or NR2A/NR2B subunit-containing receptors. This is consistent with evidence for the existence of presynaptic NMDA receptors, which

may be responsible for changes in presynaptic release probability and the changes in paired-pulse interactions described here.

The most parsimonious explanation of the present results would be that the NR2B subunits exert a tonic inhibitory restraint on the activation of NR2A subunit-containing NMDA receptors. Blockade of the NR2B subunits then results in disinhibition of NR2A subunit-containing receptors, which become the primary effectors of the increased response to NMDA. This would explain both the potentiating effect of Ro 25-6981 and the blockade of the potentiated responses by PEAQX. It is not possible to conclude from the present data whether this inhibitory subunit interaction occurs between subunits within single heterotrimeric NR1/NR2A/NR2B receptors or between discrete heterodimeric receptors containing exclusively NR1/NR2A and NR1/NR2B subunits. Additionally, the interaction may be between receptor populations located at different sites interacting by way of intracellular or retrograde messengers.

These results are partially consistent with reports that ifenprodil and Ro 25-6981 caused small inward currents produced by NMDA 1μM to be potentiated, while larger currents produced by NMDA 100μM were markedly inhibited (Kew et al., 1996; Fischer et al., 1997). However, no inhibition against any concentrations of NMDA in the intact neuronal network of the adult hippocampal slice was observed. This is most simply

explained by the very different expression of subunits and their phosphorylation within cloned receptor constructs, immature tissues and with the mature tissue used in this study but merits further investigation.

It also appears from the data in this study that the interaction between NR2B and NR2A subunits was not prevented by inhibitors of protein kinases or the serine/threonine protein phosphatases types 1 and 2A (PP1 and PP2A) (Bialojan & Takai, 1988). A finding consistent with our later observation that Ro 25-6981 was unable to block LTP induction, which requires the activation of protein kinases (Bliss & Collingridge, 1993). Although the interaction between NR2B and NR2A subunit-containing receptors was not prevented by the broad-spectrum protein kinase inhibitor staurosporine, it is nevertheless likely that constitutively active kinases are essential to phosphorylate the unknown moiety to maintain the inhibitory restraint described. The subunit interaction does appear to involve Ca^{2+}/calmodulin-dependent phosphatase 2B (calcineurin), since it was prevented by cyclosporin A. Calcineurin has previously been shown to enhance both the presynaptic release of glutamate and postsynaptic responsiveness to it (Yakel, 1997) partly by altering channel gating properties and promoting desensitisation in cultured hippocampal neurons (Lieberman & Mody, 1994; Nichols *et al.*, 1994; Sihra *et al.*, 1995; Tong *et al.*, 1995; Victor *et al.*, 1995). Furthermore, the calcineurin inhibitors cyclosporin A and FK506 are able to prevent the induction of LTD

(Mulkey *et al.*, 1994; Hodgkiss & Kelly, 1995). Interestingly, it has been reported that calmodulin-dependent inhibition of NMDA receptors will reduce the amplitude and time course of excitatory synaptic currents in 12-day-old rat hippocampal NMDA receptors (Rycroft & Gibb, 2002). NR2 subunits, unlike NR1 subunits, are known to be highly phosphorylated under basal conditions (Hall & Soderling, 1997), therefore the phosphorylation step described could be acting directly upon the NR2A and/or the NR2B subunit to produce the enhancement of responses to NMDA. It is however possible that the phosphorylation site is elsewhere on the NMDA receptor complex, which has been reported from proteomic analysis to associate with at least 77 proteins (Husi *et al.*, 2000) e.g. NR1, PSD-95, SAP 102, or at a non-NMDA site.

The present work also reveals that the potentiating effect of Ro 25-6981 is not dependent upon new protein synthesis, although it is expected that relevant proteins are constitutively present. The reason for the slow onset and apparently irreversible nature of the enhancement effect remains unclear. It may be due for example to the Ro 25-6981 binding irreversibly, to a persistent modification of the NR2B, NR2A or NR1 subunit that is then independent of Ro 25-6981 binding, to an irreversible uncoupling of the NR2B subunit from NR2A or to a change in NMDA receptor functional expression. Radiolabelled forms of Ro 25-6981 are unavailable at this time to investigate the possibility of it binding irreversibly to the NR2B subunit.

Chapter 4. The NMDA subunit specificity of LTP

Introduction

It is widely accepted that a persistent change in synaptic strength is the cellular basis of learning and memory (Alkon & Nelson, 1990; Kandel, 1997). Both long-term potentiation (LTP) and long-term depression (LTD) have now been shown to fulfil three out of the four criteria that are required to verify that they are both 'necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed'. Alterations in synaptic strength are detectable after learning, interfering with the induction and expression mechanisms results in concurrent interference in learning, and altering the pattern of synaptic weights after learning affects the ability of animals to remember a previously learned experience.   The, unfulfilled, fourth criterion is mimicry, where an implanted pattern of synaptic weights generates in the animal the behaviour of a specific recalled memory (Moser et al., 1998).

LTP, first reported in the dentate gyrus (Lomo, 1966; Bliss & Lomo, 1973), has since been demonstrated in all three excitatory pathways in the hippocampus (Schwartzkroin & Wester, 1975; Alger & Teyler, 1976) and elsewhere in the CNS (Kuba & Kumamoto, 1990). LTP is characterised by input specificity, co-operativity and associativity, leading to the proposal that LTP is a Hebbian process (Hebb, 1946), where the co-incident activity

of pre- and postsynaptic neurones is required to increase persistently synaptic strength. LTP was first shown to be involved in the cellular basis of learning and memory with experiments to block LTP in vivo coincided with deficits in hippocampal-dependent place navigation (Morris et al., 1986). LTP induction in the CA1 area is NMDA receptor dependent: it is triggered by NMDA receptor activation and blocked by NMDA receptor antagonism (Collingridge et al., 1983). AMPA receptor-mediated depolarisation of the postsynaptic cell during LTP induction, by high frequency stimulation (HFS), releases the Mg^{2+} block of NMDA receptors allowing Ca^{2+} to flow into the cell (Lynch et al., 1983; Mayer et al., 1984; Nowak et al., 1984; Malinow & Miller, 1986; Wigstrom et al., 1986). A large rise in Ca^{2+} in the postsynaptic spine sets off a cascade of events that results in LTP (Bliss & Collingridge, 1993). The induction of LTP is widely considered to be postsynaptic and the expression both pre- and postsynaptic (Kullmann & Nicoll, 1992; Manabe et al., 1993). The maintenance of LTP is characterised by an early phase which is dependent on post-translational modifications and lasts for a few hours, followed by later phases which are dependent on de novo gene expression and protein synthesis that lasts for many hours (Krug et al., 1984; Otani et al., 1989; Nguyen et al., 1994). LTP can persist for varying amounts of time dependent upon the induction protocol and the experimental model. It has recently been demonstrated that stable LTP can last for up to a year (Abraham et al., 2002).

There is much evidence that supports a role for the NR2B subunit in synaptic plasticity and specifically in LTP. Increases in the expression of the NR2B and not of the NR2A, NR2C or NR2D subunit after LTP induction and the prevention of LTP induction in an NR2B knock-down model support a role for the NR2B subunit in the early phase of LTP (Thomas *et al.*, 1994; Thomas *et al.*, 1996). Furthermore, enhanced tyrosine phosphorylation of the NR2B subunit has been demonstrated after the induction of LTP (Rosenblum *et al.*, 1996; Manabe *et al.*, 2000; Nakazawa *et al.*, 2001). LTP and LTD are bi-directional mechanisms of synaptic plasticity, induced by different patterns of afferent stimulation (Dudek & Bear, 1993). A recent investigation was made into whether these different patterns reflect the activation of specific subunits to produce either LTD or LTP. It was found that antagonists with a higher affinity for the NR2A/B subunits over the NR2C/D subunits produced greater inhibition of LTP than LTD induction (Hrabetova et al., 2000). There are also reports that the NR2B subunit is associated with age-related changes (Magnusson, 2000), the decrease in NR2B expression with increasing age leading to the blockage of LTP induction and impairments in spatial learning performance (Clayton *et al.*, 2002). Potentiating the NMDA receptors using spermine, an endogenous polyamine, could reverse this. Taking advantage of the new NR2B and NR2A subunit-selective antagonists, Ro 25-6981 and PEAQX, this investigation sought to determine the subunit specificity of NMDA-dependent LTP induction.

Results

LTP was induced by high frequency stimulation (3 identical trains of 1 second stimulation bursts at 100Hz 10 minutes apart; HFS). This produced an enhancement of fEPSP slope to $197.9 \pm 16.4\%$ ($p < 0.001$, n=6) that persisted steadily for 140 minutes from the start of the induction protocol (figure 23 and 24).

To demonstrate that the LTP induced by HFS was NMDA receptor-dependent $50\mu M$ 2-AP5 was applied during the induction protocol and prevented LTP induction (figure 25). PEAQX $0.1\mu M$ was then used, which had previously been seen in chapter 3 to block NMDA responses in an analogous manner to 2-AP5. The perfusion of this NR2A antagonist also prevented the induction of LTP (figure 26). Finally, the NR2B antagonist Ro 25-6981 was used but it was unable to prevent the induction of LTP to $170.4 \pm 15.7\%$ ($p < 0.01$, n=6) of baseline fEPSP slope, which was less than control HFS induced LTP but not significantly so (figure 27).

This result clearly demonstrates that the NR2A subunit, and not the NR2B subunit, is critically required for the induction of NMDA-dependent LTP (figure 28).
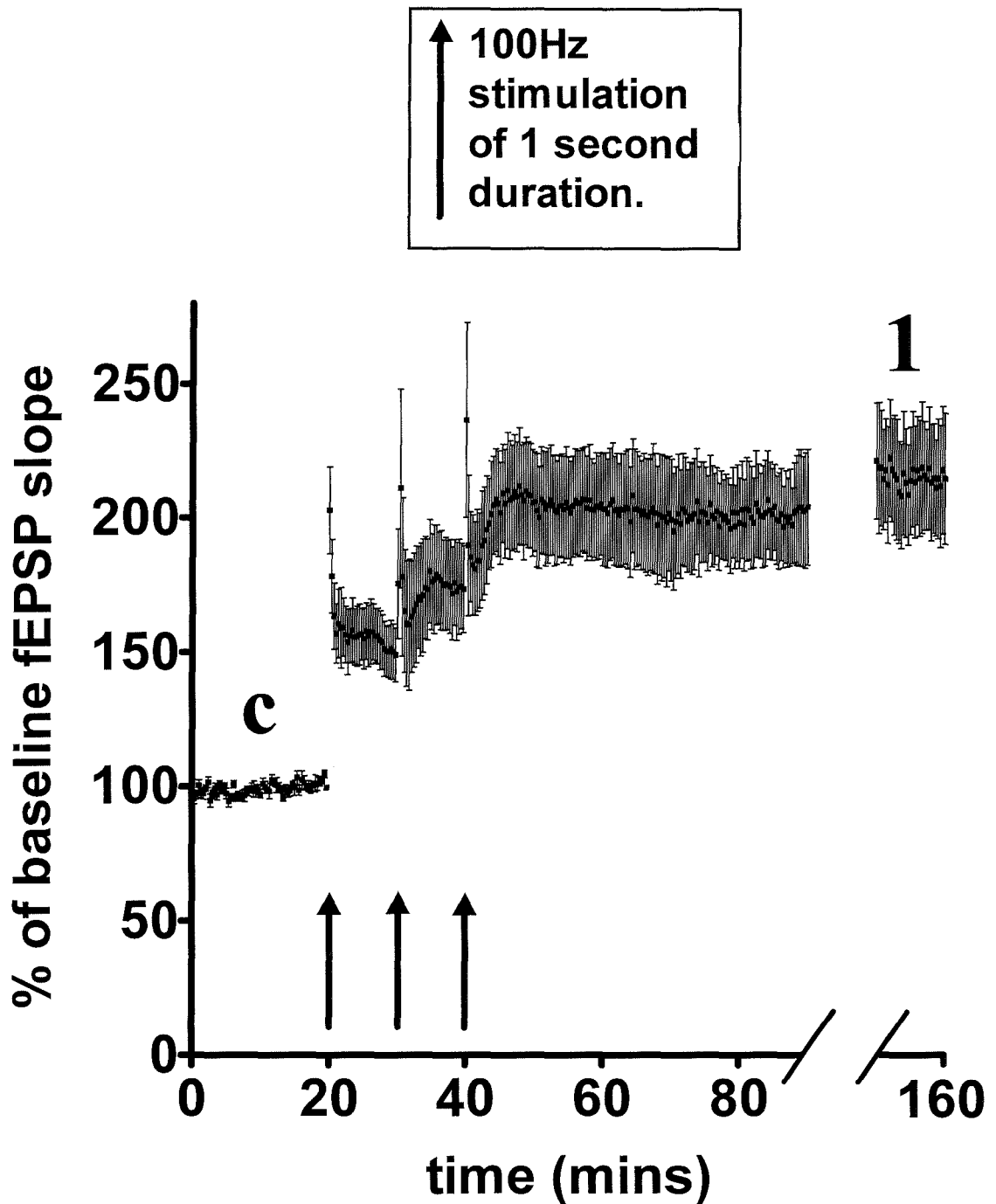
Figure 23. Effect of high frequency stimulation on fEPSP slope.

Plot showing LTP induced by three trains of stimuli using 100 pulses at 100 Hz, ten minutes apart (HFS) at 20, 30 and 40 minute time points, producing a persistent enhancement of the fEPSP slope to $197.9\pm16.4\%$ of the control size ($p<0.001$, $n=6$). Each point shows the mean±s.e.m. for $n=6$ experiments.

c

1

1mV

5ms
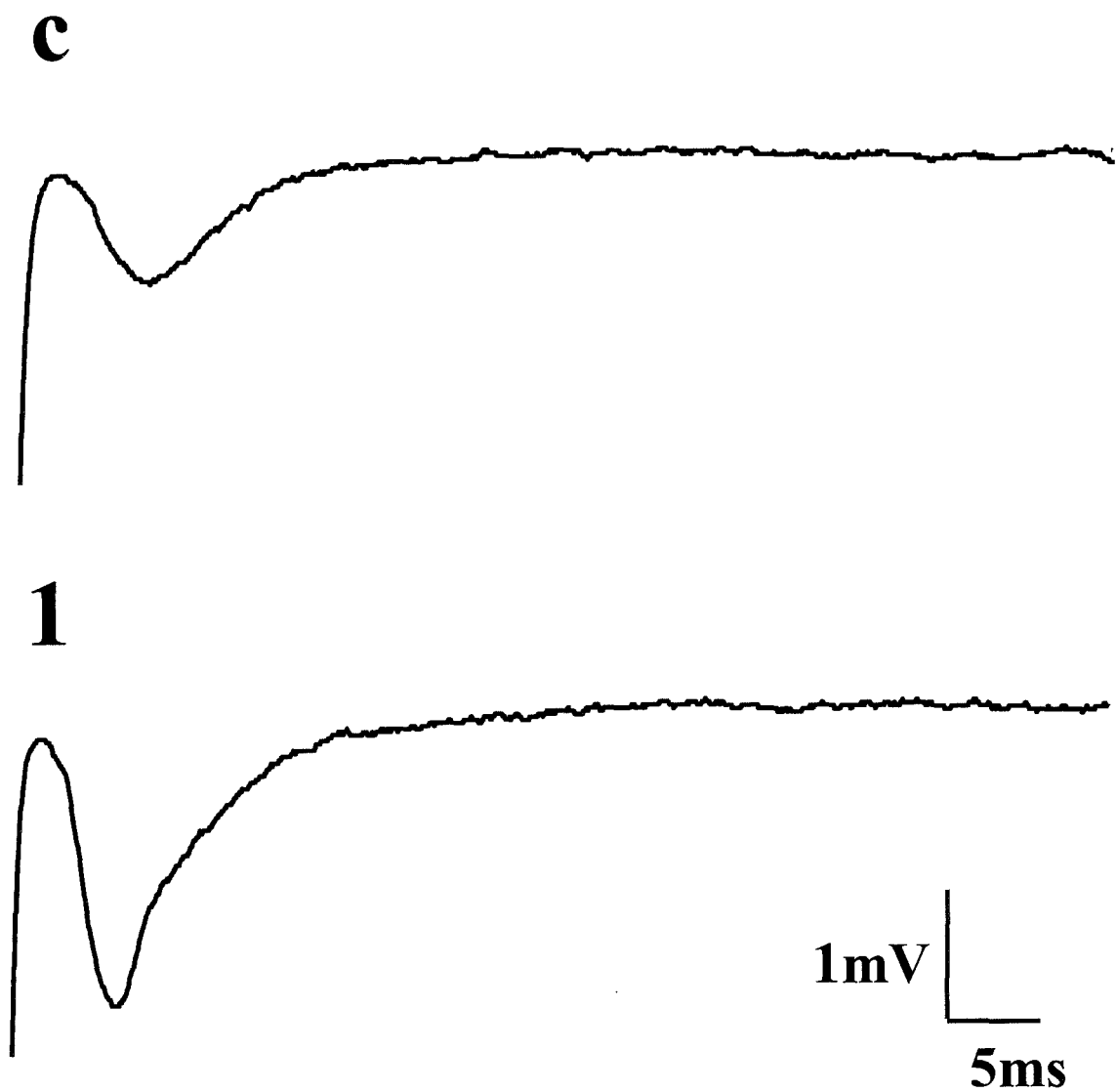
Figure 24. Representative fEPSPs demonstrating LTP.

Illustrating baseline responses (c) and potentiated responses (1) as a result of LTP induction; points correspond to figure 23.
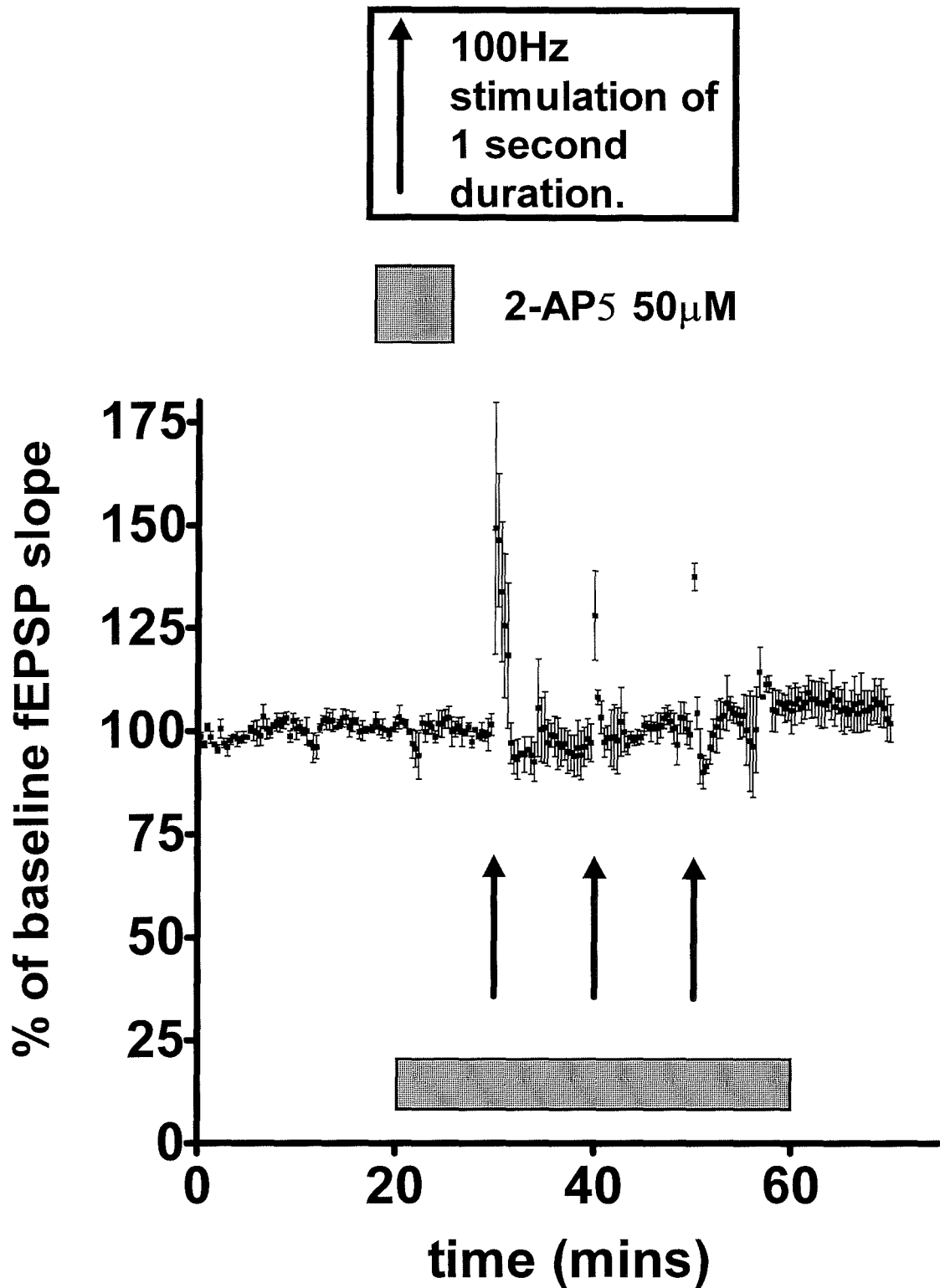
Figure 25. Blockade of NMDA-dependent LTP induction by 2-AP5.

Plot showing the perfusion of 2-AP5 50μM during the HFS induction protocol for LTP which prevented the induction of LTP, n=3. Each point shows the mean±s.e.m. for n=3 experiments.
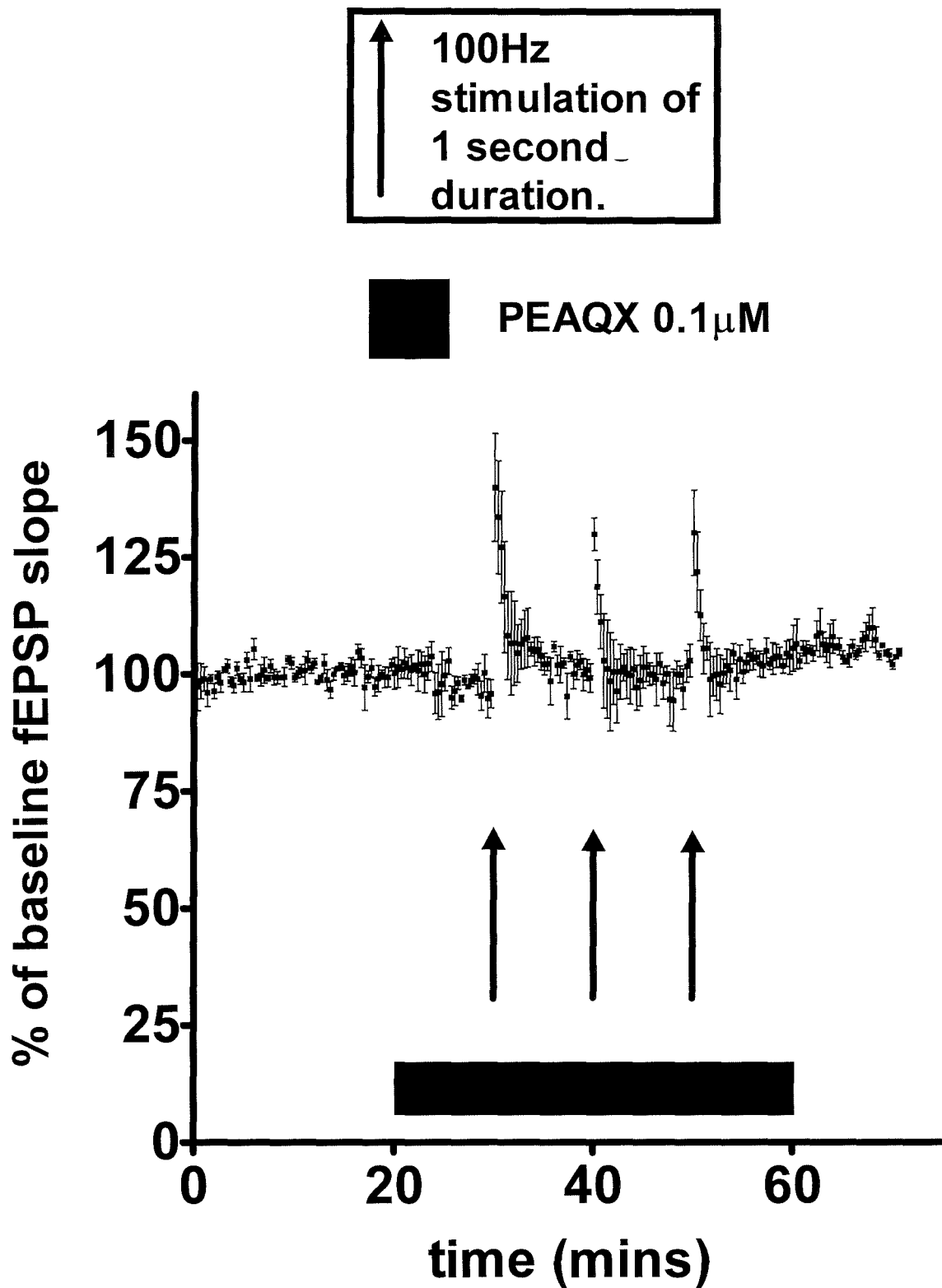
Figure 26. Blockade of NMDA-dependent LTP induction by PEAQX.

Plot showing the effect of PEAQX 0.1μM upon the induction of LTP. The perfusion of PEAQX prevented the induction of LTP. Each point shows the mean±s.e.m. for n=4 experiments.
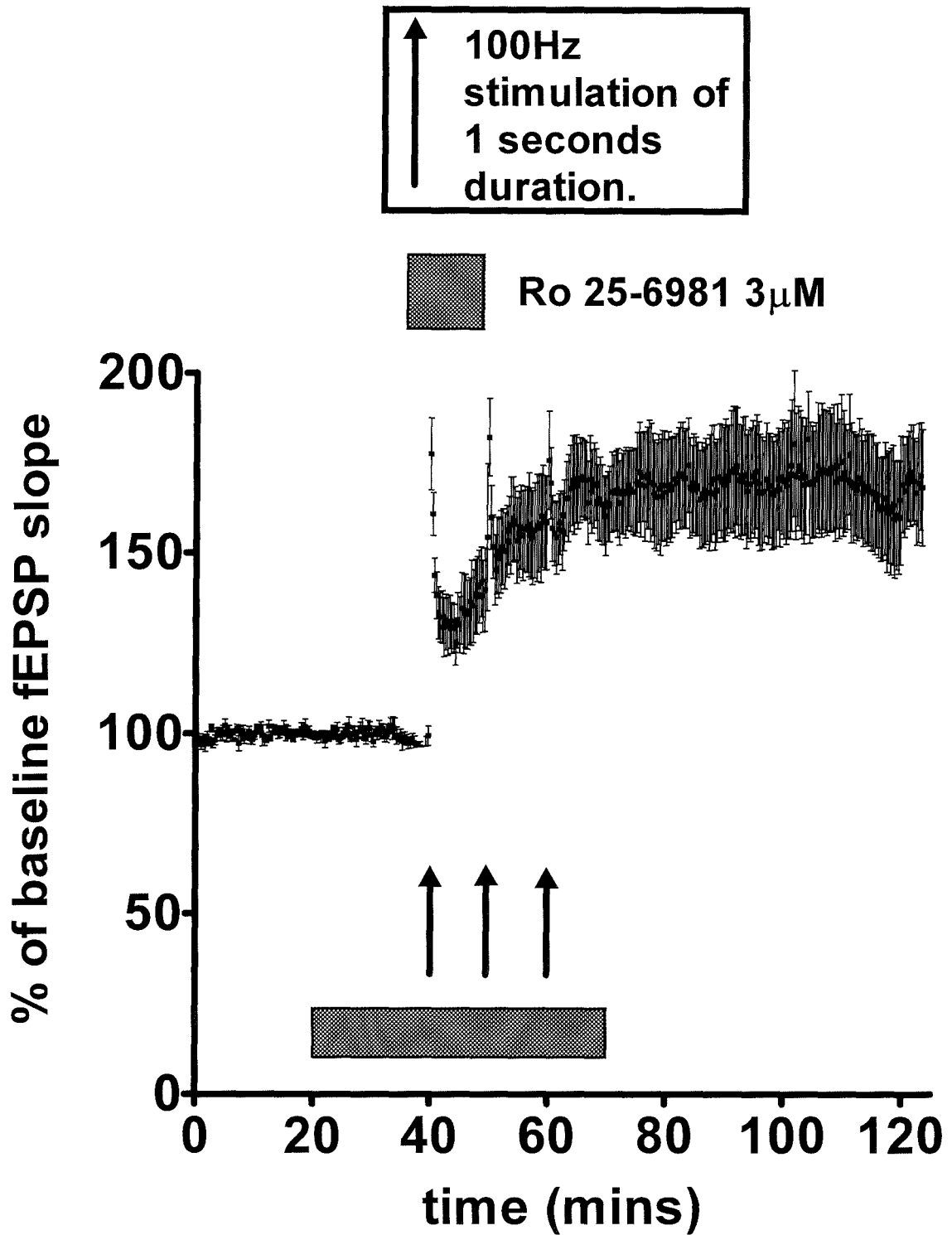
Figure 27. Effect of Ro 25-6981 on NMDA-dependent LTP induction.

Plot showing that the application of 3μM Ro 25-6981 during the HFS-LTP induction protocol was unable to prevent the induction of a significant LTP (170.4±15.7% (p<0.01, n=6) of baseline fEPSP slope. Each point shows the mean±s.e.m. for n=6 experiments.
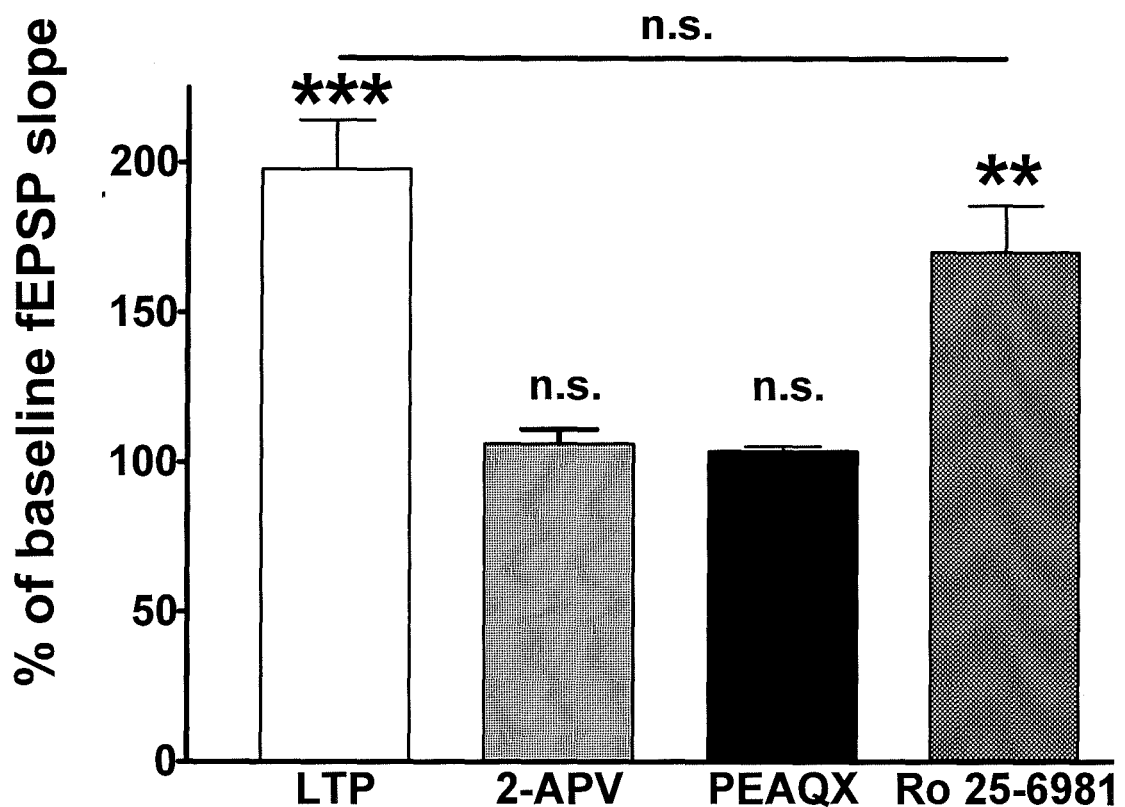
Figure 28. Role of NR2A and NR2B subunits in long-term potentiation.

Histogram showing LTP induction by high frequency stimulation (HFS, three stimulation trains of 100 pulses at 100 Hz, ten minutes apart). Bath application of either 50μM 2-AP5 or 0.1μM PEAQX prevented the induction of LTP, whereas 3μM Ro 25-6981 did not, reducing LTP to 170.4±15.7% ($p<0.01$, $n=6$) of baseline fEPSP slope. This was not significantly less than LTP in the absence of drugs ($p=0.2526$, $n=6$), but was significantly greater than the original fEPSP baseline slope (**$p<0.01$, $n=6$).

Discussion

The present study has shown that NMDA receptor-dependent LTP induction is critically dependent upon the NR2A subunit-containing receptor activation and not the NR2B subunit in the Schaffer collateral-CA1 synapse. This result is consistent with a recent report that LTP is associated with the rapid surface expression of postsynaptic NR2A subunit-containing NMDA receptors (Grosshans et al., 2002). Additionally, whilst Ro 25-6981 does not block the induction of LTP, the LTP induced was marginally reduced consistent with a recent report (Kohr et al., 2003).

Several reports have now been published investigating the subunit selectivity of LTP and LTD (Sjostrom *et al.*, 2003; Liu *et al.*, 2004; Massey *et al.*, 2004; Mallon *et al.*, 2005). Liu and colleagues reported that at hippocampal CA1 synapses of 3-4 week old rats LTP was blocked by PEAQX. Furthermore, using an LTP protocol pairing Schaffer collateral stimulation with postsynaptic depolarisation under whole-cell recording conditions LTP could be blocked by PEAQX and LTD was induced. The induction of LTD has been reported to be blocked by NR2B antagonists, including Ro 25-6981 and ifenprodil (Sjostrom *et al.*, 2003; Liu *et al.*, 2004; Massey *et al.*, 2004). However the investigation of LTD in this study was prevented because of the difficulty in induction of LTD in adult animals, in agreement with reports by other laboratories (Kemp & Bashir,

2001). Massey and colleagues reported that the induction of LTP and depotentiation in the perirhinal cortex of 7-12 week old rat was blocked by PEAQX. They also found that LTD was difficult to induce but that blockade of glutamate uptake, which led to the activation of postsynaptic, extrasynaptic NR1/NR2B receptors, overcame this obstacle. This allowed the induction of LTD, which could also be prevented by Ro 25-6981, the NR2B antagonist. However, the distribution of NMDA receptor subunits varies widely developmentally and between brain regions and synaptic pathways (Otmakhova *et al.*, 2002; Arrigoni & Greene, 2004). Therefore, different local conditions can regulate the sensitivity and characteristics of synaptic plasticity to different NR2 subunits. It has also been reported that LTP is sensitive to NR2B antagonists in the young Schaffer collateral-CA1 synapse (R. Malenka, personal communication), visual cortex (Yoshimura *et al.*, 2003), amygdala (T. Sigurdsson, personal communication) and the hippocampal commissural-CA3 synapse (Ito *et al.*, 2000). The NR2B subunit, unlike the NR2A subunit-containing receptors or AMPA receptors, seem to be of particular importance in regulating learning and memory and have recently been reported to be differentially distributed in the left-right asymmetry of the brain, which has important implications for higher order functions (Kawakami *et al.*, 2003; Shinohara *et al.*, 2003).

Chapter 5. The NR2A & NR2B subunit-selectivity of homoquinolinic acid

Introduction

The tryptophan metabolite, quinolinic acid (QA), has been recognized for many years to be a weak, endogenous NMDA receptor agonist (Stone & Perkins, 1981; Stone, 1984; 1993). Shortly after its discovery, a study of structure-activity relationships among a series of related compounds revealed that a close analogue, homoquinolinic acid (HQA), had a potent excitatory activity. Indeed, HQA appeared to be between 10 and 100-fold more potent at activating glutamate receptors (Stone, 1984). Autoradiographical and electrophysiological studies have recently been undertaken on native and recombinant receptors to evaluate the subunit-specificity of HQA. It has been reported from electrophysiological studies that HQA is a potent NMDA receptor agonist that has a higher affinity for Xenopus oocytes expressing NR2A and NR2B subunit-containing receptors, compared to NR2C and NR2D subunit-containing receptors (Buller & Monaghan, 1997). A recent study also found that HQA had an approximately 2-fold higher affinity for NR1a/NR2B and NR1a/NR2A/NR2B receptors compared to NR1a/NR2A receptors (EC_{50} 13.8, 9.04 and 25.2μM, respectively). Yet showed greater intrinsic activity for NR1a/NR2A and NR1a/NR2A/NR2B compared to NR1a/NR2B

transfected receptors (compared to glutamate responses (100%), 148±15, 125±5.6 and 93.3±4%, respectively) (Grimwood et al., 2002). In autoradiographical studies, it has been observed that ^{3}[H]-homoquinolinic acid selectively radiolabels a subpopulation of NMDA receptors in rat brain that is consistent with the distribution of the NR2B subunit and clearly dissimilar to NR2C and NR2D subunit distribution (Buller et al., 1994; De Carvalho et al., 1996; Brown et al., 1998), although there are contradictory reports where HQA was not as highly specific for NR2B-containing receptors (Grimwood et al., 2002). In radioligand binding studies ^{3}[H]-HQA also bound to a novel binding site from which it was not displaced by NMDA or glutamate but was displaced by 2-carboxy-3-carboxymethylquinoline (CCMQ), a HQA derivative (Brown et al., 1998; Grimwood et al., 2002). The identity and function of this proposed site are unknown.

This investigation was designed to establish whether there was any evidence for this unidentified site in the generation of evoked synaptic potentials in the hippocampal slice. Additionally, by using an NR2A/NR2B subunit-containing NMDA receptor agonist (HQA) to investigate subunit-pharmacology in combination with NR2A and NR2B subunit-selective antagonists (PEAQX and Ro 25-6981) this study can consider the role of NR2D subunit-containing receptors on the depression of fEPSP slope and paired-pulse interactions.

Results

HQA and QA

In order to determine the most suitable concentrations of agonists to use concentration-response curves were constructed from which it was clear that HQA and QA gave useful depressions of approximately 50% of the baseline fEPSP slope at 2.5µM and 150µM respectively (figures 29-31, 34-36). These concentrations were selected for further experiments since both increases and decreases of the depression of fEPSP slope could easily be observed. The depression elicited by the application of HQA 2.5µM and QA 150µM both coincided with a significant decrease of paired-pulse inhibition and increase of paired-pulse facilitation at all interstimulus intervals (HQA: 10ms $p<0.01$, 20ms $p<0.001$, 50ms $p<0.001$, n=10; QA: 10ms $p<0.05$; 20ms $p<0.001$; 50ms $p<0.001$; n=7) (figures 32, 33, 37, 38).
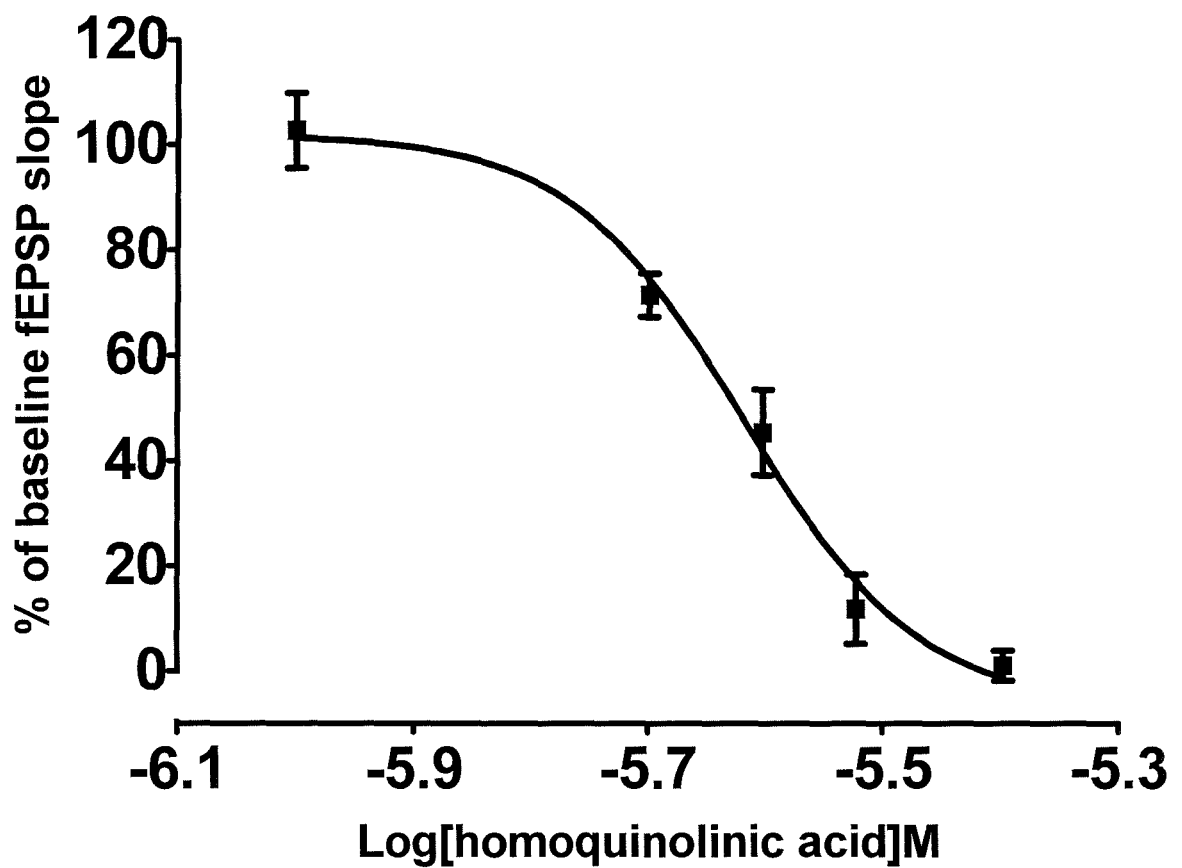
Figure 29. The concentration-response curve of HQA.

A plot showing concentration-response curve for homoquinolinic acid-produced depression of the fEPSP slope at 1, 2, 2.5, 3 and 4µM (n = 3, 5, 8, 5 and 4, respectively). The concentration inducing a 50% reduction is 2.4 µM. Each point shows the mean±s.e.m.
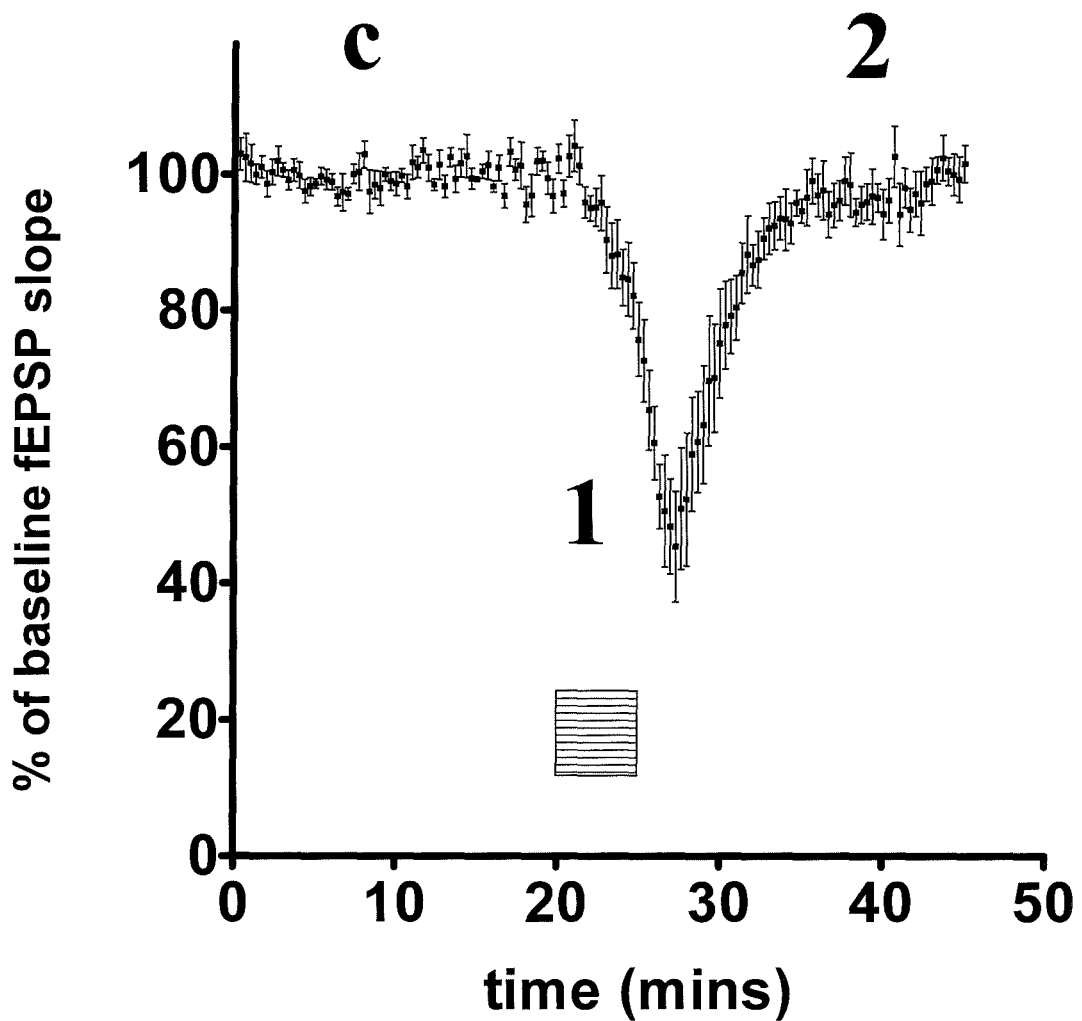
Figure 30. Effect of HQA 2.5μM on fEPSP slope.

A plot of fEPSP slope showing that homoquinolinic acid 2.5μM caused a depression of normalised fEPSP slope to approximately 50% (here 45.4% ± 8.12, $p < 0.01$, n = 8) relative to control responses. Each point shows the mean±s.e.m. for n=8 experiments.
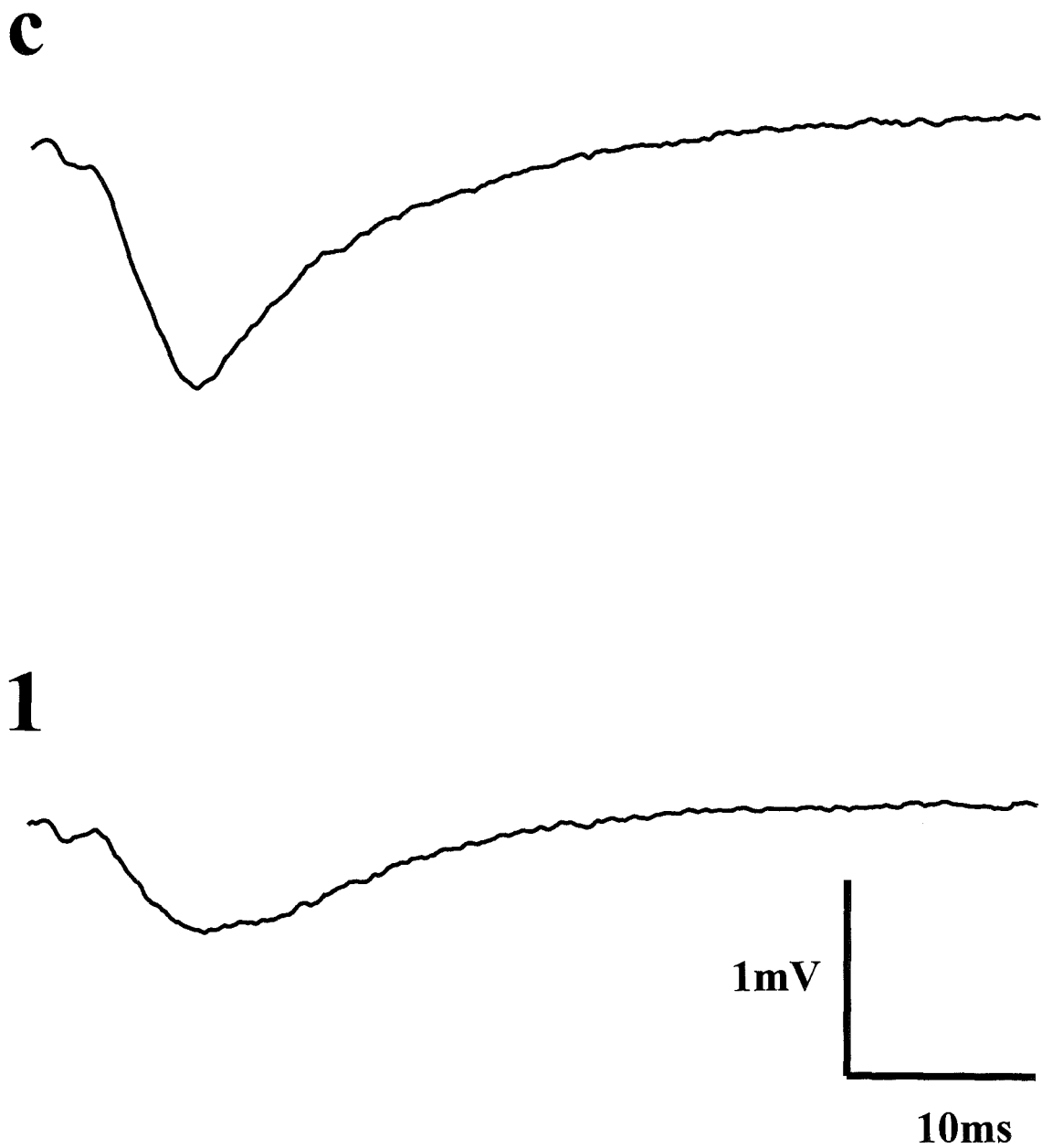
Figure 31. Representative fEPSPs showing effect of 2.5μM HQA.

fEPSPs corresponding to baseline (c) responses and during the depression elicited by HQA 2.5μM (1) in the time course of figure 30.
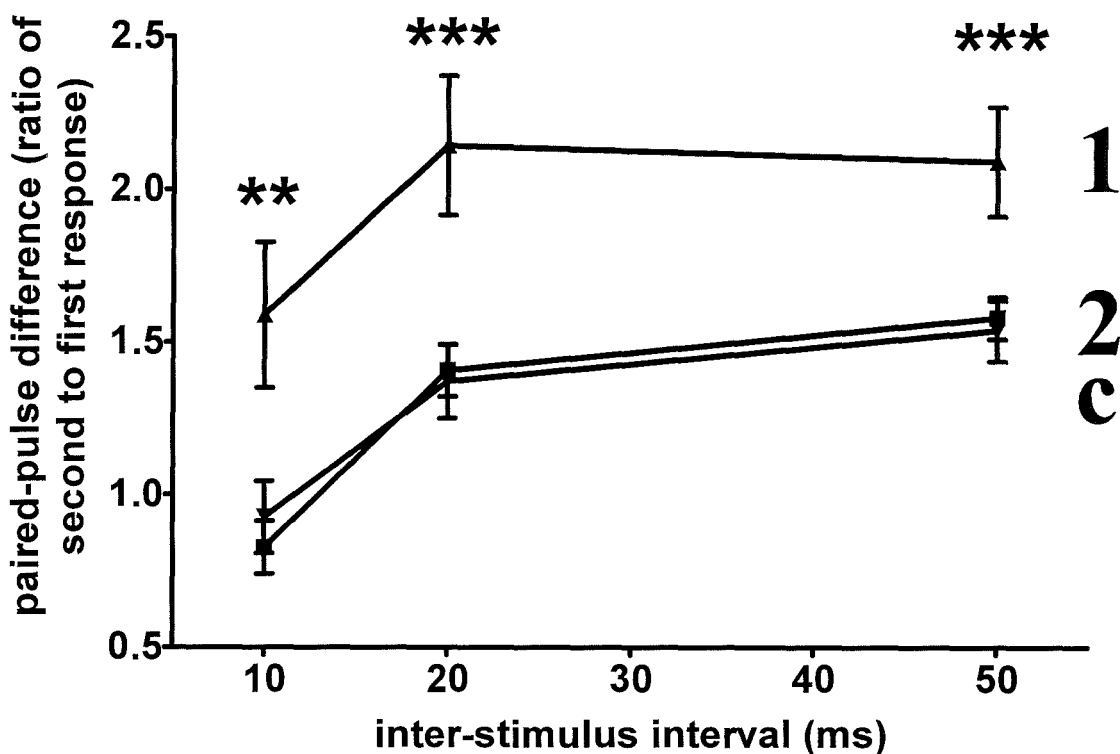
Figure 32. Effect of 2.5µM HQA on paired-pulse interactions.

Shows the paired-pulse interactions of fEPSP slope at interstimulus intervals of 10ms, 20ms and 50ms during the depression elicited by HQA 2.5µM. The results are presented as the interactions during baseline control responses (c), the effect of homoquinolinic acid 2.5µM (1) and the washout period (2) (10ms p<0.01, 20ms p<0.001, 50ms p<0.001, compared to baseline control responses, n=10), points correspond to the time course of figure 30. The data show a loss of paired-pulse inhibition at 10ms, and increased paired-pulse facilitation at 20 and 50ms caused by homoquinolinic acid. *P < 0.05, **P < 0.01, ***P < 0.001. Each point shows the mean±s.e.m. for n=10 experiments

control   **HQA 2.5µM**
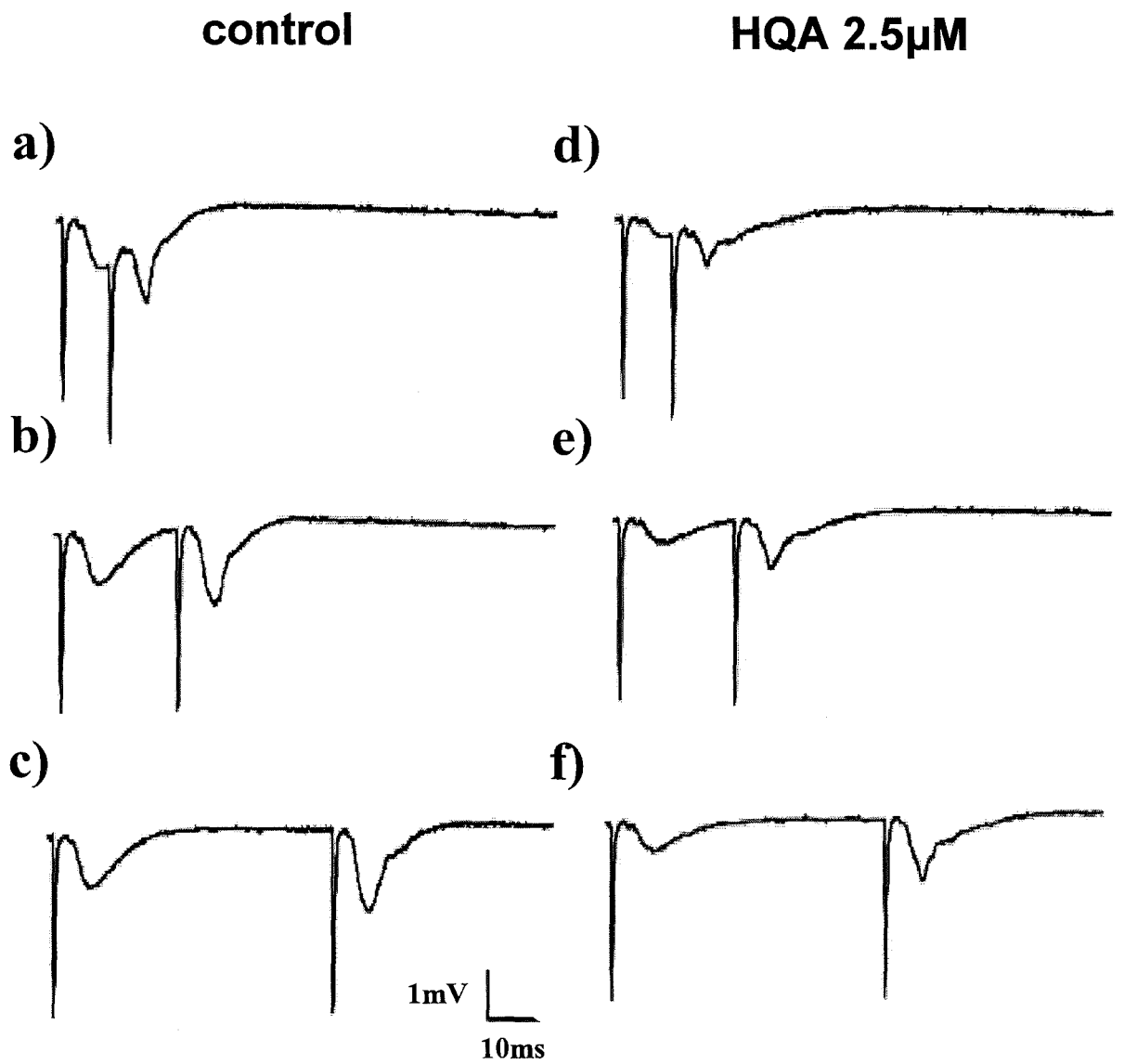
Figure 33. Example records of fEPSPs during paired-pulse interactions.

During baseline recording: (a) 10ms (b) 20ms and (c) 50ms and during the depression elicited by HQA 2.5µM: (d) 10ms (e) 20ms and (f) 50ms.
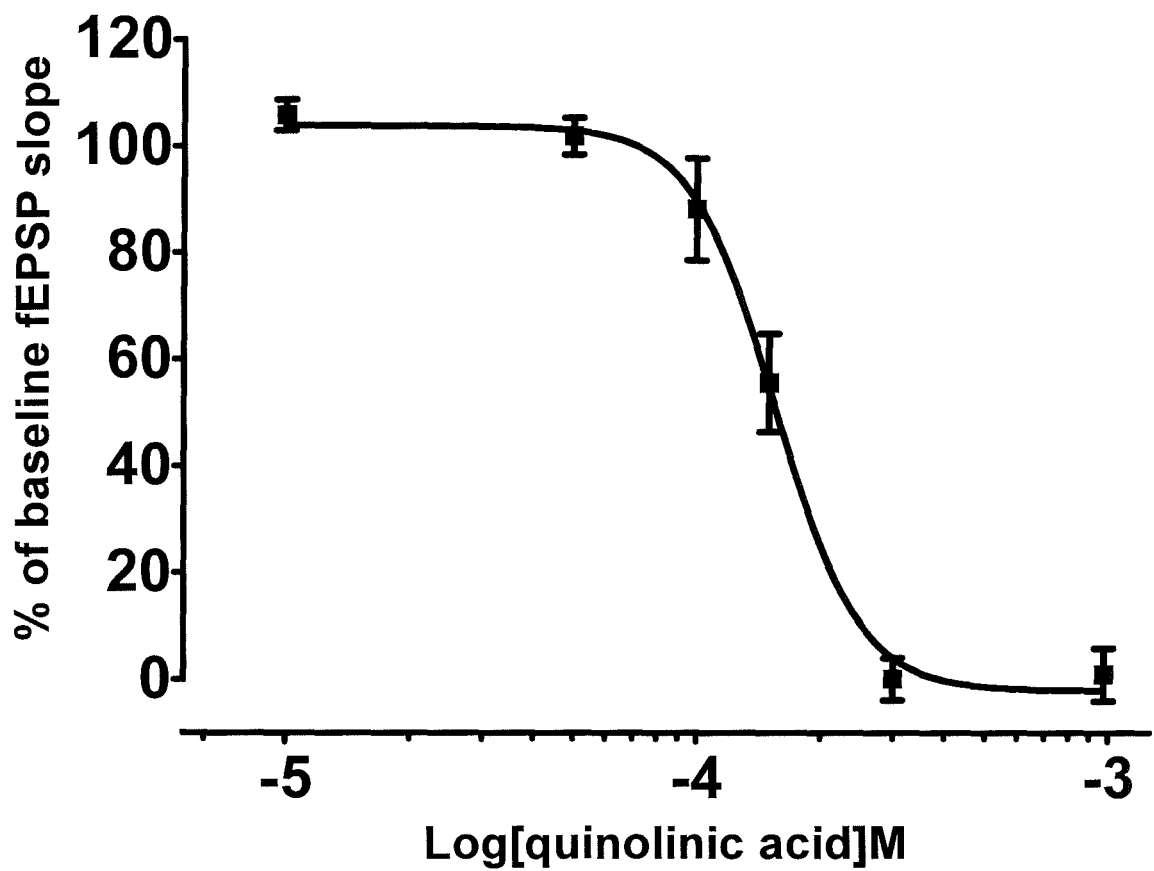
Figure 34. The concentration-response curve of QA.

A plot showing the concentration-response curve of the fEPSP slope to 5-minute applications of QA: 10, 50, 100, 150, 300 and 1000μM (n=4, 3, 6, 8, 3, and 4 respectively). The concentration inducing a 50% reduction is 157.2μM. Each point shows the mean±s.e.m.
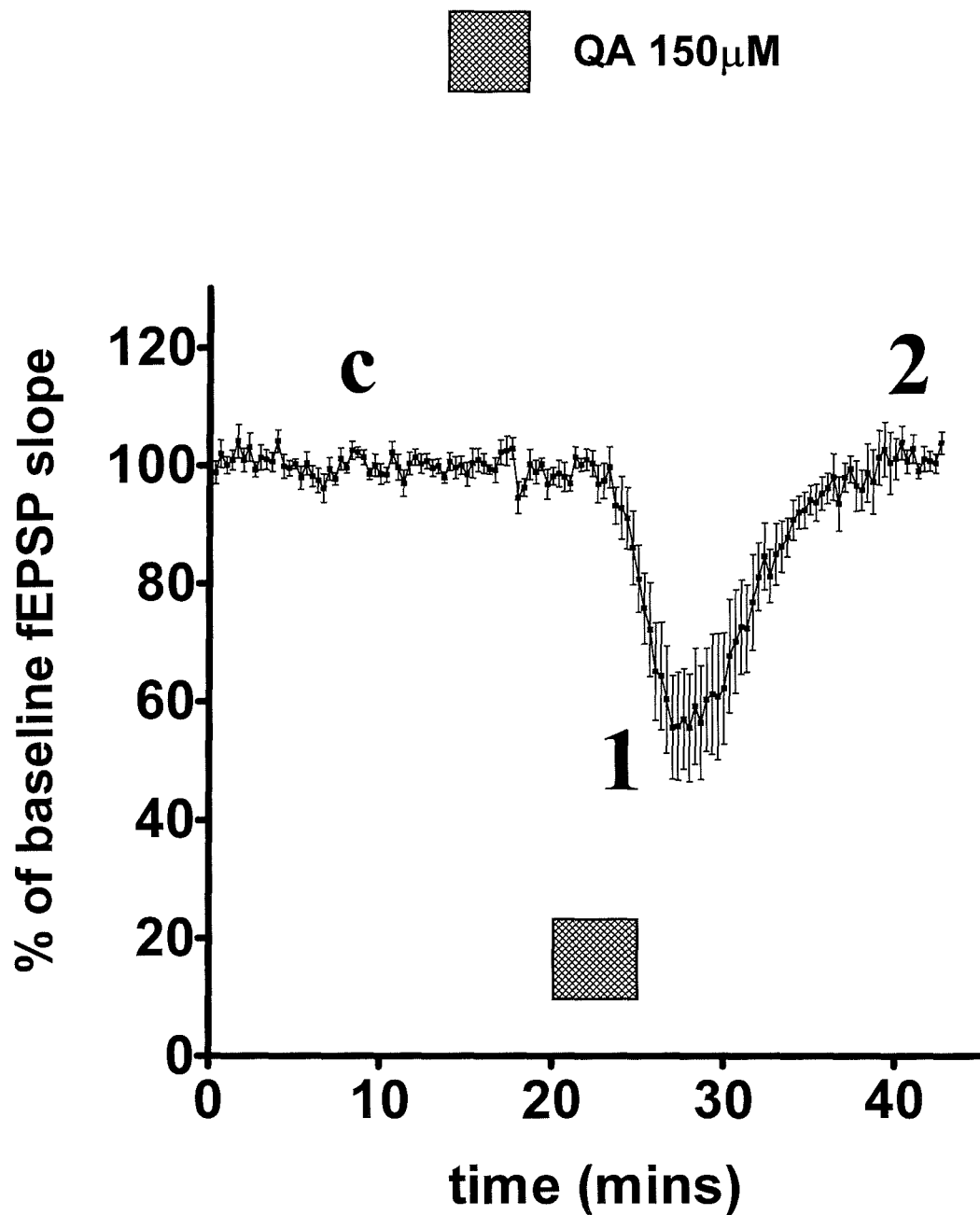
Figure 35. Effect of 150µM QA on fEPSP slope.

A plot of fEPSP slope showing that quinolinic acid 150µM caused a depression of normalised fEPSP slope to 55.7%±8.12% (p=0.0017; n=8) relative to control responses. Each point shows the mean±s.e.m. for n=8 experiments.
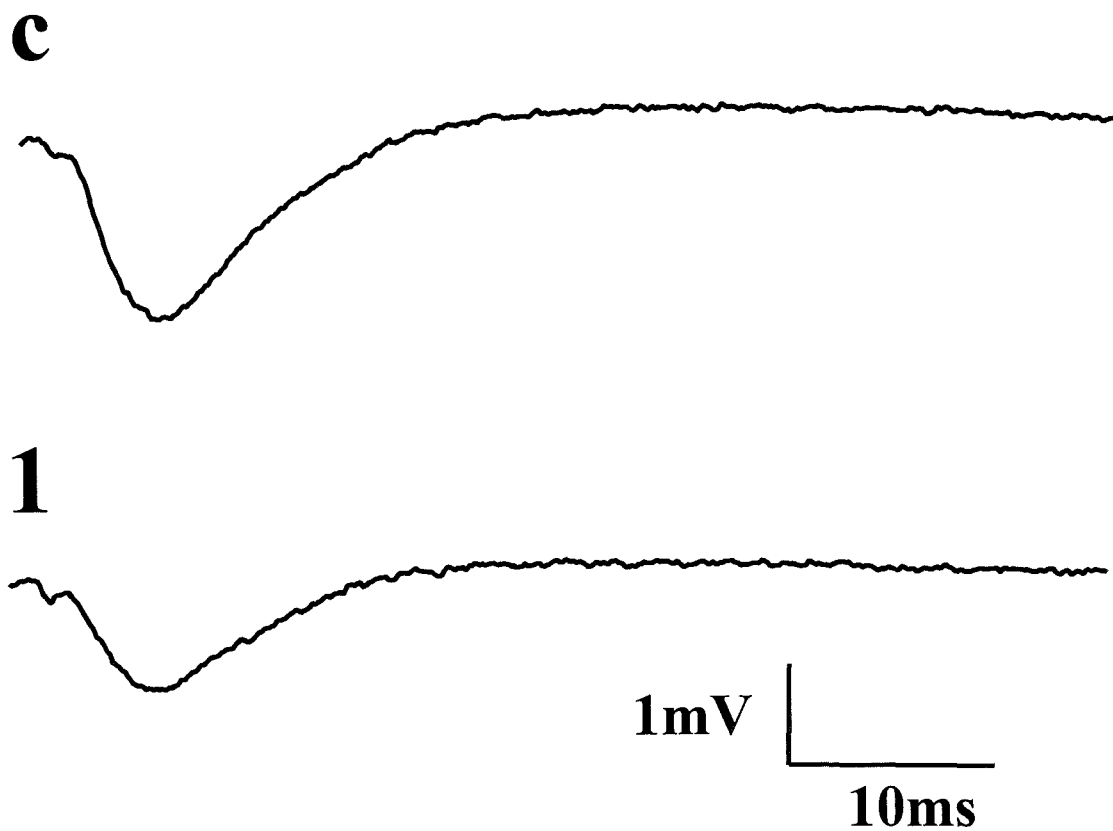
Figure 36. Representative fEPSPs showing the effect of QA 150μM.

fEPSPs corresponding to baseline (c) responses and during the depression elicited by QA 150μM (1) in the time course of figure 35.
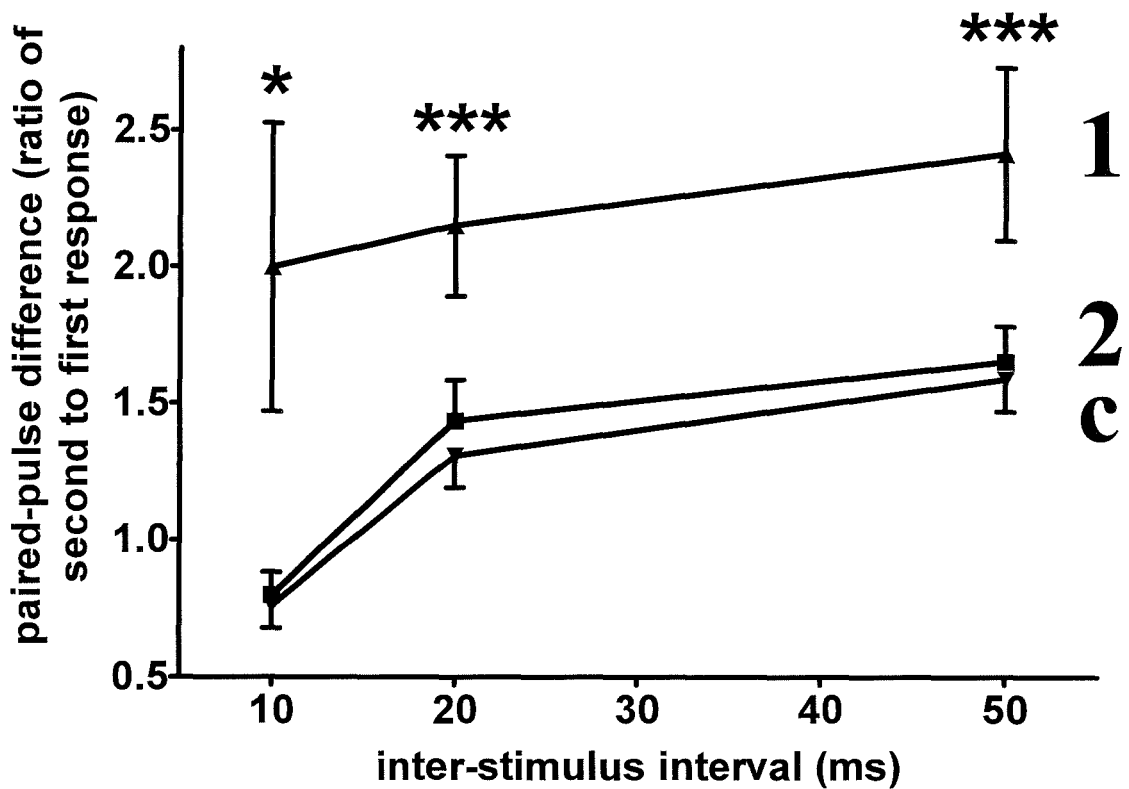
Figure 37. Effect of QA on paired-pulse interactions.

Shows paired-pulse interactions of the fEPSP slope under resting conditions, (c), in the presence of quinolinic acid 150 µM (1), and during washout (2). Quinolinic acid caused a significant decrease of paired-pulse inhibition at 10ms, and a facilitation of the paired-pulse ratio at greater intervals. Points correspond to in the time course of figure 35. *P < 0.05, **P < 0.01, ***P < 0.001 compared to baseline control responses. Each point shows the mean±s.e.m. for n=7 experiments.

control

quinolinic acid 150μM

a)

d)

b)

e)

c)

f)

1mV
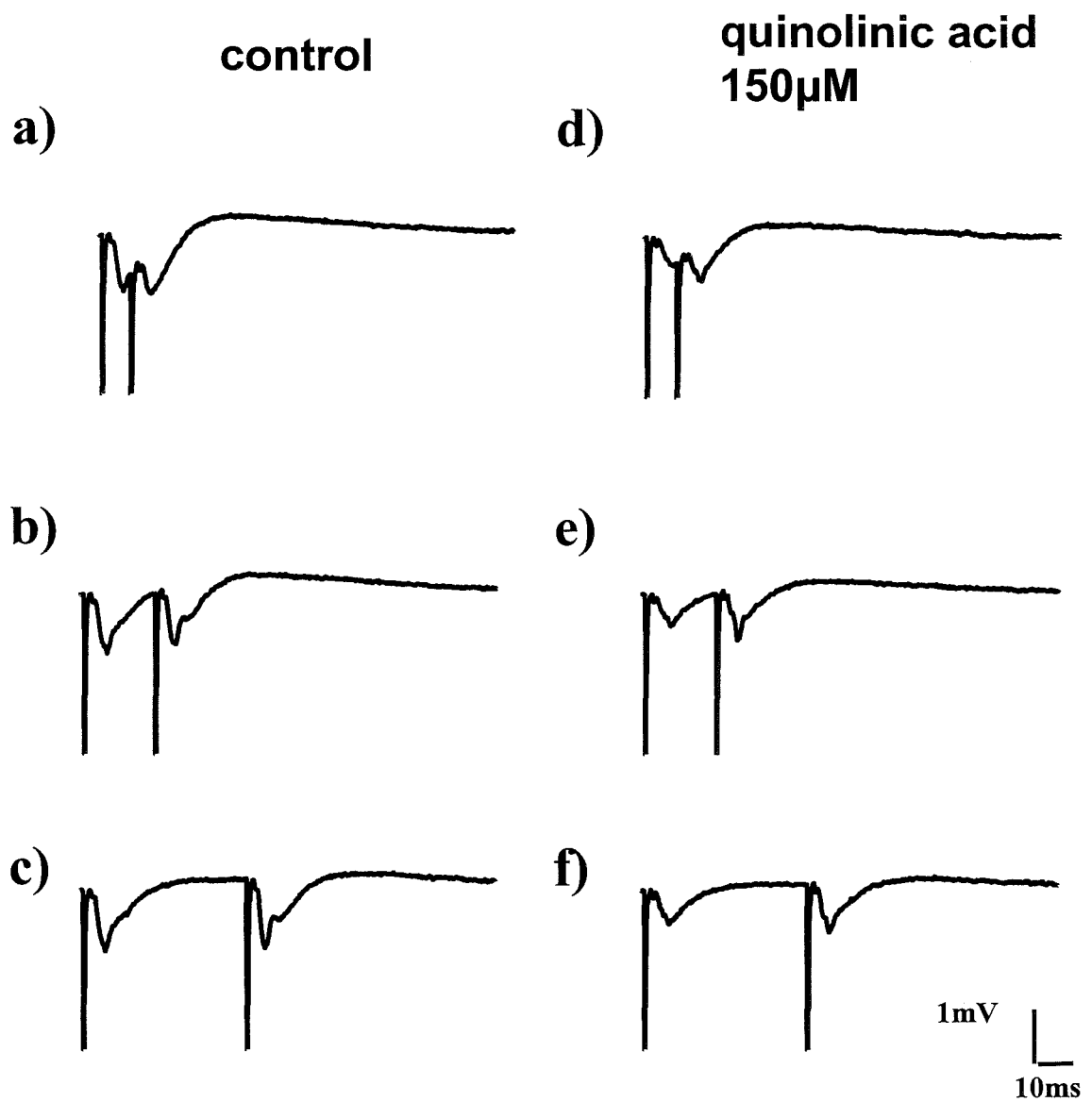
10ms

Figure 38. Example records of fEPSPs during paired-pulse interactions.

During baseline recording: (a) 10ms (b) 20ms and (c) 50ms and during the depression elicited by QA 150μM: (d) 10ms (e) 20ms and (f) 50ms.

DPCPX

Adenosine is a neuromodulator with an important role in synaptic transmission and neuronal excitability, inhibiting transmission via the activation of presynaptic A_1 receptors by decreasing release probability which enhances the paired-pulse interaction (Dunwidde, 1985; Greene, 1991). 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX) is a potent and selective adenosine A_1 receptor antagonist (Linden, 1991). Since the depolarisation of excitable tissues elicits the release of adenosine, it was important to confirm that the depression of fEPSP slope and the associated changes in paired-pulse interactions were indeed directly attributable to the activation of NMDA receptors and not to the release of endogenous adenosine.

An initial application of HQA 2.5µM caused a depression of baseline fEPSP slope to $13.13 \pm 3.88\%$ ($p < 0.001$, n=5). In the presence of DPCPX 20nM, a concentration that selectively blocks adenosine A_1 receptor, the second application of HQA 2.5µM caused a similar depression to $15.20 \pm 7.04\%$ ($p < 0.001$, n=5) compared to baseline responses (figure 39). During the initial HQA application the paired-pulse interactions were significantly enhanced at all interstimulus intervals ($p < 0.01$, n=5 at all intervals) compared to baseline control responses. The co-application of DPCPX during the second application of HQA produced an exactly similar

137

effect ($p<0.01$, $n=5$ at all intervals) compared to baseline control responses (figure 40). This suggests that the presynaptic element of the depression of fEPSP slope is not dependent upon the activation of presynaptic adenosine A_1 receptors.
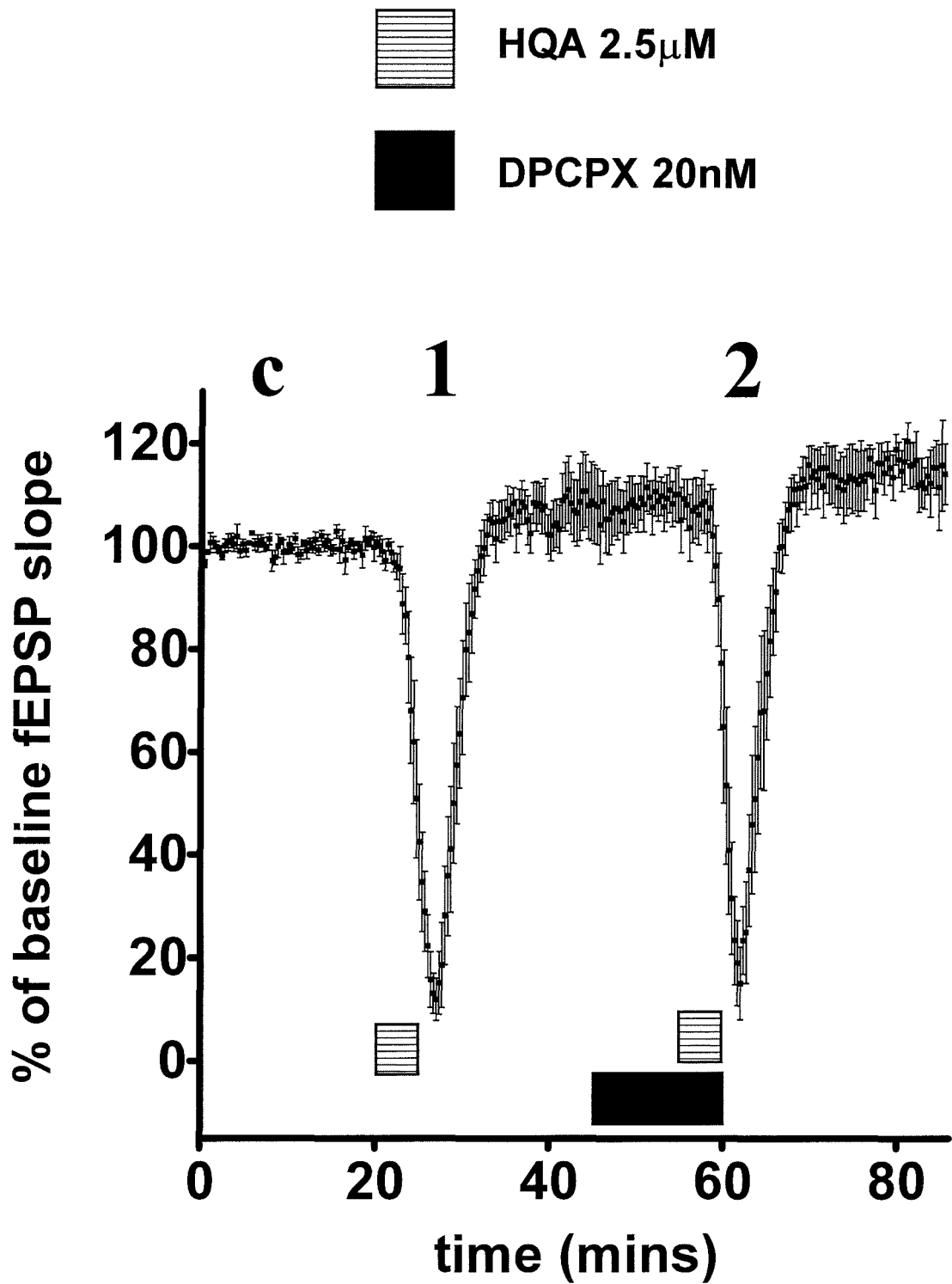
Figure 39. Effect of DPCPX upon HQA-evoked depression of fEPSP slope.

A plot of fEPSP slope showing the depression of baseline (c) fEPSP slope caused by HQA 2.5μM alone (1), and in the presence of DPCPX 20nM (2). Each point shows the mean±s.e.m. for $n=5$ experiments.
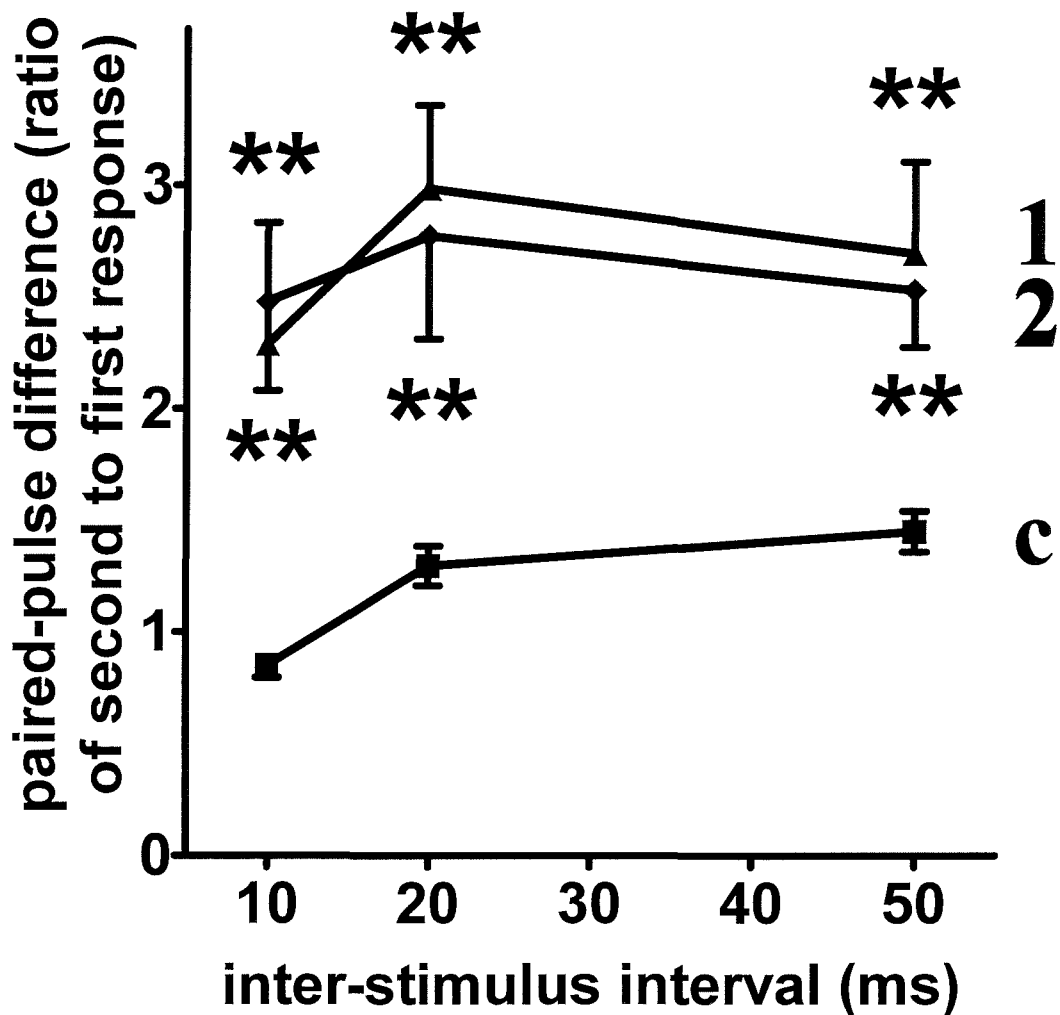
Figure 40. Effect of HQA and DPCPX upon paired-pulse interactions.

Showing the paired-pulse interactions of fEPSP slope at interstimulus intervals of 10ms, 20ms and 50ms; the results are presented as HQA 2.5μM (**1**) (p<0.01, n=5 at all intervals) and HQA 2.5μM in the presence of DPCPX 20nM (**2**) (p<0.01, n=5 at all intervals) both produced significant enhancements of paired-pulse interactions compared with baseline control responses (**c**). Each point shows the mean±s.e.m. for n=5 experiments.

Inhibition by kynurenic acid, 2-AP5 and dizocilpine

The broad-spectrum EAA antagonist, kynurenic acid (KYA) 100µM, was used at a concentration that selectively blocks NMDA and not AMPA or kainate receptors (Perkins & Stone, 1982; Stone & Burton, 1988). This blocked the depressant effect of both QA and HQA on the evoked fEPSP with no residual response that might indicate a novel binding site (figure 41). The selective and competitive NMDA receptor antagonist 2-AP5 50µM was used, which also blocked the effect of both QA 150µM and HQA 2.5µM but again left no residual effect upon the evoked fEPSP ($p<0.001$ and $p<0.001$; respectively, n=4) (figure 42).

It was thought that the concentration of HQA required to activate the novel binding site might be higher than that required for NMDA receptor activation, but that raising the agonist concentration would simply out-compete the blockade of 2-AP5. Therefore, the selective and non-competitive NMDA receptor antagonist dizocilpine (MK 801) 10µM was used, which produces a voltage and use-dependent non-competitive blockade of the NMDA receptor ion-channel. This blockade allowed the increase by 10-fold the concentration of both QA and HQA to 1.5mM and 25µM respectively so that any novel binding site should be more strongly activated. The blockade of the NMDA receptor was confirmed using NMDA itself at 10µM. However, in the presence of MK 801 these high

concentrations of agonists still did not induce any observable changes in the fEPSP. Unfortunately, it was not possible to perform control responses to these higher concentrations of QA and HQA because, without the presence of the antagonist, they produced a complete and largely irreversible loss of the fEPSP, through excitotoxic neuronal damage (figure 43).
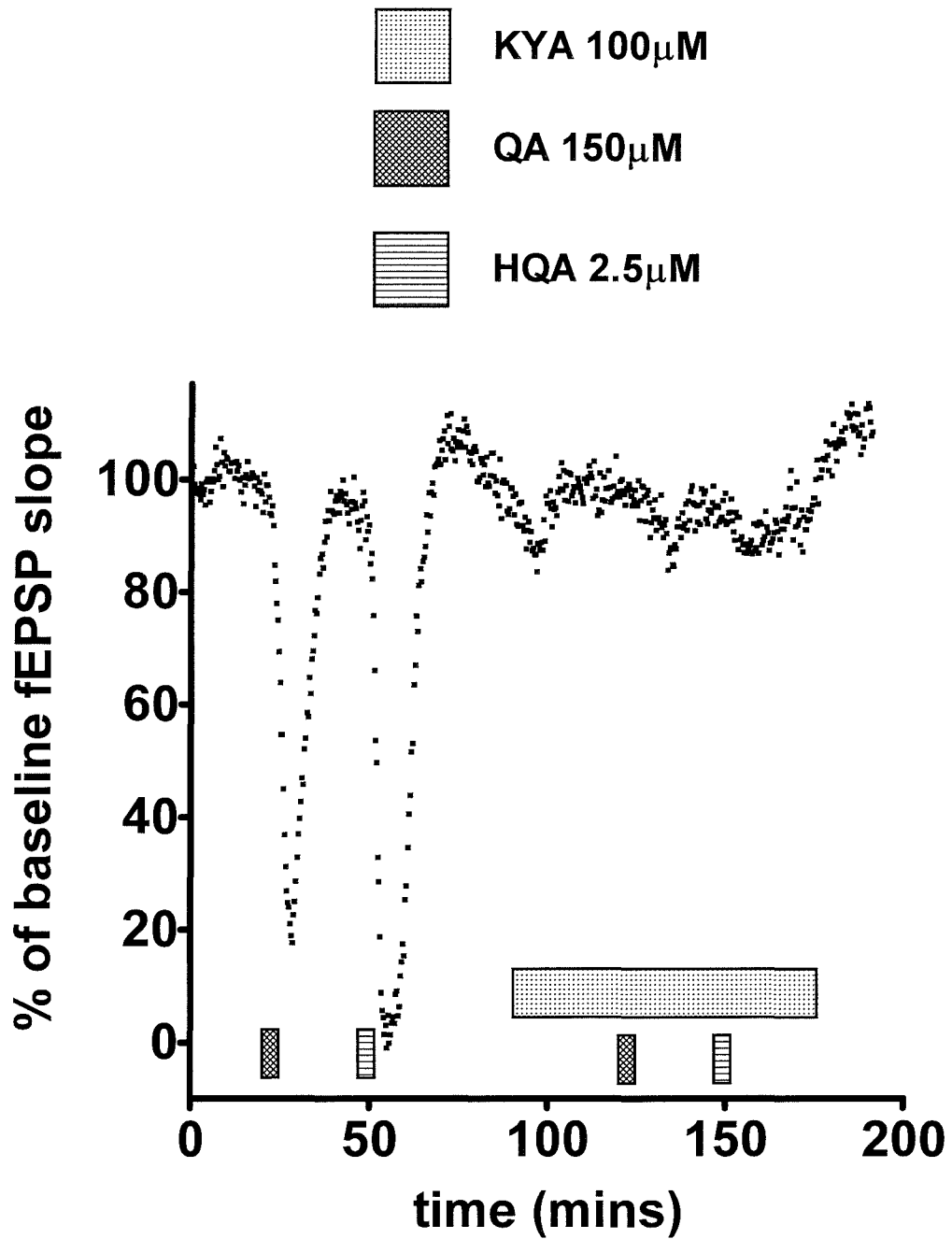
Figure 41. Effect of kynurenic acid on QA and HQA-induced depressions of fEPSP slope.

Representative plot showing the blockade by kynurenic acid 100μM of the depression of fEPSP slope caused by QA 150μM and HQA 2.5μM.
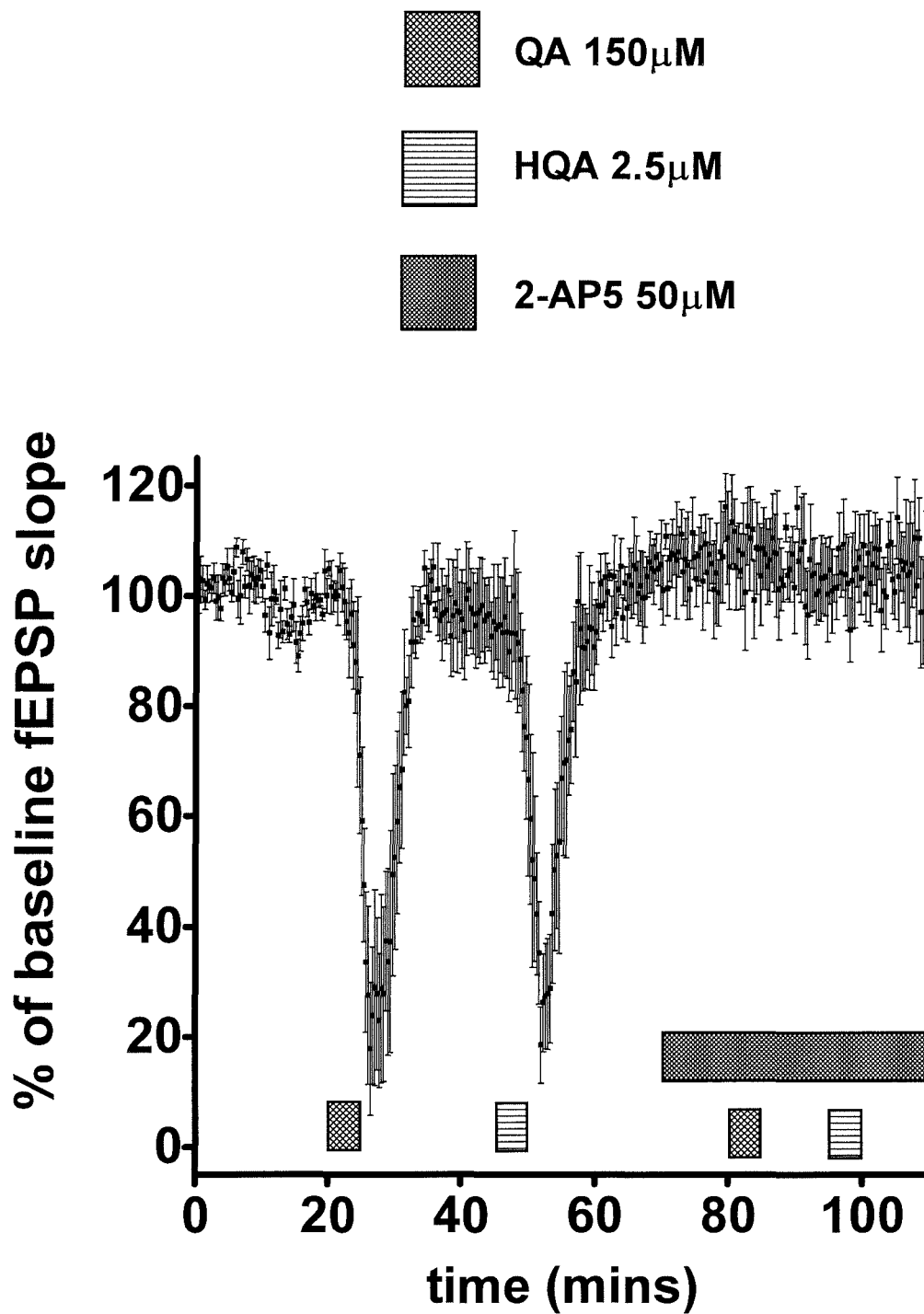
Figure 42. Effect of 2-AP5 on QA and HQA on fEPSP slope.

A plot showing the depression of fEPSP slope during the application of quinolinic acid 150μM and homoquinolinic acid 2.5μM and their subsequent inhibition by 2-AP5 50μM (P < 0.001and P < 0.001; respectively). Each point shows the mean±s.e.m. for n=4 experiments.
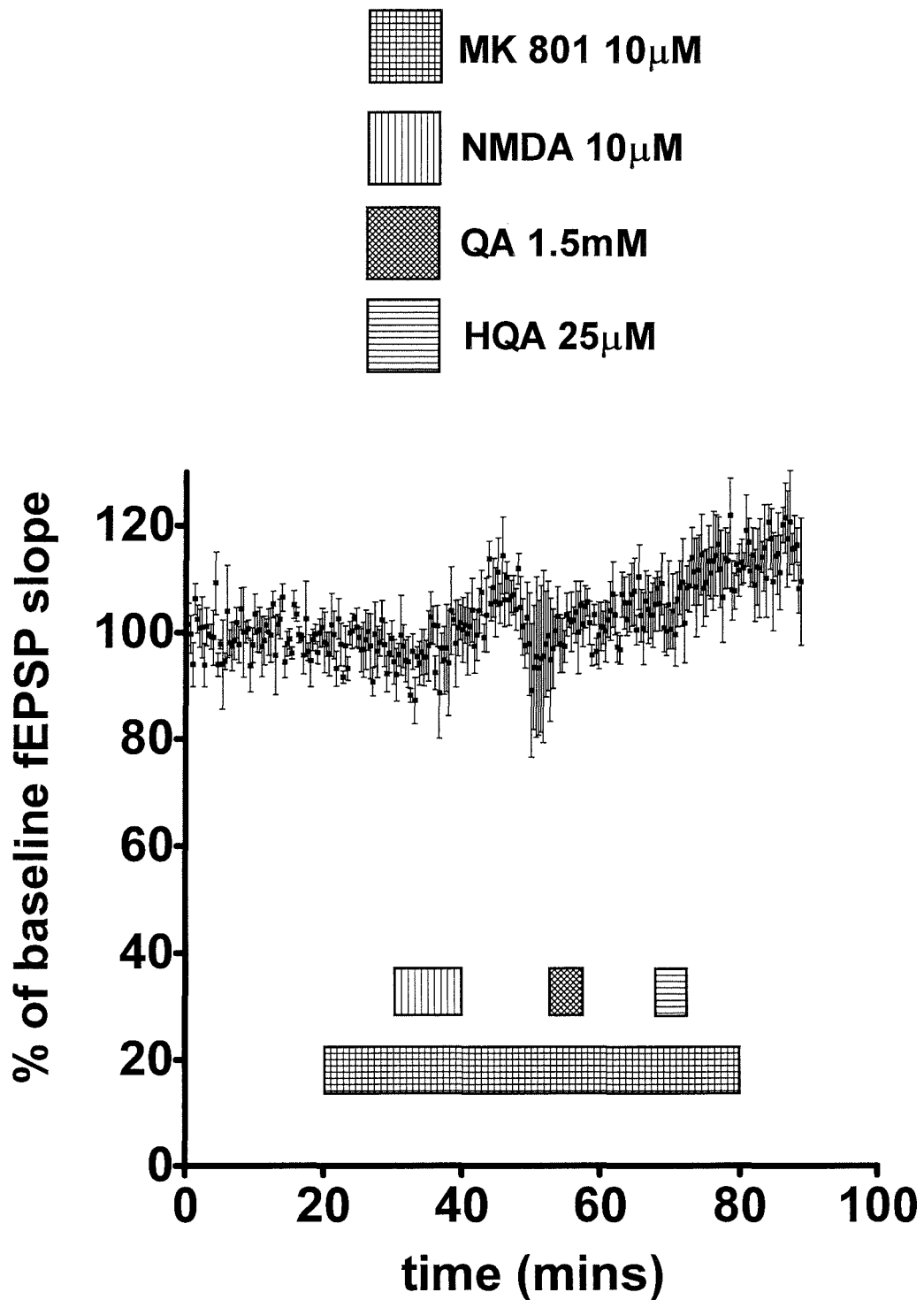
Figure 43. Effect of MK 801 on QA and HQA on fEPSP slope.

A plot showing the inhibition by MK 801 10μM of the depressant effect upon fEPSP slope by NMDA 10μM, QA 1.5mM and HQA 25μM. Each point shows the mean±s.e.m. for *n*=4 experiments.

Ro 25-6981

As illustrated in figure 44, HQA 2μM (**i**) and QA 100μM (**1**) are concentrations that produced small yet discernible depressions of baseline fEPSP slope (**c**). With the co-perfusion of Ro 25-6981 3μM, the same concentrations of QA (**2**) and HQA (**ii**) produced significant depressions of the fEPSP slope to $24.19 \pm 15.39\%$ and $-1.88 \pm 4.93\%$ respectively compared to baseline responses ($p < 0.001$ and $p < 0.001$ respectively n=5). Ro 25-6981 was then allowed to wash out and QA (**3**) and HQA (**iii**) were re-applied where a significant depression to $8.98 \pm 5.21\%$ and $-5.59 \pm 4.04\%$ of control respectively was still observed ($p < 0.001$ and $p < 0.001$ respectively n=5) (figure 44). It was noticed that the effect of Ro 25-6981 seemed to develop slowly and did not diminish during the washout phase of the experiment. It was also observed that paired-pulse interactions were significantly enhanced during the enhanced depressions of QA and HQA at all interstimulus intervals after the application of Ro 25-6981 compared to during the initial applications (QA: 10ms $p < 0.01$, 20ms $p < 0.05$, 50ms $p < 0.01$; HQA: 10ms $p < 0.001$, 20ms $p < 0.001$, 50ms $p < 0.05$, n=6; figure 45 and 46).
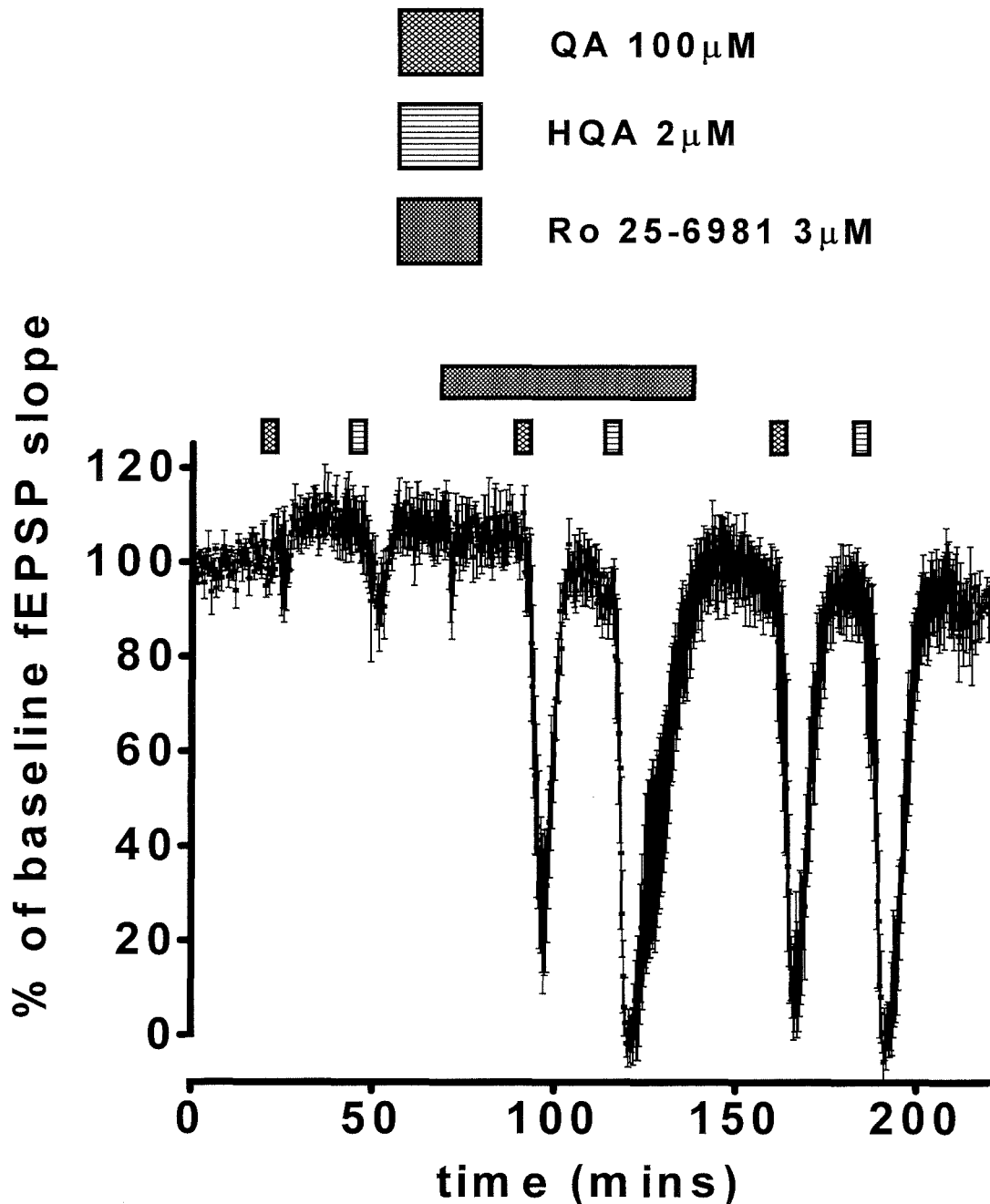
Figure 44. Effect of Ro 25-6981 with QA and HQA on fEPSP slope.

A plot showing the effect of QA 100μM (1) and homoquinolinic acid 2μM (i) compared to the baseline fEPSP response (c). During the co-perfusion of Ro 25-6981 3μM, the same concentrations of QA (2) and homoquinolinic acid (ii) produced a significant depression to 24.19±15.39% and -1.88±4.93% of the fEPSP slope respectively compared to baseline responses (p<0.001and p<0.001 respectively). Ro 25-6981 was then washed out for 20 minutes until QA (3) was re-applied and 45 minutes until homoquinolinic acid (iii) was re-applied where a significant depression to 8.98±5.21% and -5.59±4.04% respectively was observed compared to the baseline fEPSP responses (p<0.001 and p<0.001 respectively). Each point shows the mean±s.e.m.

quinolinic acid
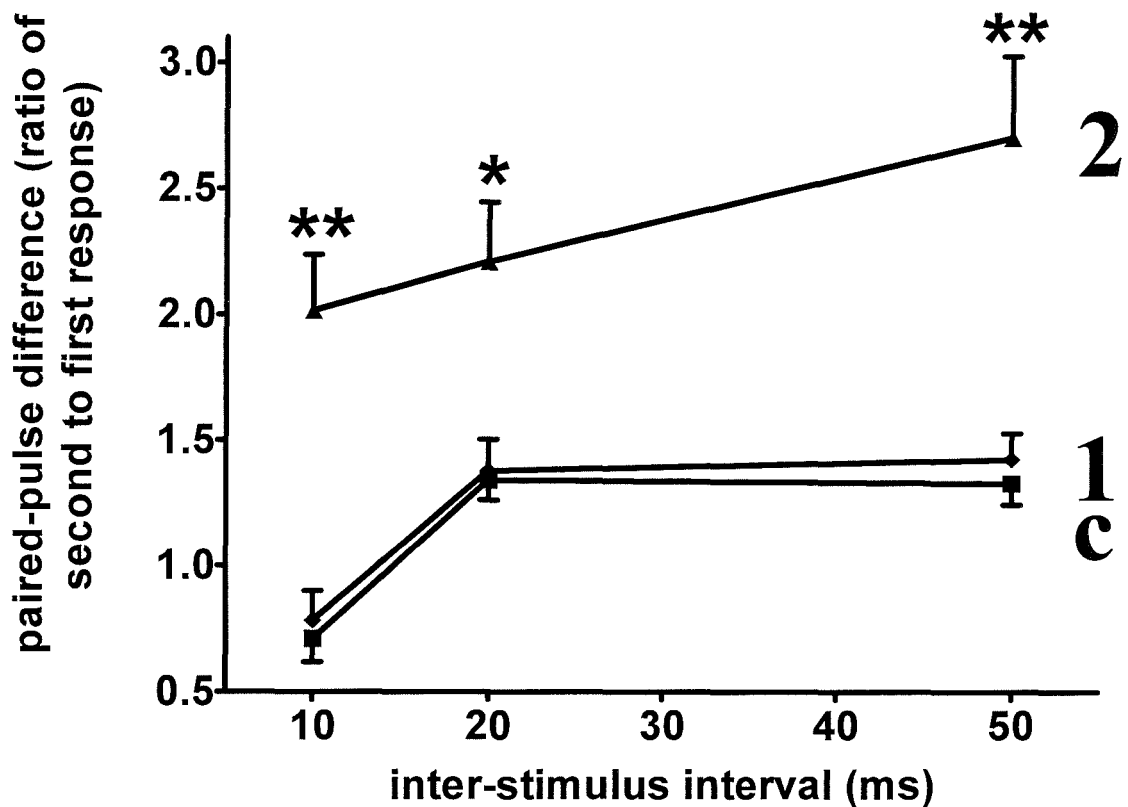
Figure 45. Effect of Ro 25-6981 and QA on paired-pulse interactions.

A plot showing the paired-pulse interactions at interstimulus intervals of 10ms, 20ms and 50ms during baseline recordings (c), the initial application of QA 100μM (1) and the second application of QA 100μM during the co-perfusion with Ro 25-6981 3μM (2) (10ms $p<0.01$, 20ms $p<0.05$, 50ms $p<0.01$, n=6). Points correspond to figure 44. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Each point shows the mean±s.e.m. for n=6 experiments.

homoquinolinic acid
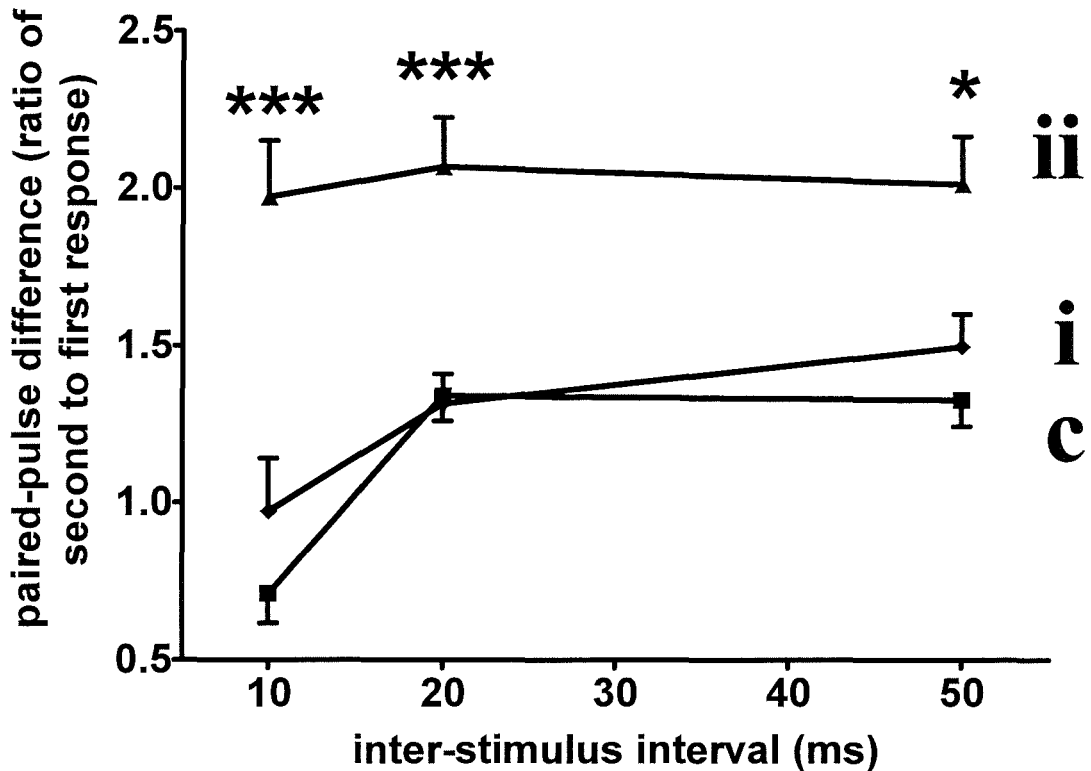
Figure 46. Effect of Ro 25-6981 and HQA on paired-pulse interactions.

Showing the paired-pulse interactions at interstimulus intervals of 10ms, 20ms and 50ms during baseline recordings (c), the initial application of HQA 2µM (i) and the second application of HQA 2µM during the co-perfusion of Ro 25-6981 3µM (ii) (10ms $p<0.001$, 20ms $p<0.001$, 50ms $p<0.05$, n=6). Points correspond to figure 44. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Each point shows the mean±s.e.m. for n=6 experiments.

Excitotoxicity

An application of HQA that significantly depressed the fEPSP slope was selected to investigate the effect of Ro 25-6981 on higher concentrations of NMDA receptor agonists. In previous studies of young cultured rat cortical neurones Ro 25-6981 had potentiated small depolarising concentrations of NMDA but blocked larger depolarising concentrations (Fischer *et al.*, 1997). A 5-minute application of HQA 4µM caused a maximal depression of fEPSP slope to 6.63±3.69% (p<0.001, n=4) that reverted to near baseline responses upon washout. Ro 25-6981 3µM was co-perfused with a second application of 4µM HQA which produced a depression of fEPSP slope to -9.27±1.13% (p<0.001, n=4). However, this depression did not fully recover to baseline responses. The fEPSP slope was irreversibly reduced to an average 34.70±0.38% compared to baseline responses (p<0.001, n=4). Maximum evokable amplitudes of fEPSP slope (100%), which were taken as an additional measure of excitotoxic damage, were significantly reduced (p=0.0011, n=4) compared to the baseline maximums recorded and they did not recover during the 70-minute washout phase of the experiment. Attempts to induce LTP at the end of the experiments also failed, again suggesting that irreversible excitotoxic damage had occurred (figure 47 and 48). This specifically ruled out the induction of NMDA-receptor dependent LTD, which was reversible by high frequency stimulation (data not shown).
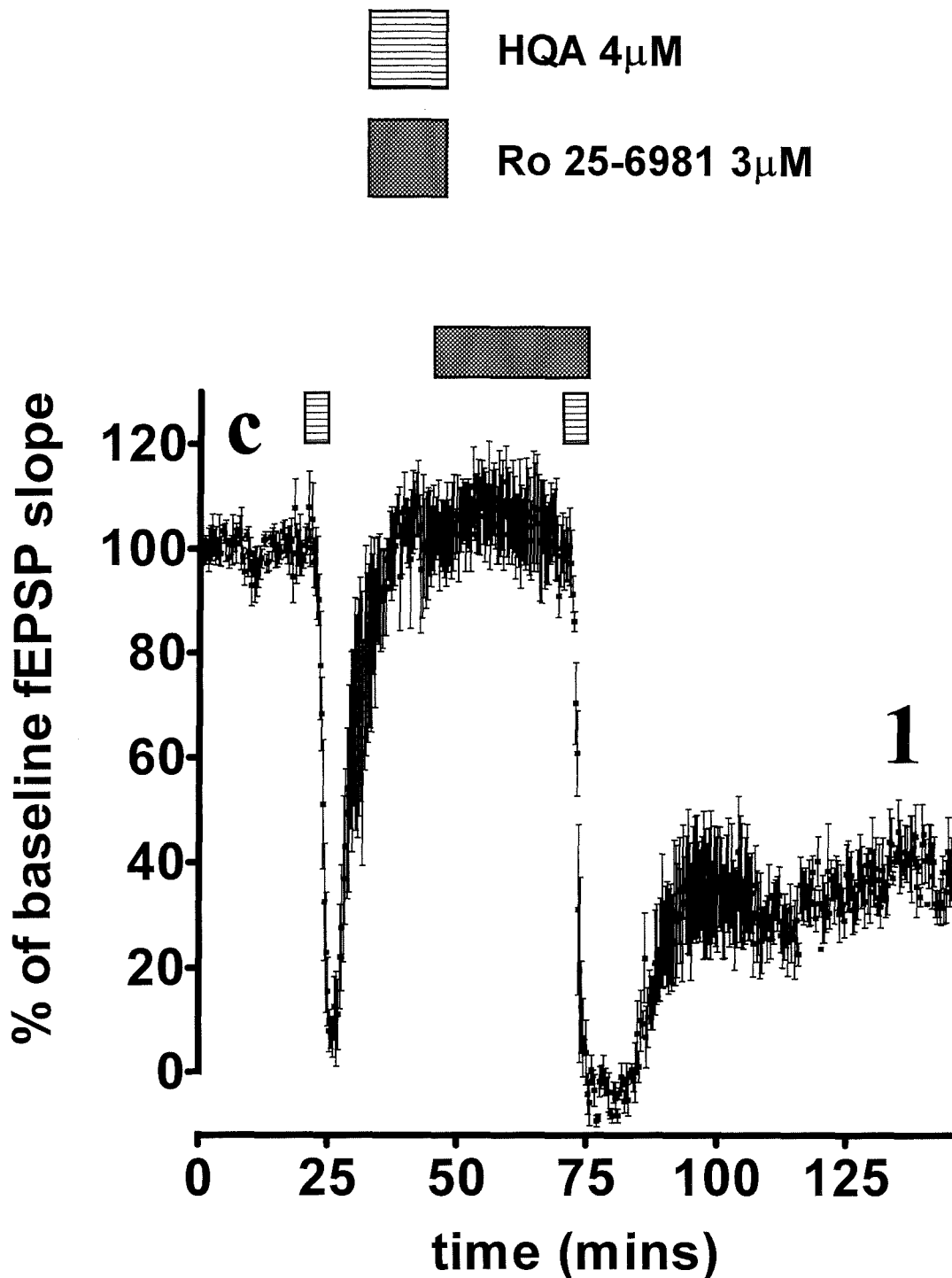
Figure 47. Effect of HQA 4μM and Ro 25-6981 on fEPSP slope.

A 5-minute application of homoquinolinic acid 4μM elicited a reversible depression of baseline fEPSP slope to 6.63±3.69% (p<0.001). Ro 25-6981 3μM was co-perfused and a second 5-minute application of homoquinolinic acid 4μM produced a depression of fEPSP slope to -9.27±1.13% (p<0.001). However, the fEPSP slope did not fully recover but was significantly and irreversibly reduced to an average 34.70±0.38% compared to the initial baseline (p<0.001). Each point shows the mean±s.e.m. for n=4 experiments.
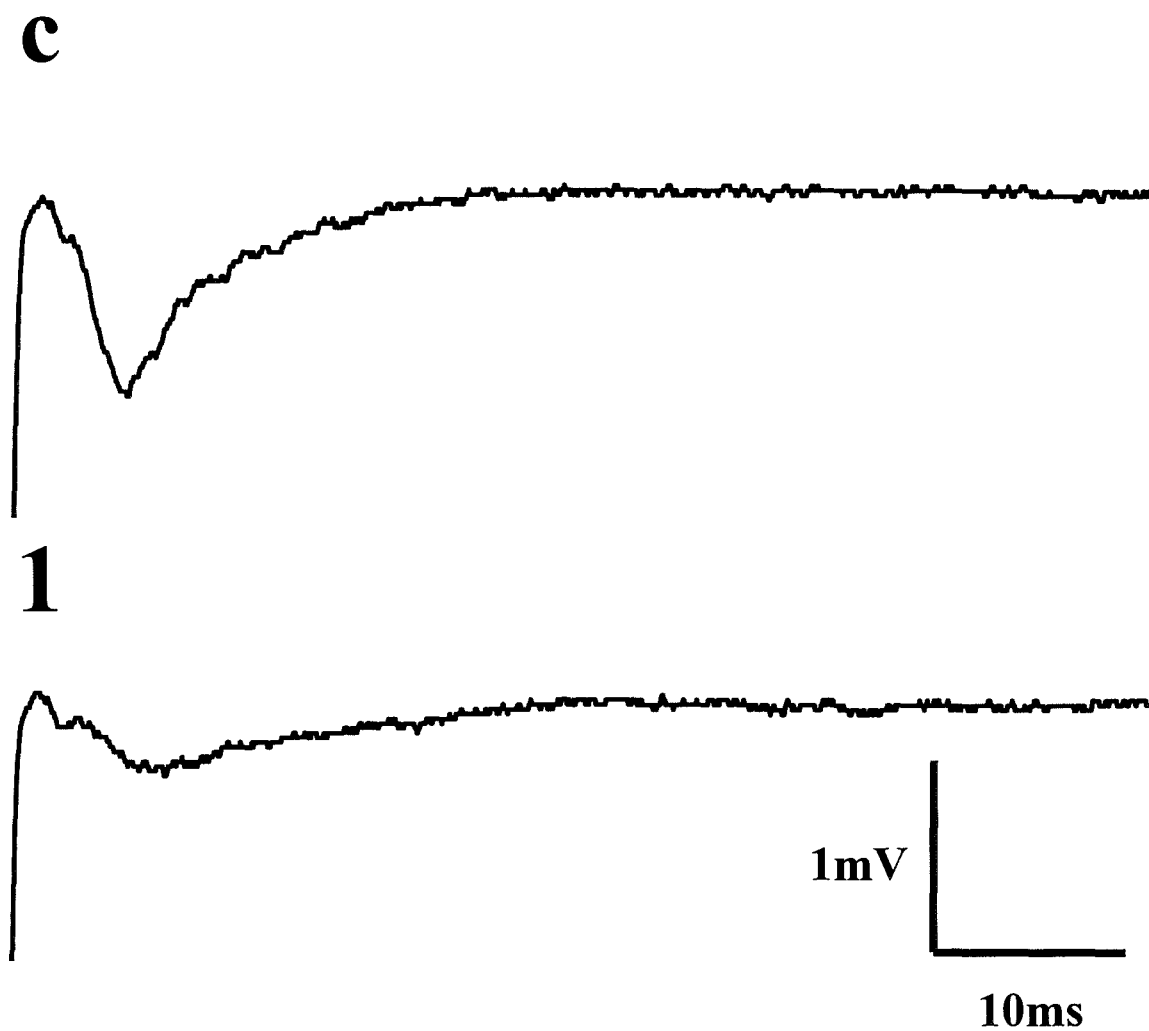
Figure 48. Representative fEPSP recordings showing excitotoxicity.

During baseline recording (c) and during the irrecoverable depression (1) corresponding to figure 47.

NR2A antagonists

HQA is reported to be a selective agonist for NR2A and NR2B subunit-containing NMDA receptors. Using Ro 25-6981, an NR2B antagonist, to produce the potentiation of the depression of fEPSP slope by low concentrations of HQA suggests that the remaining HQA-dependent effect could be modulated by the remaining NR2A component of HQA activity. In order to test this theory several candidate NR2A selective antagonists including zinc, CCMQ and PEAQX were investigated (Paoletti *et al.*, 1997; Brown *et al.*, 1998; Auberson *et al.*, 2002; Grimwood *et al.*, 2002).

CCMQ

Previous autoradiographical studies found that 2-carboxy-3-carboxymethylquinoline (CCMQ), a derivative of HQA, displaces bound [^{3}H]-HQA from its NMDA-insensitive novel binding site at a concentration of 1mM, leaving behind a binding pattern which closely resembles the distribution of NR2B subunits in the rat brain (Brown *et al.*, 1998; Grimwood *et al.*, 2002). These results and the structural homology with HQA suggest that CCMQ may be able to displace HQA from the NR2A subunit-containing NMDA receptors thereby leaving only the NR2B population, acting as an NR2A-selective ligand.

The effect of CCMQ 0.5mM upon the HQA-induced depression of fEPSP slope was investigated first. As shown in figure 49 two initial applications of HQA 2.5μM produced two similar depressions of fEPSP slope. The third application of HQA was co-applied with CCMQ but this had no effect on an identical depression of fEPSP slope. This suggests that CCMQ was not acting as an antagonist of the NR2A site activated by HQA. This study next investigated if CCMQ was an NR2A agonist and used Ro 25-6981 to block the NR2B subunit in order to help reveal any NR2A effect. As shown in figure 50 the perfusion of Ro 25-6981 alone had no effect upon baseline fEPSP slope but CCMQ elicited a small potentiation of the fEPSP slope to $108.65\pm1.71\%$ ($p=0.0058$, $n=3$). Finally, the effect of CCMQ upon the potentiation of HQA 2μM caused by Ro 25-6981 3μM was investigated in order to address the subunit specificity of this potentiation. As shown in figure 51 the application of HQA 2μM produced little response but the application of Ro 25-6981 potentiated this to a significant depression to $14.72\pm10.49\%$ ($p<0.001$, $n=3$) of baseline fEPSP slope, which was not inhibited by CCMQ. The co-perfusion of CCMQ resulted again in the same small but significant increase in fEPSP slope to $124.25\pm2.75\%$ ($p<0.05$, $n=3$). A third application of HQA 20 minutes after the end of the perfusion of Ro 25-6981 also produced a large depression to $7.18\pm3.24\%$ ($p<0.001$, $n=3$) of fEPSP slope.
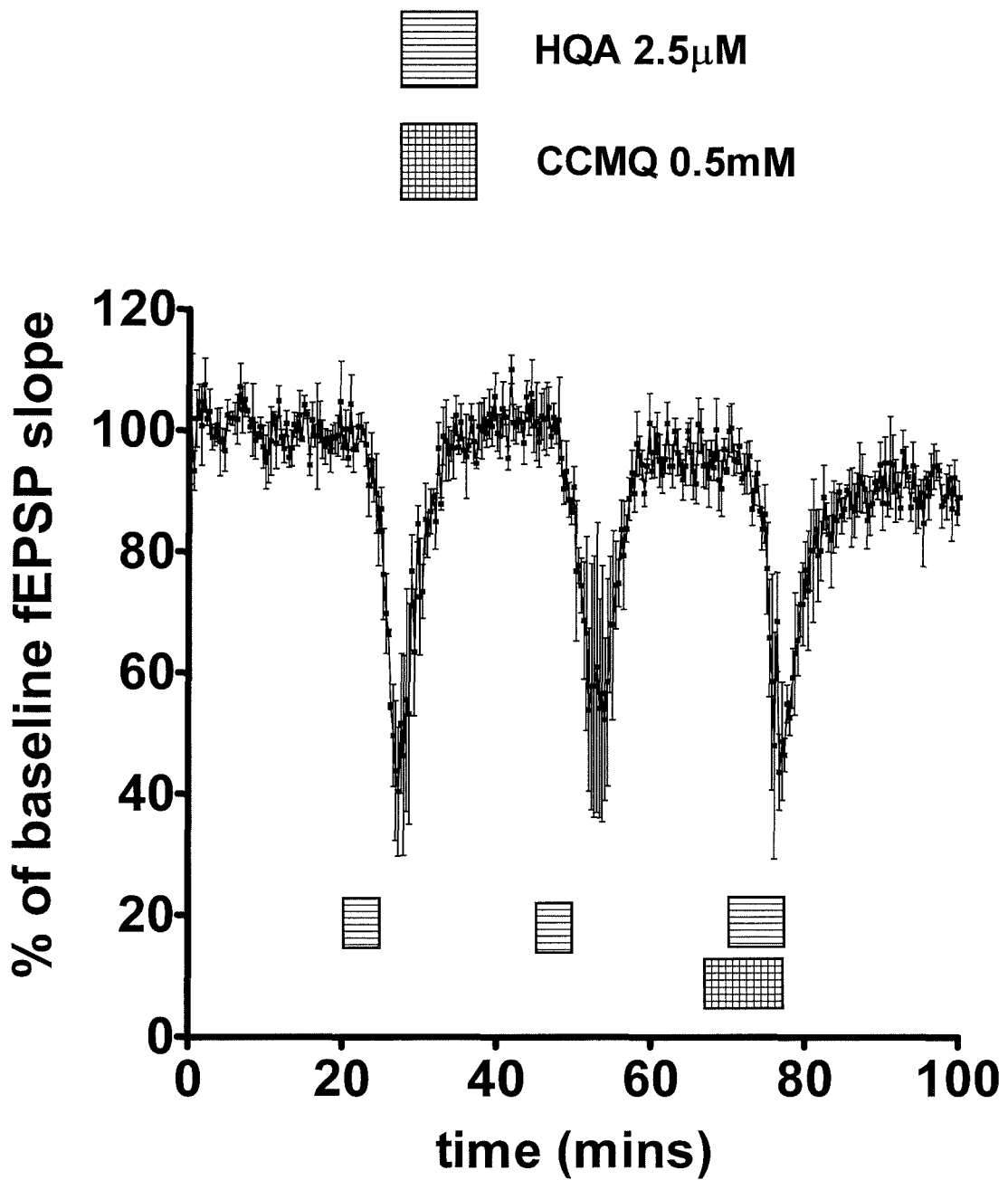
Figure 49. Effect of HQA 2.5µM and CCMQ 0.5mM on fEPSP slope.

A plot showing the effect of repeated 5-minute perfusions of homoquinolinic acid 2.5µM, that caused three consecutive depressions of the fEPSP slope. The co-perfusion of CCMQ 0.5mM with the third perfusion of homoquinolinic acid had no significant effect upon the peak magnitude or duration of the response. Each point shows the mean±s.e.m. for n=3 experiments.
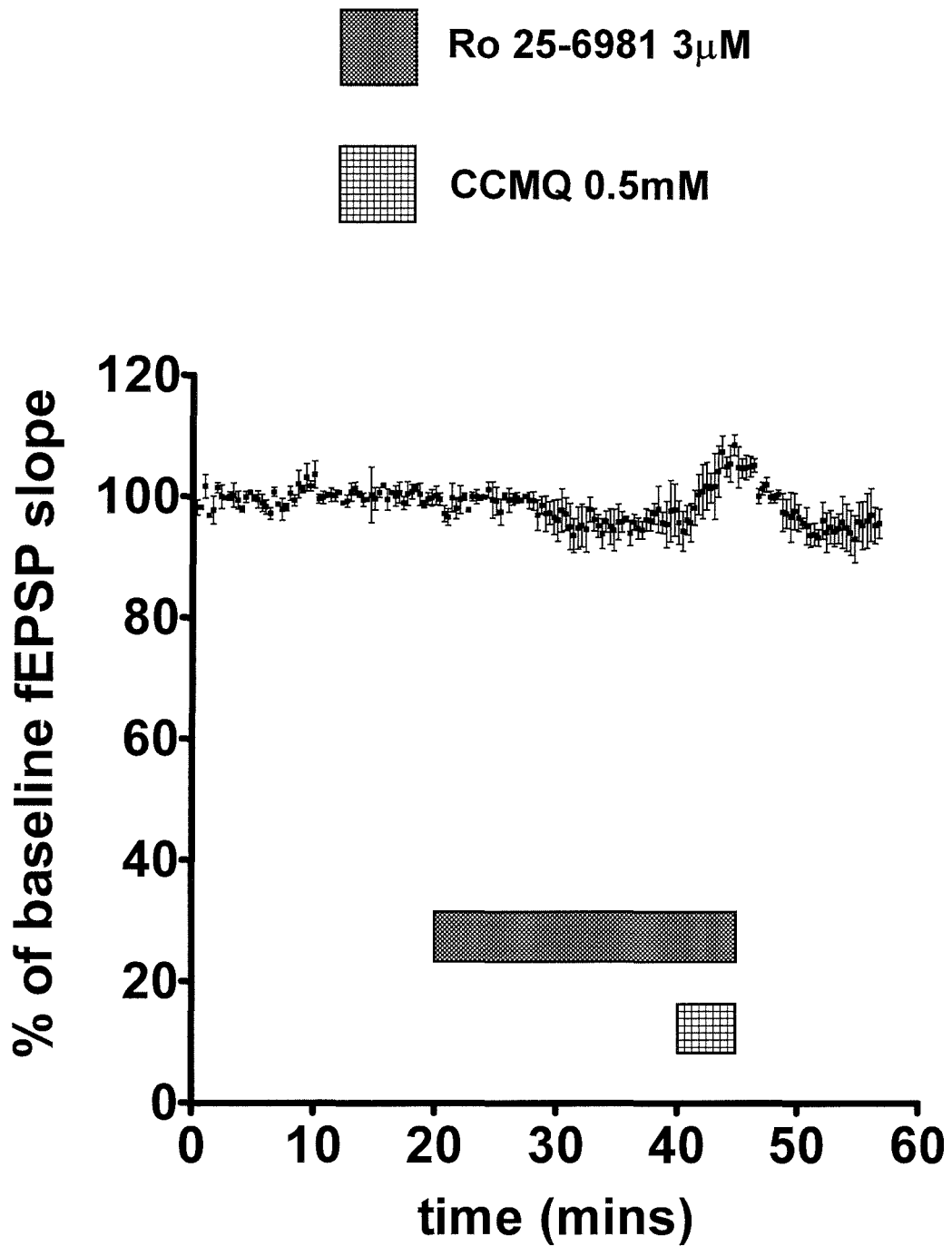
Figure 50. Effect of CCMQ and Ro 25-6981 on fEPSP slope.

A plot showing the effect of 3µM Ro 25-6981 and the subsequent co-perfusion of 0.5mM CCMQ which produced a small potentiation of the fEPSP slope to 108.65±1.71% (p=0.0058). Each point shows the mean±s.e.m. for n=3 experiments.
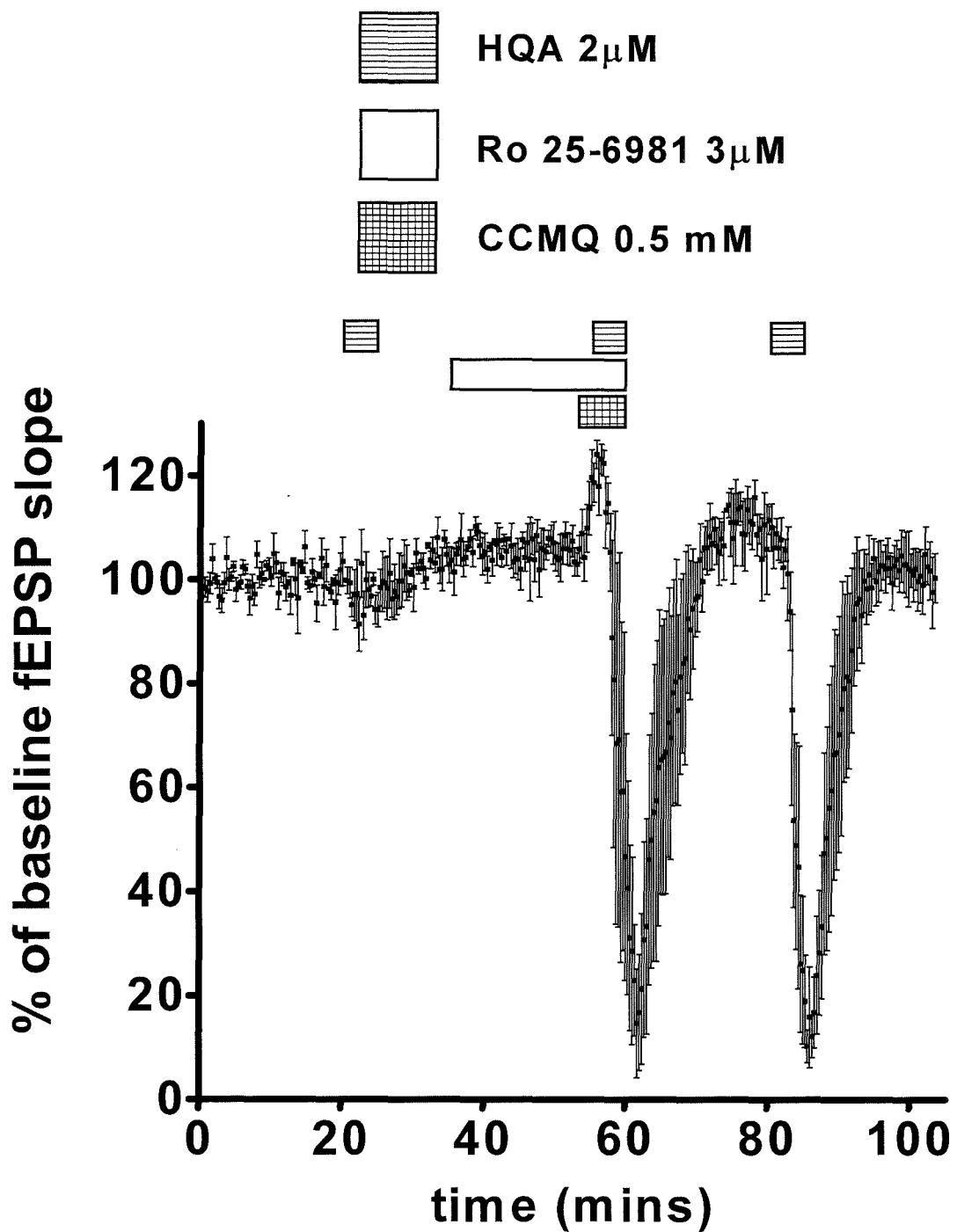
Figure 51. Effect of HQA 2µM, Ro 25-6981 and CCMQ 0.5mM on fEPSP slope.

Plot showing that the first application of HQA 2µM produced little response but the co-application of Ro 25-6981 potentiated a second HQA-application producing a significant decrease to $14.72 \pm 10.49\%$ ($p < 0.001$) of baseline fEPSP slope. The co-perfusion of CCMQ again resulted in the same small but significant increase in fEPSP slope to $124.25 \pm 2.75\%$ ($p < 0.05$). A third application of HQA 20 minutes after the end of the perfusion of Ro 25-6981 also produced a large depression to $7.18 \pm 3.24\%$ ($p < 0.001$) of fEPSP slope. Each point shows the mean $\pm$ s.e.m. for $n=3$ experiments.

Zinc

Zinc ions (Zn^{2+}) have been reported to produce a selective and non-competitive inhibition of the NR2A subunit-containing recombinant NMDA receptors, producing a voltage-independent inhibition at nanomolar concentrations (Paoletti et al., 1997). As shown in figure 52 an initial application of HQA 2.5μM caused a significant depression of fEPSP slope to 0.7±0.57% (p<0.001, n=4), a second application of HQA with Zn^{2+} 100nM also produced an identical depression of fEPSP slope to 2.6±2.2% (p<0.01, n=4). Thus, 100nM Zn^{2+} was unable to prevent the depression of fEPSP slope.
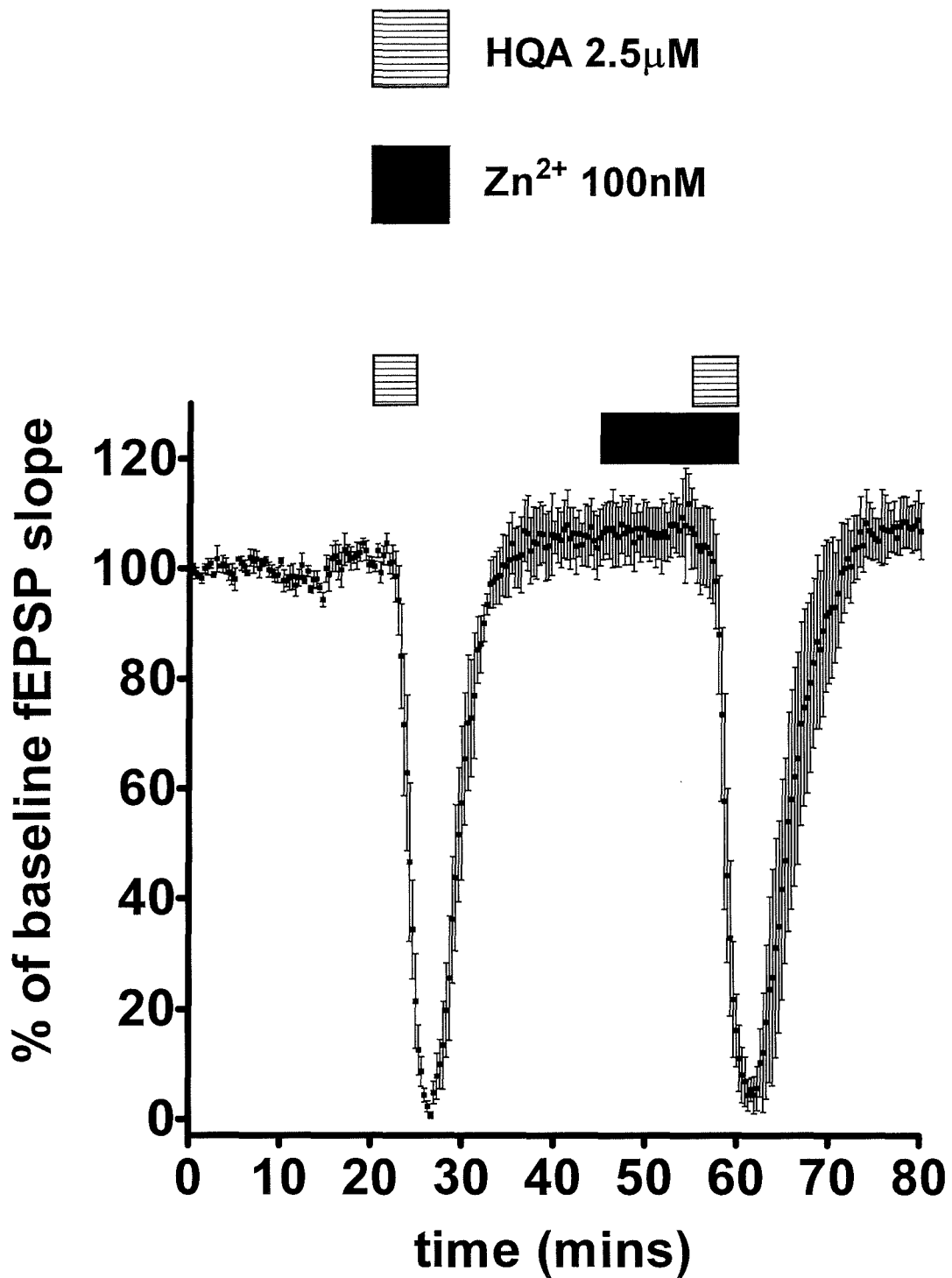
Figure 52. Effect of HQA 2.5μM and Zn^{2+} 100nM on fEPSP slope.

A plot showing that the first application of HQA 2.5μM caused a significant depression of baseline fEPSP slope to 0.7±0.57% (p<0.001). A second application of 2.5μM HQA with 100nM Zn^{2+} again produced a similar depression of fEPSP slope to 2.6±2.2% (p<0.01). Each point shows the mean±s.e.m. for n=4 experiments.

PEAQX

Having been unsuccessful with CCMQ and zinc in attempts to antagonise

the NR2A subunit, PEAQX, a novel NR2A antagonist was obtained as a

gift from Dr Yves Auberson of Novartis Pharmaceuticals. This compound

has 126-fold selectivity for the NMDA NR1A/NR2A over the

NR1A/NR2B receptors expressed in Xenopus oocytes (IC_{50} = 14nM and

1800nM respectively)(Auberson *et al.*, 2002). This investigation was

started by constructing a concentration-response curve for the inhibitory

effect of PEAQX upon the depression of fEPSP slope elicited by HQA

3μM. This was achieved by applying 3μM HQA, which produced a large

depression of the fEPSP slope, and then PEAQX was co-applied at varying

concentrations. Subsequent co-applications of the same concentration of

HQA were made and the degree of antagonism produced was observed

(figures 53 and 54). From the concentration-response curve it was

determined that using a concentration of 0.1μM provided the most selective

and complete antagonism of the NR2A subunit-containing NMDA

response. This concentration-response curve is very similar to that found

for the effect of PEAQX on NMDA-induced currents in Xenopus oocytes

expressing human NR1A/2A subunits (Appendix 2).

This study also investigated paired-pulse interactions during the interaction

of HQA and PEAQX. It was found that during the initial depression of

fEPSP slope caused by HQA $3\mu M$ the paired-pulse ratios were increased at all interstimulus intervals (10ms $p<0.001$, 20ms $p<0.01$, 50ms $p<0.001$; n=6) but that during the subsequent co-application of PEAQX $0.1\mu M$ and HQA $3\mu M$ they remained similar to baseline responses (figure 55).
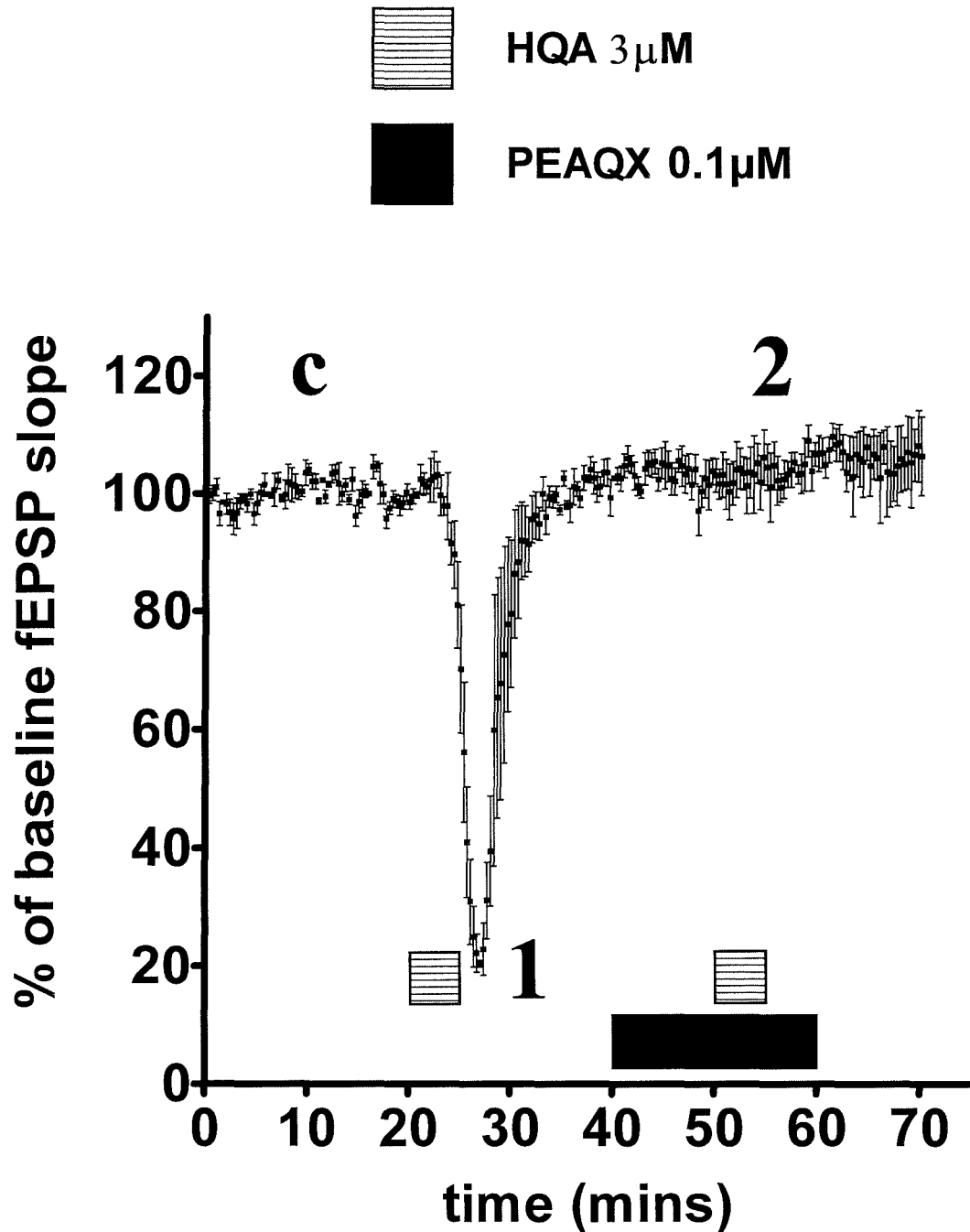
Figure 53. Effect of HQA and PEAQX on fEPSP slope.

A plot showing that the control application of HQA 3µM (1) produced a significant depression of baseline fEPSP slope (c) to 20.44±0.59% (p=0.0019). The depression of fEPSP slope expected by the second application of HQA 3µM was prevented by the co-application of PEAQX 0.1µM (2), Each point shows the mean±s.e.m. for *n*=6 experiments.
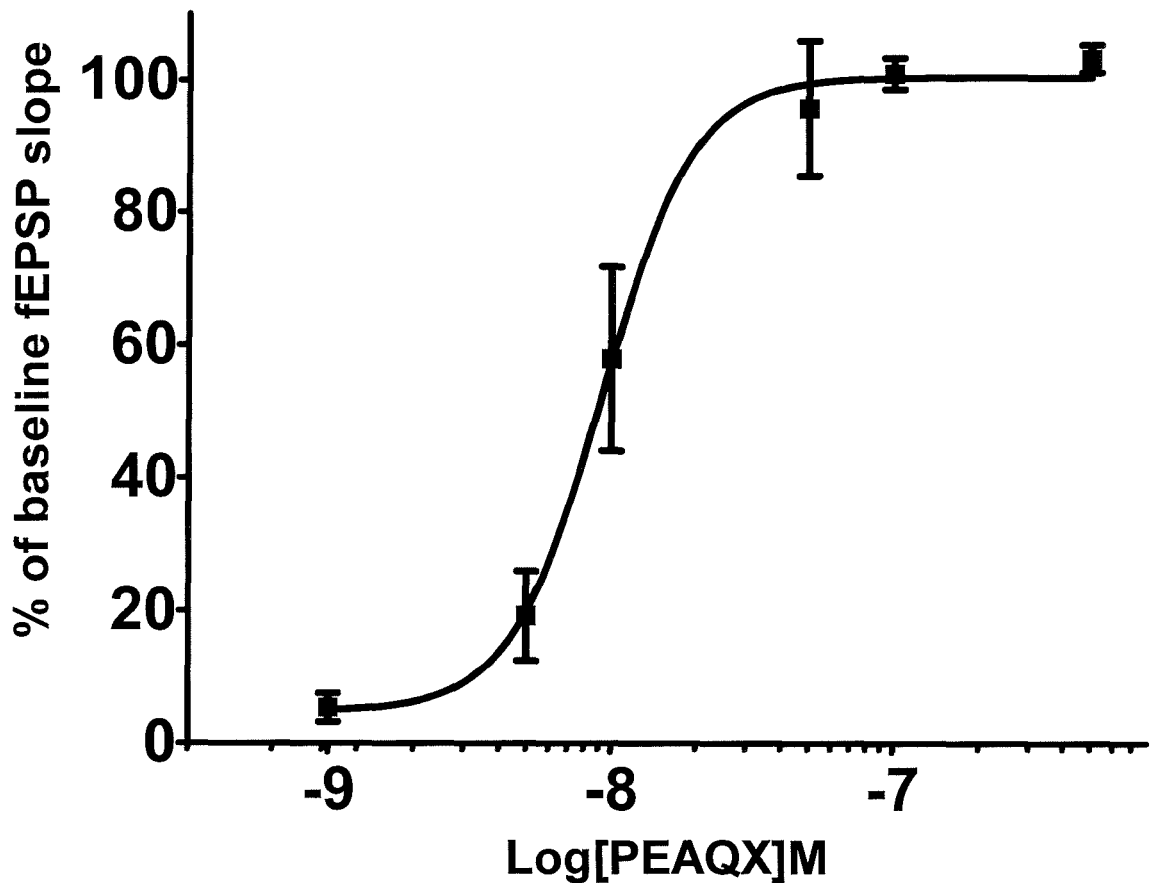
Figure 54. The concentration-response curve of PEAQX on HQA-induced depression of fEPSP slope.

A plot showing the concentration-response curve of the inhibitory action of PEAQX at concentrations of 1nM, 5nM, 10nM, 50nM, 100nM and 500nM (n=3 at all intervals, except n=6 for 100nM PEAQX) upon HQA 3μM, which normally caused a significant depression of fEPSP slope. A concentration of 9.23nM is predicted to produce a 50% inhibition of the depression elicited by HQA 3μM. Each point shows the mean±s.e.m.
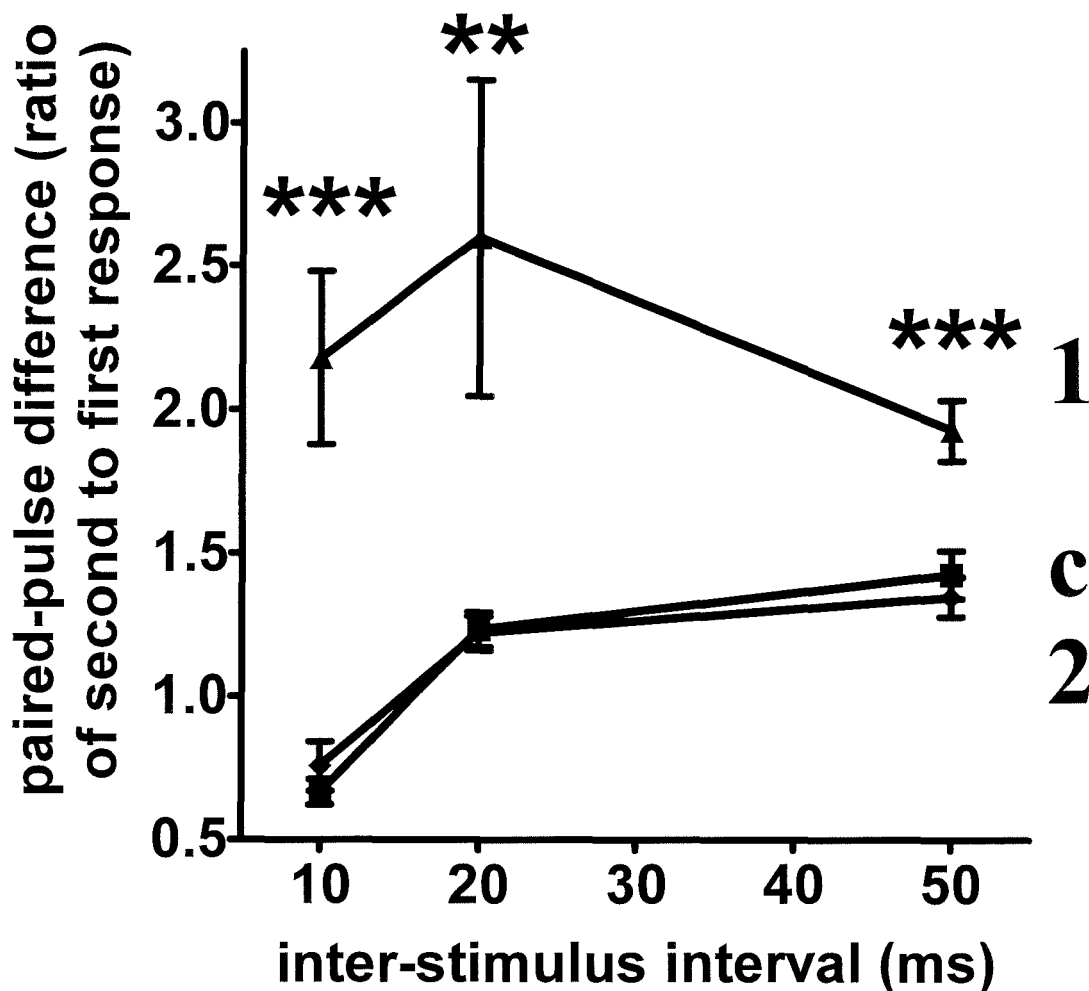
Figure 55. Effect of HQA and PEAQX on paired-pulse interactions.

A plot showing the paired-pulse interactions during baseline responses (c), which were significantly enhanced at interstimulus intervals of 10ms, 20ms and 50ms during the first application of HQA 3μM (1) and returned to control levels during the blockade of the second application of HQA 3μM by PEAQX 0.1μM (2). Points correspond to the time-course in figure 53. Each point shows the mean±s.e.m. for n=6 experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

PEAQX's inhibition of the Ro 25-6981/HQA potentiation.

However, of greater interest was the finding that the potentiated depression of fEPSP slope obtained by HQA 2μM with Ro 25-6981 3μM was also completely blocked by PEAQX 0.1μM. As shown in figure 56, a concentration of 2μM HQA (1) initially produced a small and insignificant depression of the baseline fEPSP slope (c) but following the perfusion of Ro 25-6981 3μM, a second application of HQA 2μM (2) produced a significant depression of fEPSP slope to 37.33±4.43% (p=0.0261, n=4). The co-perfusion of PEAQX 0.1μM prevented the depression of a third application of HQA 2μM (3).

It was also observed that paired-pulse interactions during the first application of HQA 2μM (1) were not significantly different from baseline responses (c). However, after the application of Ro 25-6981 3μM, the second application of HQA 2μM (2) caused a significant enhancement of paired-pulse ratios (p<0.001, n=4 at all interstimulus intervals). During the inhibition by PEAQX 0.1μM, the third application of HQA 2μM (3) saw paired-pulse interactions remain similar to baseline responses (figure 57).
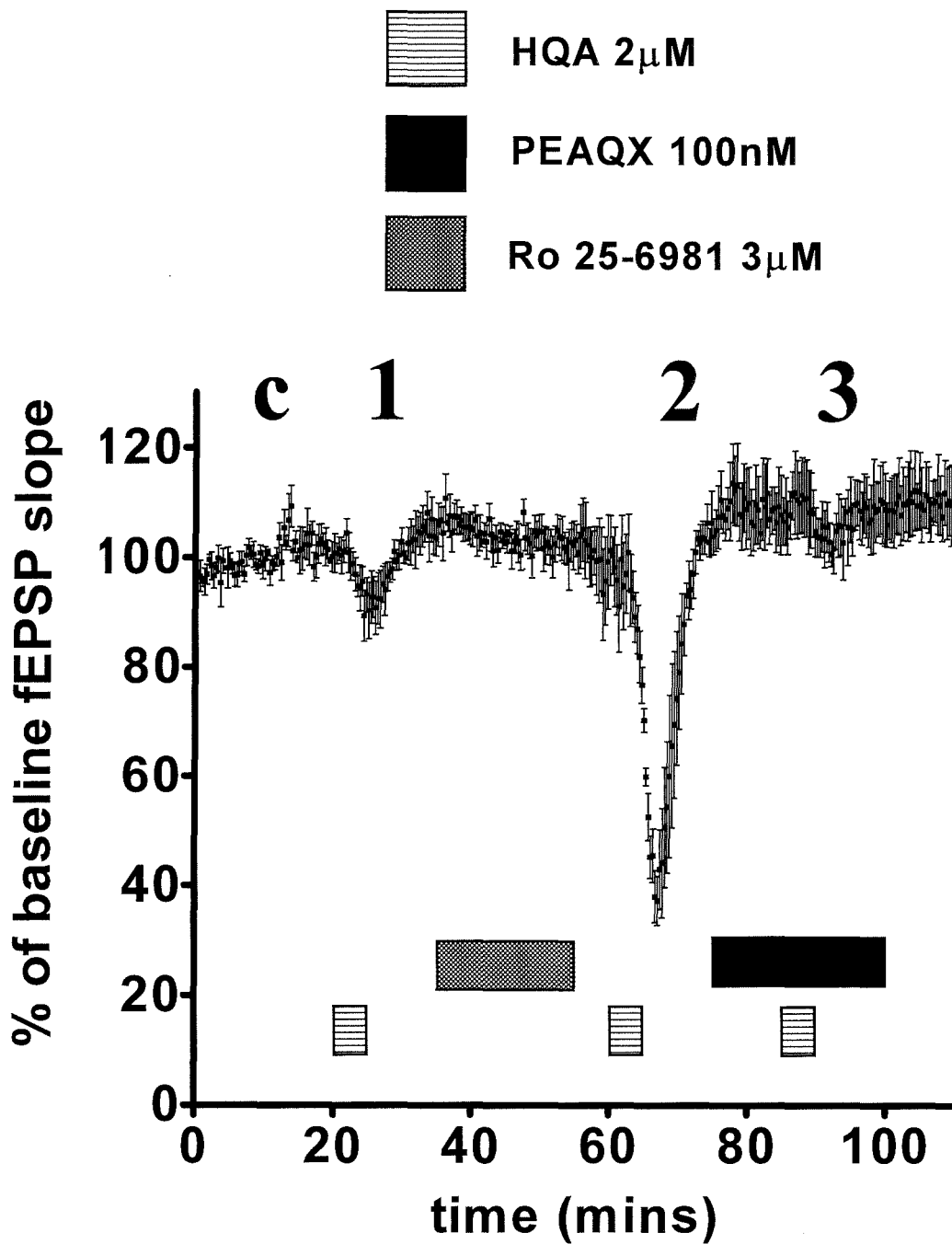
Figure 56. Effect of HQA, Ro 25-6981 and PEAQX on fEPSP slope.

A plot showing the effect of a low concentration of homoquinolinic acid 2μM (1) that initially produced a small, insignificant depression of the baseline fEPSP slope (c). Following the perfusion of Ro 25-6981 3μM, a second application of homoquinolinic acid 2μM (2) produced a significant depression of fEPSP slope to 37.33±4.43% (p=0.0261). The co-perfusion of PEAQX 0.1μM, an NR2A selective antagonist, inhibited the depression of the third application of homoquinolinic acid 2μM (3). Each point shows the mean±s.e.m. for n=4 experiments.
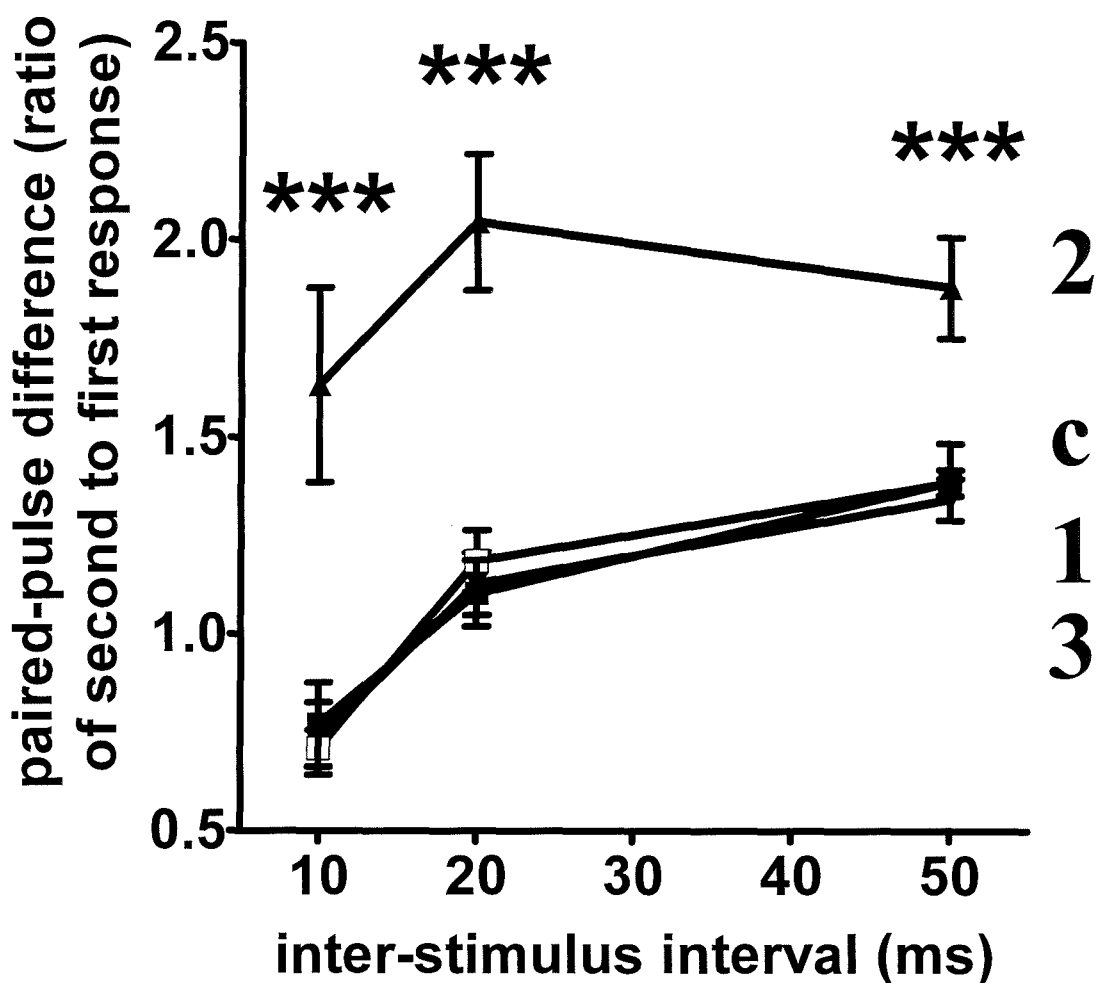
Figure 57. Effect of HQA, Ro 25-6981 and PEAQX on paired-pulse interactions.

This plot shows the paired-pulse interactions recorded during the co-perfusions of HQA $2\mu M$, Ro 25-6981 $3\mu M$ and PEAQX $0.1\mu M$. Points correspond to the time course of figure 56. Paired-pulse interactions during the first application of homoquinolinic acid $2\mu M$(**1**) were not significantly different from baseline responses (**c**). However, after the application of Ro 25-6981 $3\mu M$ the second application of homoquinolinic acid $2\mu M$ (**2**) caused an enhancement of the paired-pulse interactions at 10ms ($p<0.001$), 20ms ($p<0.001$) and 50ms ($p<0.001$) compared to baseline interactions. During the NR2A inhibition of PEAQX the third application of homoquinolinic acid $2\mu M$ (**3**) saw the paired-pulse interactions return to baseline responses (n=4). Each point shows the mean±s.e.m. for $n=4$ experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Points correspond to the time course in figure 56.

Protein synthesis

In view of the latency of onset, the long-lasting effect of Ro 25-6981 and the involvement of a protein phosphatase signal transduction step the question arose of whether any new protein synthesis was required and induced. To investigate this possibility anisomycin 30μM was used, which has previously been shown to block the maintenance of LTP and LTD at this concentration and over this timescale (Grollman, 1967; Otani *et al.*, 1989; Manahan Vaughan *et al.*, 2000; Karachot *et al.*, 2001). An initial application of HQA 2μM caused a small depression to 82.68±3.16% of baseline fEPSP slope. Anisomycin 30μM and the subsequent co-perfusion of Ro 25-6981 3μM had no significant effect on the baseline fEPSP slope. A second application of HQA 2μM produced a large and significant depression of fEPSP slope to 0.91±3.0% (p=0.0152, n=4) compared to the initial application of HQA 2μM. A similar profound depression of fEPSP slope to 6.05±4.86% (p=0.0128, n=4), again in comparison to the initial application of HQA 2μM, was obtained with a subsequent application of HQA 2μM (figure 58). This suggests that new protein synthesis is not required for the potentiation by Ro 25-6981.
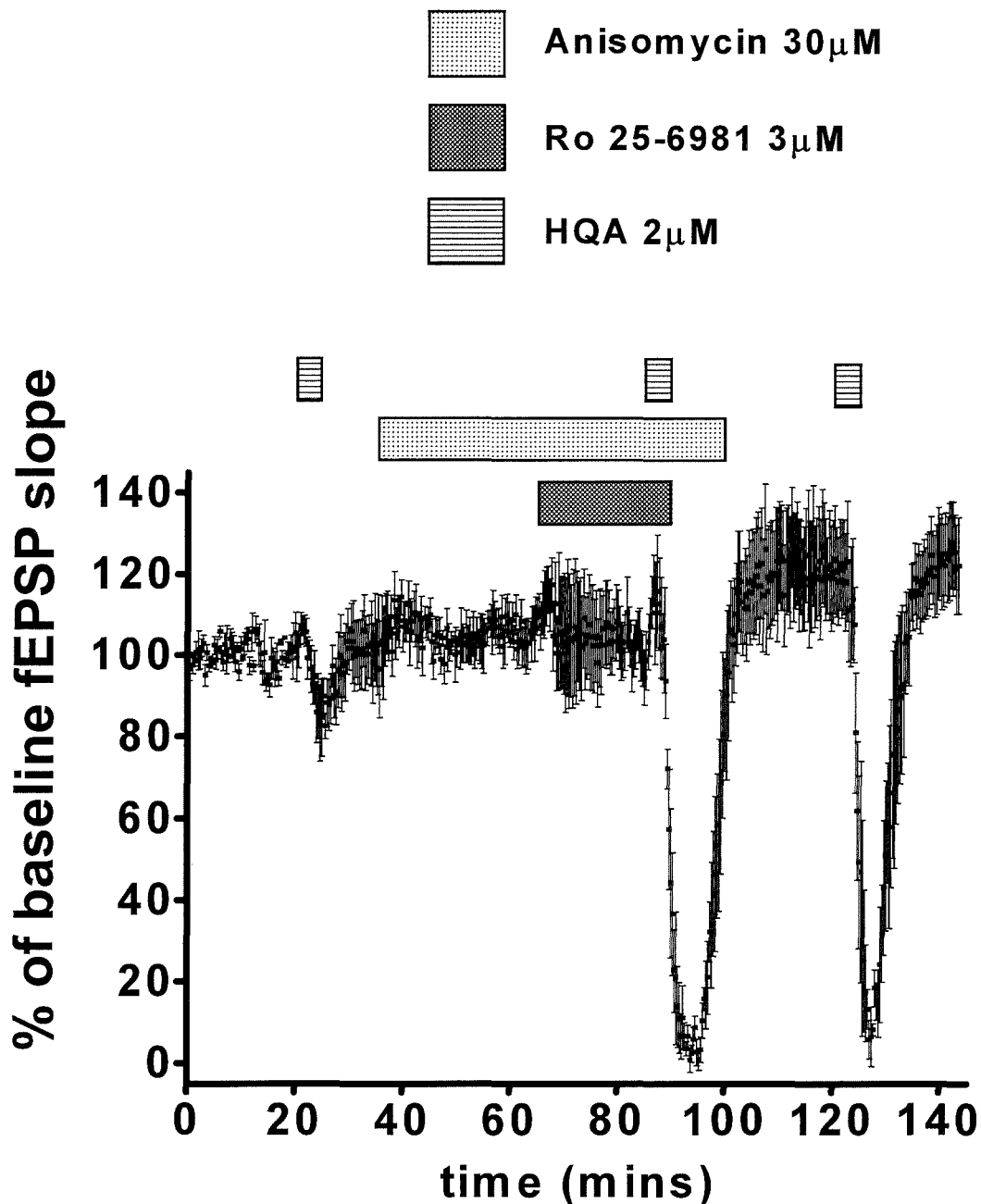
Figure 58. Effect of HQA, Ro 25-6981 and anisomycin upon fEPSP slope.

Plot showing that an initial application of homoquinolinic acid 2μM caused a small depression of baseline fEPSP slope, the perfusion of anisomycin 30μM and subsequent co-perfusion of Ro 25-6981 3μM had no significant effect on the baseline fEPSP slope. The second application of homoquinolinic acid 2μM produced a depression to 0.91±3.04% (p=0.0152,n=4) compared to the trough of the initial application of homoquinolinic acid 2μM. After washing out for 20 minutes a further application of homoquinolinic acid 2μM caused a similar depression of fEPSP slope to 6.05±4.86% (p=0.0128, n=4) again in comparison to the trough depression of the initial application of homoquinolinic acid 2μM. Each point shows the mean±s.e.m. for *n*=4 experiments.

AMPA and kainate receptors

The earlier paired-pulse data suggested that there might be a presynaptic component during the NMDA, HQA and QA evoked depressions of fEPSP slope. AMPA and kainate receptors are the main glutamatergic receptors responsible for basal synaptic transmission and the generation of currents underlying the fEPSP. AMPA and kainate were used at 100nM and 25nM respectively, concentrations that produced small depressions of the fEPSP slope. In both cases, the application of Ro 25-6981 $3\mu M$ had no effect on the responses, confirming that this effect is specific to NMDA receptor activation (figures 59 and 60). This is consistent with the absence of effect of Ro 25-6981 alone upon baseline responses.
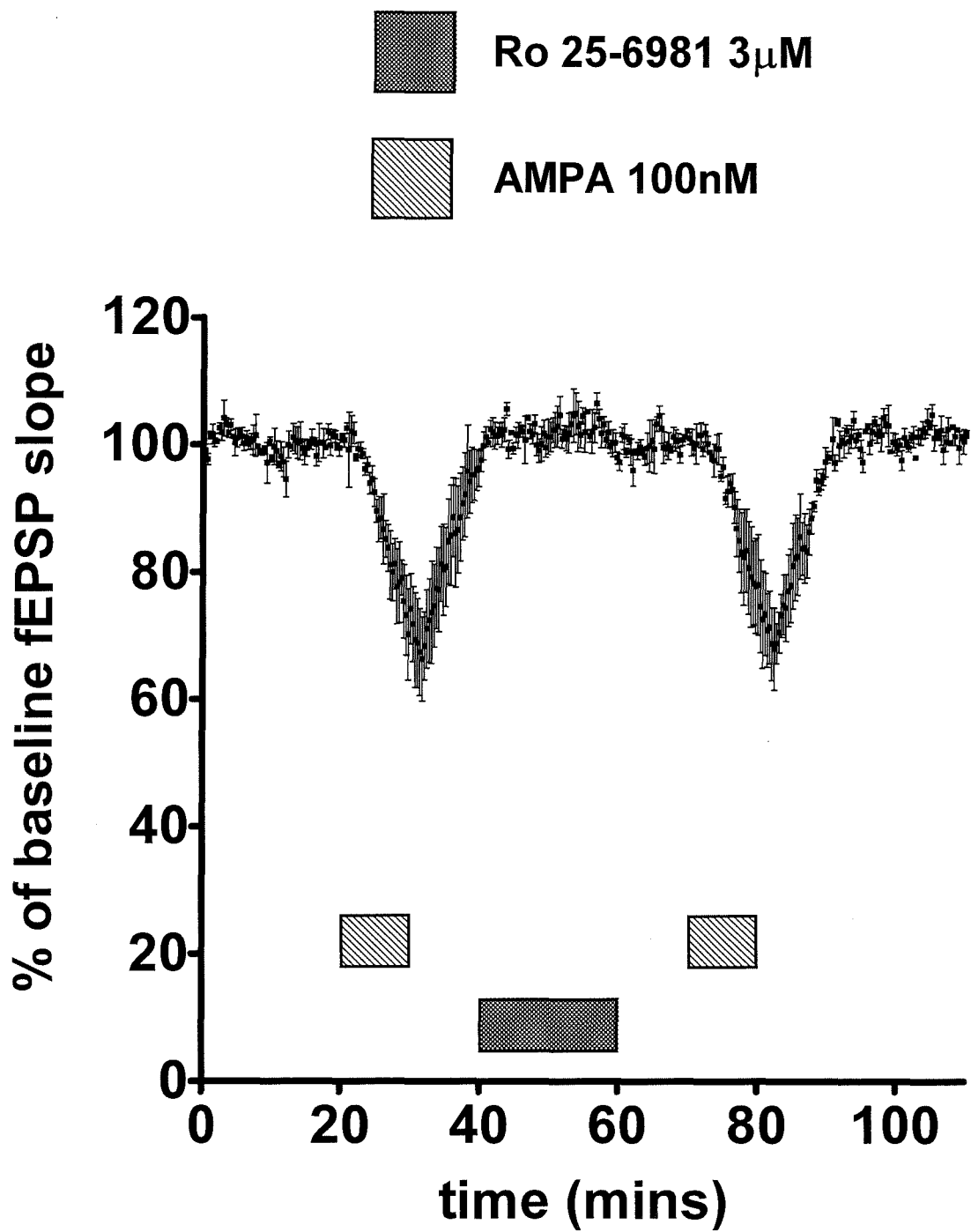
Figure 59. Effect of Ro 25-6981 and AMPA on fEPSP slope.

Plot showing the effect of 100nM AMPA applied before and after the perfusion of 3µM Ro 25-6981, the response to AMPA was unchanged. Each point shows the mean±s.e.m. for n=3 experiments.
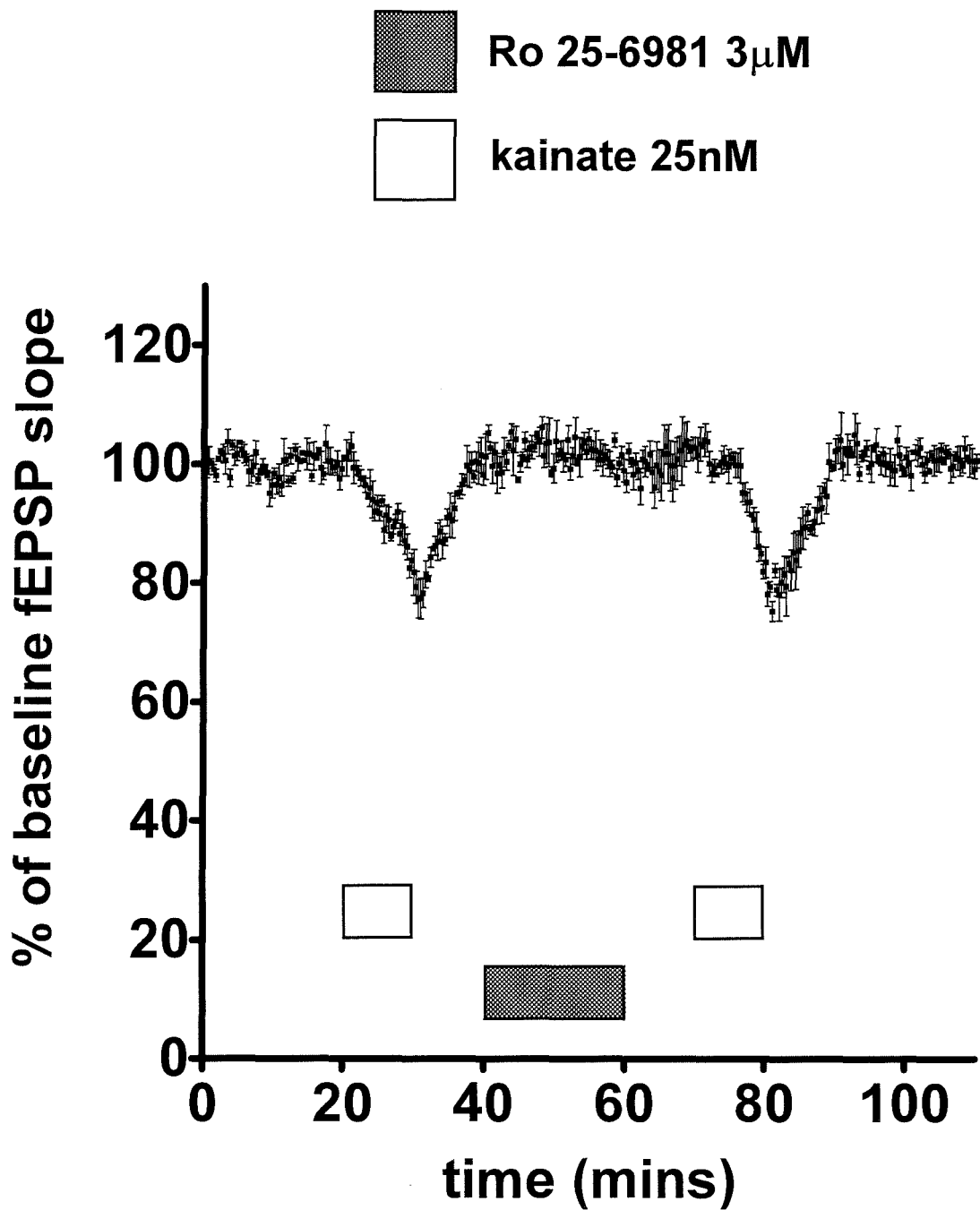
Figure 60. Effect of Ro 25-6981 and KA on fEPSP slope

Plot showing the effect of 25nM kainate applied before and after the perfusion of 3μM Ro 25-6981, the response to kainate was unchanged. Each point shows the mean±s.e.m. for $n=3$ experiments.

Discussion

In order to expose a response to the activation by homoquinolinic acid of the uncharacterised binding site reported by others the non-selective EAA antagonist kynurenic acid, the competitive NMDA receptor antagonist 2-AP5 and the non-competitive antagonist dizocilpine (MK-801) were used. 2-AP5, as with some competitive antagonists (straight chain, piperidine and piperazine structures) displays the subunit potency NR2A > NR2B > NR2D > NR2C (Laurie & Seeburg, 1994). However, neither kynurenic acid, 2-AP5, dizocilpine nor PEAQX revealed a novel action of homoquinolinic acid on the fEPSP slope. Therefore, the novel binding site for homoquinolinic acid does not affect acutely recorded synaptic potentials in the hippocampus and homoquinolinic acid appears to be a selective agonist at NMDA receptors that include the NR2A subunit.

Particularly when studied on fEPSPs, the paired-pulse paradigm represents a valuable means of assessing the actions of compounds on presynaptic terminals. The paired-pulse inhibition obtained at interpulse intervals of 10ms results from the depletion of transmitter from presynaptic stores (Burke & Hablitz, 1994; Wilcox & Dichter, 1994; Hashimoto & Kano, 1998), and is reduced by agents or procedures which decrease transmitter release. Paired-pulse facilitation, observed at longer interpulse intervals, is due to the residual intraterminal Ca^{2+} which increases transmitter release

(Hess *et al.*, 1987; Wu & Saggau, 1994; Debanne *et al.*, 1996; Kleschevnikov *et al.*, 1997). The modification of paired-pulse interactions by homoquinolinic acid and quinolinic acid suggests that at least part of its ability to depress the fEPSP slope is presynaptic in origin. Indeed, there is ample evidence corroborating the existence of presynaptic NMDA receptors in several regions of the brain (Berretta & Jones, 1996; Hamada *et al.*, 1998; Glitsch & Marty, 1999; Woodhall *et al.*, 2001), including the hippocampus (Breukel *et al.*, 1998). Alternatively, a retrograde signalling molecule released postsynaptically in response to depolarisation by homoquinolinic acid may mediate this effect indirectly or other postsynaptic effects may confound the changes in PPD and PPF (Foster & Richardson, 1997). The possibility that adenosine released in response to neuronal depolarisation could act as a retrograde inhibitor was investigated using the selective A_1 receptor antagonist DPCPX. However, DPCPX had no effect upon the magnitude of the depression caused by homoquinolinic acid or paired-pulse interactions suggesting that the presynaptic activity of homoquinolinic acid is not dependent upon the activation of presynaptic adenosine A_1 receptors.

As previously seen with NMDA, Ro 25-6981 potentiated the responses to HQA and QA on fEPSP slope and altered paired-pulse interactions. One possibility for the potentiating effect of Ro 25-6981 is that it causes an increase in the release probability of glutamate from the presynaptic

boutons. This is unlikely as there is no effect of Ro 25-6981 on baseline fEPSP or on comparable responses to AMPA and kainate. Another possibility is that Ro 25-6981 increases the local release of an endogenous NMDA agonist such as glycine, homocysteate, cysteine sulphinate or aspartate (Patneau & Mayer, 1990; Davanger et al., 1994). Whilst glycine has a high affinity and is thought to saturate NR1/NR2B receptors, it has a lower affinity for NR1/NR2A receptors, which under resting concentrations of glycine could be unsaturated and may be potentiated with rising glycine concentrations.

CCMQ was not able to block the depression of fEPSP slope at a similar concentration at which it displaced the binding of HQA in binding studies. Furthermore, the Ro 25-6981-enhanced response to HQA was similarly not blocked. This suggests that CCMQ is not acting as an antagonist in its ability to displace HQA from NR2A subunits in the previous studies. Considering the slight, yet significant, increases in fEPSP slope elicited by CCMQ under the influence of Ro 25-6981 it is possible that CCMQ is a weak NR2A agonist. This will best be confirmed using recombinant NR1/NR2A receptors expressed in Xenopus oocytes. Once this has been achieved, a study of structure-activity relationships could lead to more potent and selective NR2A agonists.

The absence of effect of zinc as an NR2A antagonist may be due to the experimental conditions where the level of NR2A inhibition is already

saturated by the concentrations of zinc contaminating the ACSF. Also there may be physiological mechanisms that regulate zinc inhibition still active in the intact slice preparation, such as tyrosine kinase src phosphorylation and chelation of zinc by thiol-reducing agents (Paoletti *et al.*, 1997; Arden *et al.*, 1998; Zheng *et al.*, 1998). In addition, differences between native heteromeric receptors and the recombinant receptors may weaken the effectiveness of zinc. Zinc is concentrated by certain neurones into synaptic vesicles and can be released in a Ca^{2+}-dependent manner at some synapses including the mossy fibre-CA3 pyramidal synapse (Dingledine *et al.*, 1999).

The effect of the NR2A antagonist PEAQX demonstrates that the NR2A subunit-containing NMDA receptors gate the currents involved in the depression of fEPSP slope and the associated paired pulse interactions. The enhancements of paired-pulse interactions are associated both with HQA alone and under the influence of Ro 25-6981, where the NR2B subunit is in both an activated and inhibited state. This suggests that the NR2B subunit cannot independently alter the presynaptic element suggested by paired-pulse results although it may be able to do so indirectly by its modulation of NR2A subunits, which alone seems to modulate directly the enhancement of paired-pulse interactions and the depression of the fEPSP slope.

Chapter 6. General Discussion

Negative coupling of NR2B to NR2A subunit-containing receptors

This study has suggested that acutely activated, tonically activated or constitutively active NR2B subunits maintain an inhibitory restraint upon the activation of the NR2A subunit-containing NMDA receptors by way of a protein phosphatase 2B (calcineurin) transduction step. The NR2A subunit-containing receptor seems to be the principal receptor involved in postsynaptic depolarisation and presynaptic alteration of release probability. The NR2B subunit has no observable function other than to gate the activity of the NR2A subunit-containing NMDA receptors. A recent report investigating the properties of NR1/NR2B/NR2D receptors expressed in newborn rat hippocampal granule cells has also observed evidence of negative coupling (Pina Crespo & Gibb, 2002). Additionally, NR3A subunits have been shown to negatively modulate NMDA receptors (Das *et al.*, 1998). Although the effect of Ro 25-6981 is dependent on the presence of an NMDA receptor agonist to observe its effect, it is a persistent change in the weight of the synapse over a timescale reported to be associated with a potentiation of NMDA (Watt *et al.*, 2004). Furthermore, it may involve co-incident pre- and postsynaptic activity suggestive of a Hebbian process. The NMDA receptor has a central role in

the induction of LTP and LTD, the negative coupling demonstrated in this study has significant implications for determining both the threshold for LTP and LTD induction and perhaps the magnitude of plasticity induced. The cellular mechanism by which this enhancement is produced is not entirely understood. Such an enhancement has very exciting implications for our understanding of NMDA receptor physiology and pharmacology.

Multimeric NMDA receptor composition

Nicotinic acetylcholine receptors are pentamers, voltage-gated potassium channels are tetramers and gap junction proteins mediating coupling at electrical synapses are hexameric (or hexamonic). Glutamate receptors were often supposed to be pentamers because they, like the acetylcholine receptor are ligand gated. Equal evidence exists for both a tetrameric or a pentameric structure from receptor subunit studies (Behe *et al.*, 1995; Ferrer Montiel & Montal, 1996; Premkumar & Auerbach, 1997; Laube *et al.*, 1998; Mano & Teichberg, 1998; Rosenmund *et al.*, 1998). However, these studies are designed to identify the number of functional binding sites in a receptor but are based upon the assumption that each binding site in a receptor operates independently of other subunits. This study has suggested that there is a negative coupling between the NR2A and the NR2B receptor subunits and it has been reported by others of a negative coupling in receptors containing NR3A and NR2A subunits (Ciabarra *et al.*, 1995; Das *et al.*, 1998) and NR2B and NR2D subunits (Pina Crespo &

Gibb, 2002). This observation suggests that the analysis of dose-response curves (Laube *et al.*, 1998) and patterns of main and sub-conductance states (Behe *et al.*, 1995; Premkumar & Auerbach, 1997) may be more complicated and the heteromeric structure of the native NMDA receptor may have been miscalculated.

Presynaptic NMDA receptors

Demonstrating the role of presynaptic receptors definitively ideally requires the fulfilment of five criteria (McGehee & Role, 1996; MacDermott *et al.*, 1999):

1. Evidence for the presence of presynaptic receptors.

2. Activation of these receptors exogenously produces the presynaptic effect that alters release probability (that mimics physiological control).

3. Presence of an endogenous ligand at the synapse that can be released under physiological conditions for activation of the presynaptic receptor.

4. Antagonists that block the presynaptic ionotropic receptors block synaptic plasticity.

5. Activation of the presynaptic receptor leads to the observed change in release through a characterised mechanism.

In answer to these criteria:

1. AMPA, kainate and metabotropic glutamate receptors have all been established as presynaptic autoreceptors in the hippocampus (Vazquez *et al.*, 1994; Chittajallu *et al.*, 1996; Alici *et al.*, 1997), modulating excitatory transmission by altering glutamate release probability. NMDA receptors have been amply demonstrated on the postsynaptic membrane (Petralia *et al.*, 1994; Racca *et al.*, 2000) and there is accumulating evidence demonstrating a presynaptic locus. Presynaptic NMDA autoreceptors have been reported in the cerebellum (Glitsch & Marty, 1999), spinal cord (Liu et al., 1997), hippocampus (Breukel *et al.*, 1998), suprachiasmatic nucleus (Hamada et al., 1998) and entorhinal cortex (Berretta & Jones, 1996). Furthermore, presynaptic NMDA receptors have been reported to be NR2B subunit-containing and tonically activated, causing an increase in glutamate release probability, dependent upon Ca^{2+} influx, in the entorhinal cortex (Woodhall et al., 2001). Additionally, immunohistochemical studies have found evidence for presynaptic NMDA receptors (Liu *et al.*, 1994; Paquet & Smith, 2000).

2. This report has suggested, through the changes observed in paired-pulse interactions, that NMDA receptor activation by HQA, QA and NMDA generated an effect with a presynaptic element. However, whilst PPF and PPD are generally accepted to be presynaptic phenomenon, it does not follow that postsynaptic effects cannot influence their appearance in field recordings (Foster & Richardson, 1997).

3. The endogenous ligand at these synapses is glutamate, which could act under physiological conditions to activate a presynaptic receptor. Furthermore, this study has demonstrated this effect by using another endogenous ligand quinolinic acid.

4. This investigation has demonstrated that both 2-AP5 and PEAQX can block NMDA-receptor dependent LTP. Furthermore, it has been reported recently that Ro 25-6981 is able to inhibit LTD (Sjostrom *et al.*, 2003; Liu *et al.*, 2004; Massey *et al.*, 2004)

5. The inhibition of this effect by cyclosporin A, which modulates protein phosphatase 2B (calcineurin) activity, demonstrates a distinct and well-characterised signal transduction pathway.

The lack of sufficiently compelling evidence on point 2 in this study means that there is not full qualification for a presynaptic NMDA receptor.

Therefore, the possible involvement of a postsynaptic receptor with a retrograde signalling mechanism must be considered. In NMDA receptor dependent synaptic plasticity, postsynaptically induced processes such as LTD and LTP are still able to trigger presynaptic changes through retrograde messengers such as nitric oxide activating PKG (Reyes Harde *et al.*, 1999) and endocannibinoids acting upon CB1 receptors (Chevaleyre & Castillo, 2003; Sjostrom *et al.*, 2003) or release from other sources such as glia (Parpura *et al.*, 1994).

Whilst changes in paired-pulse interactions are widely regarded to be due to presynaptic alterations in release probability; when studied on fEPSPs and especially where there are changes in the size of the postsynaptic potential in response to the test agents many factors affect them which can affect their interpretation. Including, changes in presynaptic release probability (Andreasen & Hablitz, 1994), activity of presynaptic autoreceptors on inhibitory or excitatory terminals (Davies *et al.*, 1990; Stanford *et al.*, 1995), modulation of postsynaptic conductances and the presence of GABA-ergic inhibition following the fEPSP and the depression cause by NMDA agonist action (Duguid & Smart, 2004).

Also, considering the intracellular data showing a purely postsynaptic manifestation of these effects a further study of the contribution of different NMDA subunits is required on clear presynaptic phenomenon such as spontaneous miniature EPSPs or by concurrently measuring by patch-

clamp whole cell recording corresponding presynaptic and postsynaptic neurones.

It is clear that NR2A and NR2B subunit-containing NMDA receptors are found postsynaptically, as NR1/NR2A, NR1/NR2B or NR1A/NR2A/NR2B heteromers, at the synaptic site whilst NR1/NR2B receptors are found exclusively at the extrasynaptic site (Li *et al.*, 1998; Stocca & Vicini, 1998; Tovar & Westbrook, 1999). A tonically activated state of the agonist-preferring NR2B subunit-containing receptor could be achieved by being located near to the release site of glycine and glutamate on the presynaptic bouton. However, the NR2B subunit in both it's activated and inhibited state is associated with enhancements in paired-pulse inhibition whereas, only the blockade of the NR2A subunit prevents the enhancement of paired-pulse interactions. Alternatively, the NR2B subunit may be in a constitutively active state that is independent of agonist binding as has been observed with other receptors (Scheer & Cotecchia, 1997; Behan & Chalmers, 2001; Richard *et al.*, 2001). Interestingly NR2B subunit-containing receptors have a higher glutamate and glycine affinity, are less sensitive to tonic proton inhibition, and are insensitive to desensitisation, which suggests that they would be more sensitive detectors of glutamate than NR2A subunit-containing receptors, especially at extrasynaptic sites. Applications of HQA, QA and NMDA by themselves and under the influence of NR2B antagonism are associated with depressions of fEPSP

slope and increases in paired-pulse ratios. These are both blocked by PEAQX, the NR2A antagonist, suggesting that the presynaptic element may be NR2A subunit-mediated. It is difficult from the current study to make any definitive resolution of the receptor populations found pre- and postsynaptically or the subunit makeup of such receptors.

It may be possible to investigate these further using single channel analysis of outside-out patches from these tissues. Using selective antagonists, it can be determined whether the negative coupling of NR2B upon NR2A is between subunits in a single heterotrimeric NR1/NR2A/NR2B receptor (rather than between discrete heterodimeric NR1/NR2A and NR1A/NR2B receptors). This may also address the location of these subunits at pre- or postsynaptic sites. There is evidence for the co-localisation of NR2A and NR2B subunits in the hippocampus, and specifically within the CA1, CA2 and CA3 regions (Fritschy et al., 1998).

Excitotoxicity

This report has demonstrated that, far from being neuroprotective, Ro 25-6981 actually precipitates an excitotoxic insult from otherwise benign, depolarising concentrations of NMDA and HQA. This observation is contradictory to previous reports of NR2B antagonists being neuroprotective in excitotoxic studies (Gotti *et al.*, 1988; Fischer *et al.*, 1997; Lynch *et al.*, 2001; Gill *et al.*, 2002) but some reports have

corroborated this study (Zou & Crews, 2003). The age of tissue used in investigations could account for the reason for the difference in these results. In previous studies immature tissues were used whereas this study has used adult tissues in a well-proven experimental model of excitotoxicity (Schurr et al., 1995). As mentioned in the introduction, the expression levels and distribution of NMDA subunits change dramatically during development, especially in respect of the NR2A and the NR2B subunits. This developmental change is responsible for the alteration in susceptibility of tissues to excitotoxicity and also to the induction of synaptic plasticity (Dudek & Bear, 1993; Liu et al., 1996). The use of immature tissues to investigate excitotoxicity is dictated to some extent by methodological constraints. The rationale for using these tissues in the investigation of stroke, which is an almost exclusive threat to the adult population, is difficult to justify, especially here, considering the disparity between these results. Additionally, the study of tissue from different areas of the brain and even different regions of the hippocampus will dictate the influence of NMDA receptors and the expression of different types and quantity of NR2 subunits. A recent study of cultured hippocampal neurones reported that the selective activation of synaptic NR2A subunit-containing NMDA receptors is associated with the potent activation of cAMP response element binding protein (CREB) and subsequent brain-derived neurotrophic factor (BDNF) gene expression. This leads to an anti-apoptotic cell-signalling pathway. However, the activation of NR2B

sensitive extrasynaptic NMDA receptors by bath application of NMDA agonists or exposure to hypoxic/ischaemic conditions, which cause the reversal of glutamate transporters (Rossi et al., 2000), is associated with the Ca^{2+}-dependent transient activation of CREB. This leads to a CREB shut-off signal of the synaptic pro-survival pathway and leads to cell death (Hardingham et al., 2002). It can be concluded that excessive overactivation of the NR2A-subunit associated channel could be directly responsible for the large Ca^{2+} influx that triggers excitotoxicity. The NR2B subunit acting to gate the activity of the NR2A subunit-containing receptor and when it is inhibited this restraint is lost.

An obvious question arises as to whether there a correlation between decreased levels of NR2B subunit expression, mRNA levels and genetic expression in adult patients susceptible to stroke or in geriatric patients who have an age-related susceptibility to stroke and in other relevant disease states. Indeed, the NR2B subunit is already implicated in an impressive number of age related diseases and developmentally, the mRNA and the protein expression of the NR2B subunit decreases significantly between the ages of 3 and 30 months in mice (Magnusson, 2000; Magnusson et al., 2002) which is similar to the decrease in the human hippocampus (Law et al., 2003). It is possible to further develop this study by investigating the effect of selective subunit NMDA antagonists in hypoxia, ischaemia and seizure disorder, which are known to have NMDA receptor components.

The potential for the development of drugs to modulate this NR2B-NR2A interaction is very exciting.

Phosphatase hypothesis

This report has suggested that NR2B subunits maintain an inhibitory control over NR2A subunit-containing receptors. It could achieve this by being acutely activated, tonically activated or remaining constitutively active physiologically, maintaining the phosphorylation state of an unidentified protein. The superfusion of Ro 25-6981 blocks the NR2B subunit leading to the calcineurin-mediated dephosphorylation of an unknown protein residue resulting in the potentiation of NR2A subunit-containing NMDA receptor responses. NR2 subunits are known to be highly phosphorylated under basal conditions whilst NR1 subunits are very weakly phosphorylated (Lau & Huganir, 1995; Hall & Soderling, 1997; Smart, 1997). Therefore, the dephosphorylation step described here may be acting directly upon either the NR2A or the NR2B subunit to produce the enhancement of responses to NMDA and HQA. However, it may also be acting on the NR1 subunit or associated proteins such as PSD-95 and SAP 102 or an unrelated and possibly novel site. Interestingly, there is strong evidence for a tyrosine residue that is phosphorylated and leads to potentiation of the NMDA receptor and is found on the NR2A or NR2B subunit-containing receptor, as seen with insulin potentiation and LTP (Chen & Leonard, 1996; Kohr & Seeburg, 1996; Rosenblum *et al.*, 1996;

Smart, 1997; Zheng *et al.*, 1998; Christie *et al.*, 1999). The absence of effect of okadaic acid, a potent inhibitor of the serine/threonine protein phosphatases types 1 and 2A, rules out these serine residues being involved. The next step is to identify if there are changes in the levels of phosphorylation of these subunits. This can be achieved by a combination of immunoprecipitation using NR1, NR2A and NR2B subunit specific antibodies to these proteins, then immunoblotting with anti-phosphotyrosine and anti-phosphoserine specific antibodies and estimating changes in levels of phosphorylation compared to controls with a chemiluminescent secondary antibody. There are many studies that correlate increases in phosphorylation with increases in receptor activity and that dephosphorylation is associates with reductions in receptor activity (Smart, 1997). However, it was found in this study that the activity of calcineurin, which de-phosphorylates is associated with the potentiation of responses to NMDA. This is blocked by Cyclosporin A, a phosphatase inhibitor. Many of the previous studies were carried out using immature tissue as with previous excitotoxicity reports as mentioned previously. In addition to the developmental switch of subunit expression (Tovar & Westbrook, 1999) the basal levels of phosphorylation of the adult NMDA receptor do not reach maturity until 3-4 weeks post-natally (Cudmore & Gurd, 1991).

Synaptic plasticity

The entry of calcium into the neurone contributes ~16% of the NMDA receptor current but the subsequent activation of Ca^{2+}-dependent enzymes and signal transduction pathways induce potent effects (Schneggenburger et al., 1993). Both LTP and LTD can be induced by NMDA receptor activation, resulting in an influx of Ca^{2+} that triggers plasticity. The relative level, kinetics and location of the Ca^{2+} influx determining whether LTP or LTD is induced; a large, fast, synaptic influx inducing LTP and a relatively modest, prolonged, extrasynaptic influx inducing LTD (Lisman, 1989; Artola & Singer, 1993; Neveu & Zucker, 1996; Yang et al., 1999; Cho et al., 2001; Liu et al., 2004; Massey et al., 2004). The receptor kinetics of the NR2B subunit-containing receptor is relatively slower than the NR2A subunit-containing receptor resulting in calcium influxes that are consistent with LTD and LTP induction respectively (figure 61). This suggests that there is a role of NR2B in LTD that requires an NMDA-mediated influx of Ca^{2+} consistent with the NR2B subunit-containing NMDA receptor kinetics. Indeed, it has recently been reported that neocortical LTD is inhibited by NR2B antagonists affecting presynaptic receptors (Sjostrom et al., 2003), that LTD in the perirhinal cortex is inhibited by the NR2B antagonist Ro 25-6981 but not by the NR2A antagonist PEAQX (Massey, 2003; Massey et al., 2004) and that LTD in the hippocampus is NR2B dependent (Liu et al., 2004). The NR2A

subunit-containing receptor has kinetics consistent with those required for a large and rapid rise in intracellular calcium concentrations leading to LTP induction. During LFS protocols that induce LTD the NR1/NR2B receptors make a larger contribution to the total charge transfer and Ca^{2+} influx whereas during HFS protocols that induce LTP the NR1/NR2A mediates the greater total charge transfer and Ca^{2+} influx (Erreger et al., 2005). This study has demonstrated that the NR2A subunit is critically required in the induction of LTP. Furthermore, it is known that LTP requires protein kinase activation (Malenka et al., 1989) while LTD requires protein phosphatase activation (Mulkey et al., 1993; Mulkey et al., 1994). This study has also shown that the protein phosphatase 2B (calcineurin) inhibitor cyclosporin A is able to prevent the effect of Ro 25-6981. This is consistent with reports of calcineurin being critical in the induction of LTD (Mulkey et al., 1994). This study cannot rule out the involvement of the NR2B subunit in Schaffer collateral-CA1 LTP, especially considering the interaction of NR2B with the critical NR2A subunit-containing NMDA receptors described, the marginal reduction in LTP induced and the many reports implicating the NR2B subunit in LTP.

This report has suggested that NR2B subunit-containing NMDA receptors, which have been reported to be tonically or constitutively active (Sah et al., 1989; Berretta & Jones, 1996; Breukel et al., 1998; Woodhall et al., 2001) and are critically involved in the induction of LTD (Sjostrom et al., 2003),

negatively gate under physiological conditions the NR2A subunit-containing NMDA receptor. It is widely reported and our studies have also found that successful NMDA dependent LTD induction is very difficult to achieve whilst LTP induction is relatively easy (Kemp & Bashir, 2001; Massey *et al.*, 2004). It could be that such tissues, rather than being in a resting state, are in a maximal LTD state and are therefore only able to be potentiated towards an LTP state from which level they can then be easily depotentiated. This could be explained by differential expression of NR2 subunits in NMDA receptors. This study has not demonstrated that there is a way to increase the levels of the NR2B-mediated restraint and this may be because the level of restraint is saturated. A caveat to this link is the exclusivity to the NMDA receptor and the requirement of NMDA agonist to observe this phenomenon. The induction of LTP and LTD critically require the NR2A and NR2B subunits respectively but they are further associated with the trafficking of AMPA receptors to and from the postsynaptic membrane and an associated change in the synaptic weight of neuronal transmission independent of NMDA receptor agonists (Malinow & Malenka, 2002; Watt *et al.*, 2004). This is missing from the interaction this study has shown but if bridged might lead to the induction of LTP proper.

Donald Hebb's neurophysiological hypothesis states that '*When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.*' (Hebb, 1946).

A modification of this hypothesis has led to the Bienenstock-Cooper-Munro (BCM) theory of the sliding threshold of synaptic plasticity (Bienenstock *et al.*, 1982). It hypothesizes that active synapses grow stronger or weaker when postsynaptic activity exceeds or falls below a 'modification threshold', θ_m. Presynaptic activity triggers synaptic depression or potentiation depending on the concurrent level of postsynaptic activity. The history of integrated postsynaptic activity determines the threshold level at which plasticity is induced; the value of θ_m varies as a function of the history of integrated postsynaptic activity. As average activity falls or rises, so does the value of θ_m.

This hypothesis led to the observations in the visual cortex that experience drives NMDA receptor subunit switching from LTD-induction-preferring NR1/NR2B heterodimeric to LTP induction-preferring NR1/NR2B/NR2A heterotrimeric receptors (Kirkwood *et al.*, 1996; Philpott *et al.*, 2001). NR2A and NR2B subunit-containing receptors provide the biological basis for the BCM sliding threshold of synaptic plasticity. This model is further strengthened by the recent reports of NR2A and NR2B antagonist sensitive

induction of LTP and LTD respectively (Liu *et al.*, 2004; Massey *et al.*, 2004; Mallon *et al.*, 2005). The negative coupling of NR2B to NR2A subunit-containing receptors may represent a further mechanism for fine-tuning the strength of NMDA mediated synaptic transmission and for maintaining a threshold for induction of synaptic plasticity that only requires changes in the NR2B subunit. Perhaps by lateral diffusion between synaptic and extrasynaptic sites, in agreement with recent studies where the NR2A subunit does not appear to be very mobile (Groc *et al.*, 2004; Triller & Choquet, 2005). Furthermore, as well as the induction threshold for LTP or LTD induction there is also the magnitude of change in strength elicited (Castellani *et al.*, 2001). It was found in this study that whilst LTD could not be induced, the level of LTP that was reliably induced was of a particularly high magnitude. A level at which it was then possible to depotentiate from (data not shown). It may be that the experience-deprived environment the rats are familiar with, means that the biological basis for the sliding threshold is set such that they are in a maximal state of LTD and that only LTP can be induced. It has been reported that rats from enriched environments are more likely to exhibit LTD (T.V.P. Bliss, personal communication). The investigation of this could help to discover more about the biological basis for the magnitude of synaptic plasticity induced and the effects of environment (Castellani *et al.*, 2001).

NR2A

⊖

⊕ Adenylate cyclase

(staurosporine) LTP

(PEAQX)

cAMP

High [Ca²⁺]ᵢ

PKA

CaMKII – Ⓟ

(okadaic acid)

CaM

Phosphatase 1

⊖

Inhibitor 1

Inhibitor –Ⓟ
1

Low[Ca²⁺]ᵢ

CaMKII

(Ro 25-6981)

⊕

(cyclosporin A)

NR2B

Calcineurin

LTD
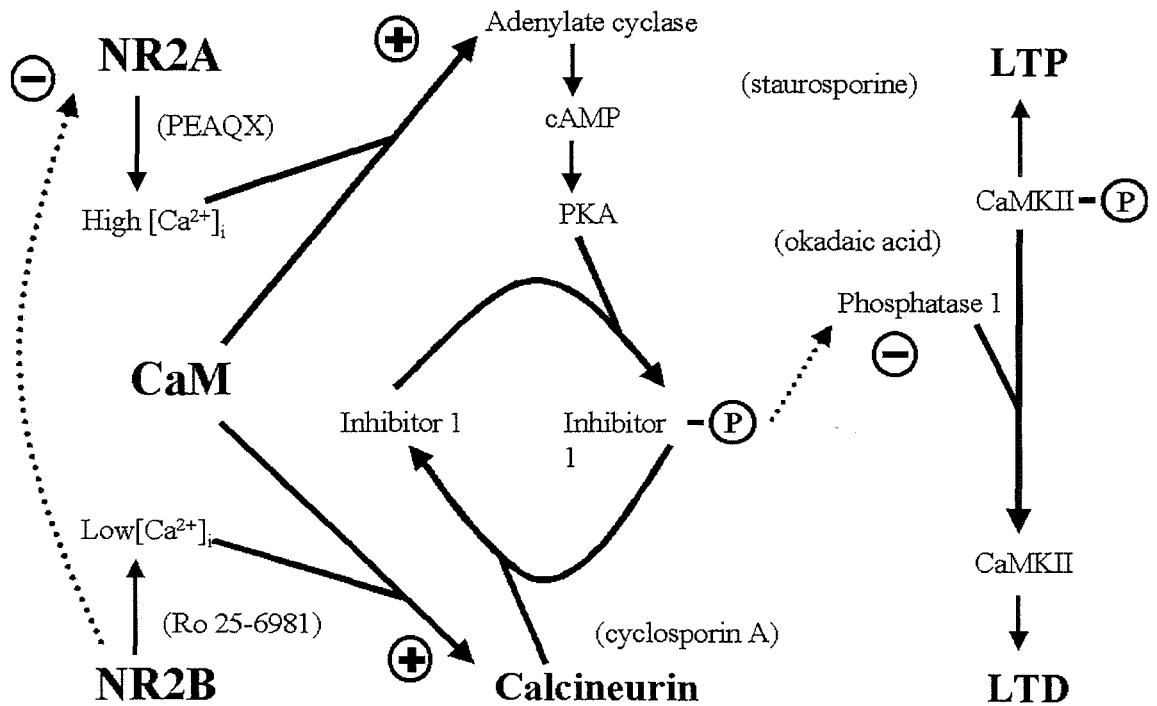
Figure 61. Phosphatase hypothesis.

This scheme summarises the intracellular transduction systems that account for the differing pharmacology and physiology of the NR2A and NR2B subunit-containing receptors, the associated phosphoprotein steps and their influence over LTP and LTD. In brackets are the relevant antagonists (Mulkey *et al.*, 1994; Revest & Longstaff, 1998).

Appendix 1

Methods

Experiments were carried out by Professor T.W. Stone to further investigate the actions of Ro 25-6981 using intracellular recording techniques.

Intracellular recordings were made using sharp glass microelectrodes (o.d. 1.5mm) (Clark Electromedical, Harvard Instruments). These were pulled on a Narashige vertical puller in a manually controlled two-stage process to produce pipettes with a shank length of approximately 20mm. The electrodes were filled with 1M potassium acetate, yielding a resistance of 80-100MΩ when measured in the extracellular space of the brain slices via the Neurolog amplifier. Potentials were amplified by a Neurolog 102 DC amplifier or by an Axoclamp-2A operated in bridge balance mode, and filtered between DC and 20KHz. The electrode was advanced in 2.5μm steps using a remotely controlled World Precision Instruments motorised stepper. When a neurone was approached, indicated by a rise in electrical noise, a brief increase of electrode capacitance ('buzz') was induced in order to facilitate entry. Cells were allowed to recover from penetration for at least 10 minutes before examining them for electrical properties and stability. Neurones were then used for experimentation if they displayed a

stable resting potential (±10% over 20 minutes) greater than 60mV and a spike of at least 70mV in response to an intracellular depolarising current pulse. Current pulses (0.1-1nA) were used to monitor membrane input resistance throughout the experiments. Records were calibrated using the amplifier's own calibration pulse (10mV), and were recorded directly onto a high frequency thermal chart recorder (Grass instruments Dash IV), although the filter settings were placed at DC to 10Hz to reduce interfering transients and action potentials.

Results

NMDA

Four neurones were tested with a combination of NMDA and Ro 25-6981. In each case, NMDA was superfused at a concentration of 2.5uM for 10 minutes, with 20 minutes between each application. At least two control applications were made. At this concentration, the cells showed little or no response, the two applications of NMDA immediately prior to Ro 25-6981 yielding barely discernible depolarisations of 0.19±0.048, 0.26±0.059mV (n=4). Ten minutes after the second of these controls, superfusion of Ro 25-6981 (3uM) was commenced for 20 minutes. The next application of NMDA (parallel with the second 10 minutes of Ro 25-6981) produced a similarly weak effect (0.26±0.066mV). However, the next application, which was begun 20 minutes after ending the superfusion of Ro 25-6981, produced a substantial depolarisation (10.67±1.25mV; p<0.00001, n=4) (figures 62 and 63). This suggests that the negative coupling of NR2B to NR2A subunit-containing NMDA receptors is occurring at postsynaptic sites.

a

~1mV~ NMDA

b

c

d

e

f

300s

100mV
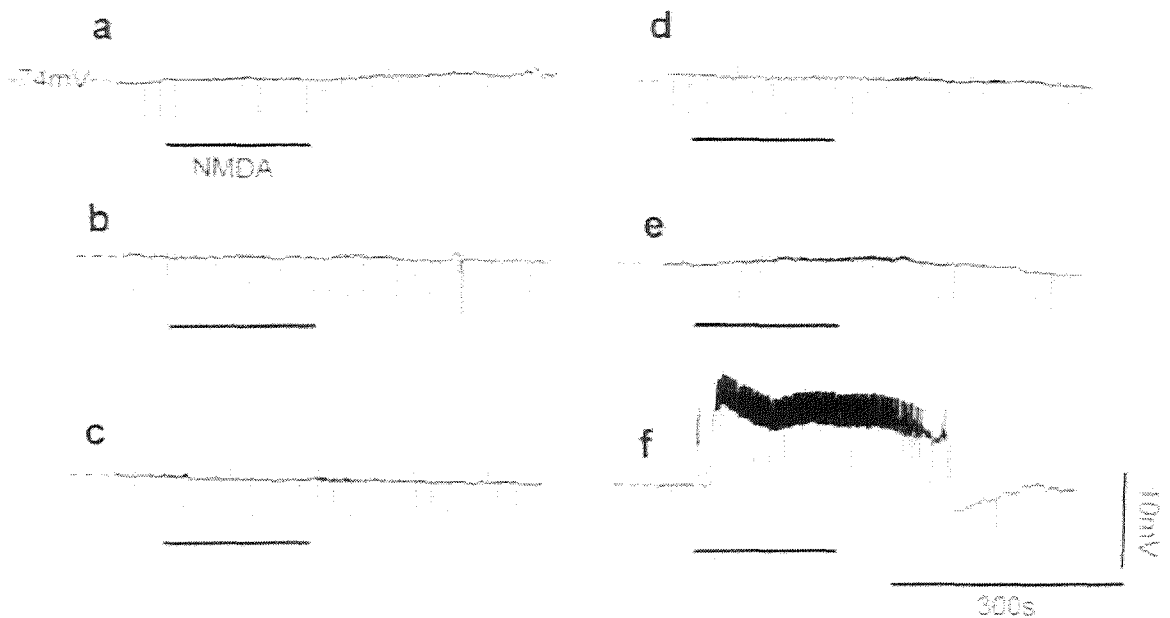
Figure 62. Intracellular recordings of NMDA responses and the effect of Ro 25-6981

Intracellular recordings from a pyramidal neurone of resting potential −74 mV, with input resistance monitored by hyperpolarising pulses of 0.3nA amplitude, 300 ms duration every 20 s. Recordings are from one of six neurons tested with 2.5μM NMDA and 3μM Ro 25-6981. In panels a–c, NMDA was superfused alone at 2.5μM for ten minutes, with twenty minutes between each application. Ten minutes after record c, Ro 25-6981 (3μM) was applied for twenty minutes. The next application of NMDA (d) (parallel with the second ten minutes of Ro 25-6981) still produced no clear response. The next application (e, twenty minutes after ending Ro 25-6981) indicates an increased frequency of excitatory synaptic potentials elicited by NMDA, while in panel (f), NMDA produced a substantial depolarisation with superimposed action potentials (truncated).
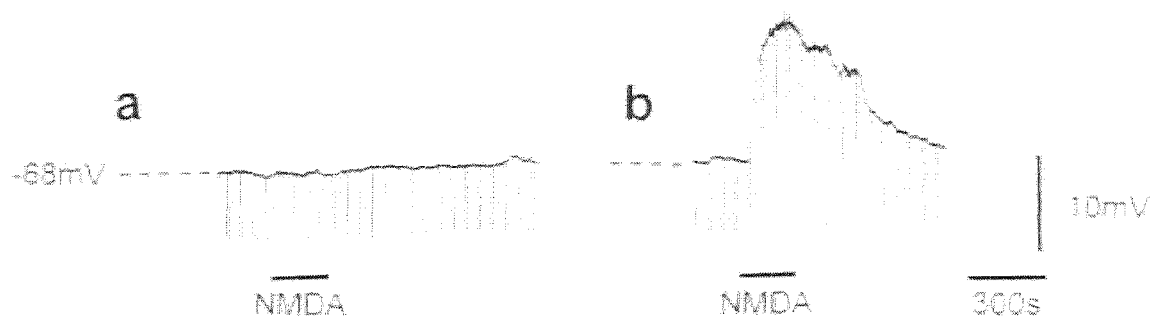
Figure 63. Intracellular recordings showing the effect of NMDA alone and after the application of Ro 25-6981.

Intracellular recordings from a pyramidal neurone of resting potential –68 mV, with input resistance monitored by hyperpolarising pulses of 0.3nA amplitude, 300 ms duration every 20 s. Recordings are from a second of the six neurons tested with 2.5µM NMDA and 3µM Ro 25-6981. NMDA was superfused alone at 2.5µM for ten minutes, with twenty minutes between three repeated applications (a). Ten minutes after record the third application had ended, Ro 25-6981 (3µM) was applied for twenty minutes. The next application of NMDA (parallel with the second ten minutes of Ro 25-6981) still produced no clear response. The next application (twenty minutes after ending Ro 25-6981) indicated an increased frequency of excitatory synaptic potentials elicited by NMDA, while in panel b, a further application of NMDA (fifty minutes after ending Ro 25-6981) produced a substantial depolarisation with superimposed action potentials (truncated).

Appendix 2

PEAQX is an NMDA receptor antagonists with a preference for the 1A/2A, rather than 1A/2B receptor composition.

Y. P. Auberson[*], S. Bischoff, K. Lingenhoehl, H. Neijt, M. F. Pozza, M. Schmutz

PEAQX is a water-soluble quinoxalinedione with a high affinity for the glutamate-binding site of NMDA receptors ($[^3H]$CGP39653 binding assay: IC_{50} = 11nM). At a concentration of 10 μM, PEAQX showed no additional activity in a battery of 68 radioligand binding assays for CNS receptors.

In vitro, PEAQX possesses a 126-fold selectivity for the NMDA 1A/2A over 1A/2B receptor composition expressed in *Xenopus* oocytes (Figure 1, IC_{50} = 14nM and 1800nM respectively). Experiments on embryonic cortical cells and on neocortical slices confirm these results on native receptors: Cultured embryonic cortical cells express NR2A subunits at a very low level, resulting in a low potency of PEAQX (IC_{50} = 1 μM). NR2A subunits are expressed to a much larger extent in adult slices, and the effect of PEAQX is therefore stronger in this model (IC_{50} = 68nM).

Figure 1: Effect of PEAQX on NMDA-induced currents in *Xenopus* oocytes expressing human NMDAR 1A/2A or 1A/2B. Structure of (1RS,1'S)-PEAQX.
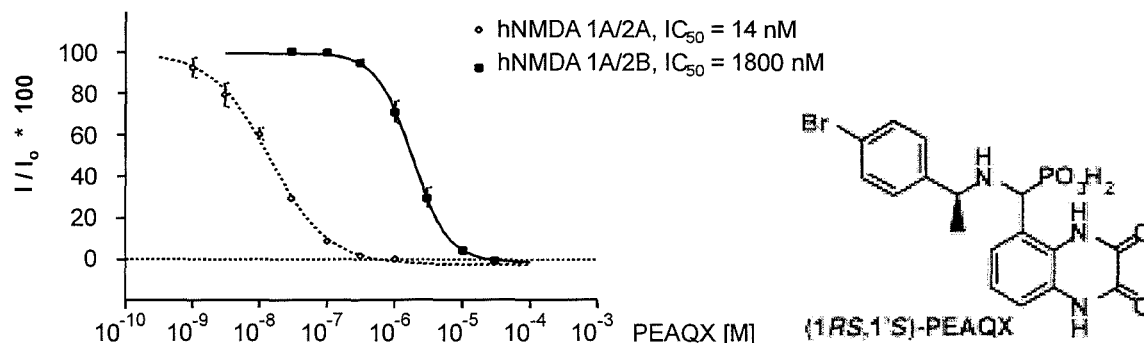
In vivo, PEAQX is a potent anticonvulsant in both DBA/2 mice (tonic convulsions: ED_{50} = 1.2 mg/kg, 1h i.p.) and in the mouse maximal electroshock test (ED_{50} = 5 mg/kg, 1h i.p.), with a long duration of action ($\geq$ 24h). Anticonvulsant activity after intravenous administration indicates a rapid brain penetration. The side effect profile of PEAQX is clearly different from non-selective or NMDA 1A/2B selective NMDA antagonists, as it causes no motor stimulation at anticonvulsant doses, does not disrupt the prepulse inhibition in the rat startle model, and does not substitute for PCP in rats trained in a two lever operant task.

[*] K-136.6.82, Novartis Pharma AG, 4002 Basel, Switzerland.
Tel. +41 61 696 8404, Fax +41 61 696 8676, yves.auberson@pharma.novartis.com

References

Abraham, W.C., Logan, B., Greenwood, J.M. & Dragunow, M. (2002) Induction and experience-dependent consolidation of stable long-term potentiation lasting months in the hippocampus. *J Neurosci*, **22**, 9626-9634.

Alger, B.E. (1984) Characteristics of a slow hyperpolarizing synaptic potential in rat hippocampal pyramidal cells *in vitro*. *J Neurophys*, **52**, 892-910.

Alger, B.E. (1991) Gating of GABAergic inhibition in hippocampal pyramidal cells. *Ann N Y Acad Sci*, **627**, 249-263.

Alger, B.E. & Nicoll, R.A. (1982) Pharmacological evidence for two kinds of GABA receptors on rat hippocampal pyramidal cells studied *in vitro*. *J Physiol*, **328**, 125-141.

Alger, B.E. & Teyler, T.J. (1976) Long-term and short-term plasticity in the CA1, CA3, and dentate regions of the rat hippocampal slice. *Brain Res*, **110**, 463-480.

Alici, K., Gloveli, T., Schmitz, D. & Heinemann, U. (1997) Effects of glutamate receptor agonists and antagonists on Ca2+ uptake in rat hippocampal slices lesioned by glucose deprivation or by kainate. *Neuroscience*, **77**, 97-109.

Alkon, D.L. & Nelson, T.J. (1990) Specificity of molecular changes in neurons involved in memory storage. *FASEB J*, **4**, 1567-1576.

Andreasen, W.W. & Hablitz, J.J. (1994) Paired-pulse facilitation in the denate gyrus: a patch-clamp study in rat hippocampus in vitro. *J Neurophysiol*, **72**, 326-336.

Anson, L.C., Chen, P.E., Wyllie, D.J., Colquhoun, D. & Schoepfer, R. (1998) Identification of amino acid residues of the NR2A subunit that control glutamate potency in recombinant NR1/NR2A NMDA receptors. *J Neurosci*, **18**, 581-589.

Anson, L.C., Schoepfer, R., Colquhoun, D. & Wyllie, D.J. (2000) Single-channel analysis of an NMDA receptor possessing a mutation in the region of the glutamate binding site. *J Physiol*, **527 Pt 2**, 225-237.

Arden, S.R., Sinor, J.D., Potthoff, W.K. & Aizenman, E. (1998) Subunit-specific interactions of cyanide with the NMDA receptor. *J Biol Chem*, **273**, 21505-21511.

Arrigoni, E. & Greene, R.W. (2004) Schaffer collateral and perforant path inputs activate different subtypes of NMDA receptors on the same CA1 pyramidal cell. *Br J Pharmacol*, **142**, 317-322.

Artola, A. & Singer, W. (1993) Long-term depression of excitatory synaptic transmission and its relationship to long-term potentiation. *Trends Neurosci*, **16**, 480-487.

Auberson, Y.P., Allgeier, H., Bischoff, S., Lingenhoehl, K., Moretti, R. & Schmutz, M. (2002) 5-Phosphonomethylquinoxalinediones as competitive NMDA receptor antagonists with a preference for the human 1A/2A, rather than 1A/2B receptor composition. *Bioorg Med Chem Lett*, **12**, 1099-1102.

Ault, B., Evans, R.H., Francis, A.A., Oakes, D.J. & Watkins, J.C. (1980)
Selective depression of excitatory amino acid induced
depolarizations by magnesium ions in isolated spinal cord
preparations. *J Physiol*, **307**, 413-428.

Barnard, E.A. & Henley, J.M. (1990) The non-NMDA receptors: types,
protein structure and molecular biology. *Trends Pharmacol Sci*, **11**,
500-507.

Bear, M.F. & Abraham, W.C. (1996) Long-term depression in
hippocampus. *Annu Rev Neurosci*, **19**, 437-462.

Behan, D.P. & Chalmers, D.T. (2001) The use of constitutively active
receptors for drug discovery at the G protein-coupled receptor gene
pool. *Curr Opin Drug Discov Devel*, **4**, 548-560.

Behe, P., Stern, P., Wyllie, D.J., Nassar, M., Schoepfer, R. & Colquhoun,
D. (1995) Determination of NMDA NR1 subunit copy number in
recombinant NMDA receptors. *Proc R Soc Lond B Biol Sci*, **262**,
205-213.

Benardo, L.S. (1994) Separate activation of fast and slow inhibitory
postsynaptic potentials in rat neocortex *in vitro*. *J Physiol*, **476**, 203-
215.

Benardo, L.S. (1995) Recruitment of inhibition by enhanced activation of
synaptic NMDA responses in the rat cerebral cortex. *Brain Res*, **627**,
314-324.

Berretta, N. & Jones, R.S. (1996) Tonic facilitation of glutamate release by presynaptic N-methyl-D-aspartate autoreceptors in the entorhinal cortex. *Neuroscience*, **75**, 339-344.

Bialojan, C. & Takai, A. (1988) Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *Biochem J*, **256**, 283-290.

Bienenstock, E.L., Cooper, L.N. & Munro, P.W. (1982) Theory for the development of neuron selectivity:orientation specificty and binocular interaction in visual cortex. *J. Neurosci*, **2**, 32-48.

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

Bliss, T.V. & Lomo, T. (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol*, **232**, 331-356.

Breukel, A.I., Besselsen, E., Lopes da Silva, F.H. & Ghijsen, W.E. (1998) A presynaptic N-methyl-D-aspartate autoreceptor in rat hippocampus modulating amino acid release from a cytoplasmic pool. *Eur J Neurosci*, **10**, 106-114.

Brown, J.C., Tse, H.W., Skifter, D.A., Christie, J.M., Andaloro, V.J., Kemp, M.C., Watkins, J.C., Jane, D.E. & Monaghan, D.T. (1998) [3H]homoquinolinate binds to a subpopulation of NMDA receptors and to a novel binding site. *J Neurochem*, **71**, 1464-1470.

Buller, A.L., Larson, H.C., Schneider, B.E., Beaton, J.A., Morrisett, R.A. & Monaghan, D.T. (1994) The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition. *J Neurosci*, **14**, 5471-5484.

Buller, A.L. & Monaghan, D.T. (1997) Pharmacological heterogeneity of NMDA receptors: characterization of NR1a/NR2D heteromers expressed in Xenopus oocytes. *Eur J Pharmacol*, **320**, 87-94.

Burke, J.P. & Hablitz, J.J. (1994) Presynaptic depression of synaptic transmission mediated by activation of metabotropic glutamate receptors in rat neocortex. *J Neurosci*, **14**, 5120-5130.

Cajal, S.R., Y. (1968) *The structure of Ammon's horn*, Springfield.

Castellani, G.C., Quinlan, E.M., Cooper, L.N. & Shouval, H.Z. (2001) A biophysical model of bidirectional synaptic plasticity: dependence on AMPA and NMDA receptors. *PNAS*, **98**, 12772-12777.

Chatterton, J.E., Awobuluyi, M., Premkumar, L.S., Takahashi, H., Talantova, M., Shin, Y., Cui, J., Tu, S., Sevarino, K.A., Nakanishi, N., Tong, G., Lipton, S.A. & Zhang, D. (2002) Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature*, **415**, 793-798.

Chen, N., Luo, T., Wellington, C., Metzler, M., McCutcheon, K., Hayden, M.R. & Raymond, L.A. (1999) Subtype-specific enhancement of NMDA receptor currents by mutant huntingtin. *J Neurochem*, **72**, 1890-1898.

Chen, S.-J. & Leonard, J.P. (1996) Protein tyrosine kinase-mediated potentiation of currents from cloned NMDA receptors. *J Neurochem*, **67**, 194-200.

Chenard, B.L., Bordner, J., Butler, T.W., Chambers, L.K., Collins, M.A., De Costa, D.L., Ducat, M.F., Durmont, M.L. & Fox, C.B. (1995) (1S,2S)-1-(4-Hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol: A Potent New Neuroprotectant Which Blocks N-Methyl-D-Aspartate Responses. *J Med Chem*, **38**, 3138-3145.

Chenard, B.L., Shalaby, I.A., Koe, B.K., Ronau, R.T., Butler, T.W., Prochniak, M.A., Schmidt, A.W. & Fox, C.B. (1991) Separation of alpha 1 adrenergic and N-methyl-D-aspartate antagonist activity in a series of ifenprodil compounds. *J Med Chem*, **34**, 3085-3090.

Cheng, C., Fass, D.M. & Reynolds, I.J. (1999) Emergence of excitotoxicity in cultured forebrain neurons coincides with larger glutamate-stimulated [Ca(2+)](i) increases and NMDA receptor mRNA levels. *Brain Res*, **849**, 97-108.

Cheung, H.H., Takagi, N., Teves, L., Logan, R., Wallace, M.C. & Gurd, J.W. (2000) Altered association of protein tyrosine kinases with postsynaptic densities after transient cerebral ischemia in the rat brain. *J Cereb Blood Flow Metab*, **20**, 505-512.

Chevaleyre, V. & Castillo, P.E. (2003) Heterosynaptic LTD of hippocampal GABAergic synapses: a novel role of endocannabinoids in regulating excitability. *Neuron*, **38**, 461-472.

Chittajallu, R., Vignes, M., Dev, K.K., Barnes, J.M., Collingridge, G.L. & Henley, J.M. (1996) Regulation of glutamate release by presynaptic kainate receptors in the hippocampus. *Nature*, **379**, 78-81.

Chizh, B.A., Headley, P.M. & Tzschentke, T.M. (2001) NMDA receptor antagonists as analgesics: focus on the NR2B subtype. *Trends Pharmacol Sci*, **22**, 636-642.

Cho, K., Aggleton, J.P., Brown, M.W. & Bashir, Z.I. (2001) An experimental test of the role of postsynaptic calcium levels in determining synaptic strength using perirhinal cortex of rat. *J Physiol (Lond)*, **532**, 459-466.

Choi, D.W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron*, **1**, 623-634.

Choi, D.W. (1991) Excitotoxicity. In Meldrum, B. (ed.) *Excitatory amino acid antagonists*. Blackwell Scientific, Oxford, pp. 216-236.

Choi, D.W. & Rothman, S.M. (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annu Rev Neurosci*, **13**, 171-182.

Christie, B.R., Wenthold, R.J. & Monaghan, D.T. (1999) Insulin causes a transient phosphorylation of NR2A and NR2B receptor subunits in rat hippocampus. *J. Neurochem*, **72**, 1523-1528.

Ciabarra, A.M., Sullivan, J.M., Gahn, L.G., Pecht, G., Heinemann, S. & Sevarino, K.A. (1995) Cloning and characterization of chi-1: a

developmentally regulated member of a novel class of the ionotropic glutamate receptor family. *J Neurosci*, **15**, 6498-6508.

Clayton, D.A., Mesches, M.H., Alvarez, E., Bickford, P.C. & Browning, M.D. (2002) A hippocampal NR2B deficit can mimic age-related changes in long-term potentiation and spatial learning in the Fischer 344 rat. *J Neurosci*, **22**, 3628-3637.

Collingridge, G.L., Kehl, S.J. & McLennan, H. (1983) The action of an N-methylaspartate antagonist on synaptic processes in the rat hippocampus. *J Physiol*, **338**, 27P.

Collingridge, G.L. & Lester, R.A. (1989) Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol Rev*, **41**, 143-210.

Collingridge, G.L. & Singer, W. (1990) Excitatory amino acid receptors and synaptic plasticity. *Trends Pharmacol Sci*, **11**, 290-296.

Creager, R., Dunwiddie, T. & Lynch, G. (1980) Paired-pulse and frequency facilitation in the CA1 region of the in vitro rat hippocampus. *J Physiol*, **299**, 409-424.

Cudmore, S.B. & Gurd, J.W. (1991) Postnatal age and protein tyrosine phosphorylation at synapses in the developing rat brain. *J Neurochem*, **57**, 1240-1248.

Curtis, D.R. & Johnston, G.A. (1974) Amino acid transmitters in the mammalian central nervous system. *Ergeb Physiol*, **69**, 97-188.

Curtis, D.R., Phillis,J.W.,Watkins,J.C. (1959) Chemical excitation of spinal neurones. *Nature*, **183**, 611-612.

Curtis, D.R., Phillis,J.W.,Watkins,J.C. (1960) The chemical excitation of spinal neurones by certain acidic amino acids. *J Physiol*, **150**, 656-682.

Danysz, W., Wroblewski, J.T., Brooker, G. & Costa, E. (1989) Modulation of glutamate receptors by phencyclidine and glycine in the rat cerebellum: cGMP increase *in vivo*. *Brain Res*, **479**, 270-276.

Das, S., Sasaki, Y.F., Rothe, T., Premkumar, L.S., Takasu, M., Crandall, J.E., Dikkes, P., Conner, D.A., Rayudu, P.V., Cheung, W., Chen, H.S., Lipton, S.A. & Nakanishi, N. (1998) Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. *Nature*, **393**, 377-381.

Davanger, S., Storm-Mathisen, J. & Ottersen, O.P. (1994) Colocalization of glutamate and glycine in bipolar cell terminals of the human retina. *Exp Brain Res*, **98**, 342-354.

Davies, C.H. & Collingridge, G.L. (1993) The physiological regulation of synaptic inhibition by GABAB autoreceptors in rat hippocampus. *J Physiol*, **472**, 245-265.

Davies, C.H., Davies, S.N. & Collingridge, G.L. (1990) Paired-pulse depression of mono-synaptic GABA-mediated inhibitory postsynaptic responses in rat hippocampus. *J Physiol*, **424**, 513-531.

Davies, J. & Watkins, J.C. (1979) Selective antagonism of amino acid-induced and synaptic excitation in the cat spinal cord. *J Physiol*, **297**, 621-635.

De Carvalho, L.P., Bochet, P. & Rossier, J. (1996) The endogenous agonist quinolinic acid and the non endogenous homoquinolinic acid discriminate between NMDAR2 receptor subunits. *Neurochem Int*, **28**, 445-452.

De Keyser, J., Sulter, G. & Luiten, P.G. (1999) Clinical trials with neuroprotective drugs in acute ischaemic stroke: are we doing the right thing? *Trends Neurosci*, **22**, 535-540.

Debanne, D., Guerineau, N.C., Gahwiler, B.H. & Thompson, S.M. (1996) Paired-pulse facilitation and depression at unitary synapses in rat hippocampus: quantal fluctuation affects subsequent release. *J Physiol*, **491 (Pt 1)**, 163-176.

Deisz, R.A. & Prince, D.A. (1989) Frequency-dependent depression of inhibition in guinea-pig neocortex in vitro by GABAB receptor feed-back on GABA release. *J Physiol*, **412**, 513-541.

Dingledine, R., Borges, K., Bowie, D. & Traynelis, S.F. (1999) The glutamate receptor ion channels. *Pharmacol Rev*, **51**, 7-61.

Doble, A. (1995) Excitatory amino acid receptors and neurodegeneration. *Therapie*, **50**, 319-337.

Dudek, S.M. & Bear, M.F. (1992) Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. *Proc Natl Acad Sci U S A*, **89**, 4363-4367.

Dudek, S.M. & Bear, M.F. (1993) Bidirectional long-term modification of synaptic effectiveness in the adult and immature hippocampus. *J Neurosci*, **13**, 2910-2918.

Duguid, I.C. & Smart, T.G. (2004) Retrograde activation of presynaptic NMDA receptors enhances GABA release at cerebellar interneuron-Purkinje cell synapses. *Nat Neurosci*, **7**, 525-533.

Dunah, A.W., Wang, Y., Yasuda, R.P., Kameyama, K., Huganir, R.L., Wolfe, B.B. & Standaert, D.G. (2000) Alterations in subunit expression, composition, and phosphorylation of striatal N-methyl-D-aspartate glutamate receptors in a rat 6-hydroxydopamine model of Parkinson's disease. *Mol Pharmacol*, **57**, 342-352.

Dunwidde, T.V. (1985) The physiological role of adenosine in the nervous system. *Int. Rev. Neurobiol.*, **27**, 63-139.

Eriksson, M., Nilsson, A., Froelich Fabre, S., Akesson, E., Dunker, J., Seiger, A., Folkesson, R., Benedikz, E. & Sundstrom, E. (2002) Cloning and expression of the human N-methyl-D-aspartate receptor subunit NR3A. *Neurosci Lett*, **321**, 177-181.

Erreger, K., Dravid, S.M., Banke, T.G., Wyllie, D.J. & Traynelis, S.F. (2005) Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and siganalling profiles. *J Physiol*, **563**, 345-358.

Evans, R.H., Francis, A.A. & Watkins, J.C. (1978) Mg2+-like selective antagonism of excitatory amino acid-induced responses by alpha, epsilon-diaminopimelic acid, D-alpha-aminoadipate and HA-966 in isolated spinal cord of frog and immature rat. *Brain Res*, **148**, 536-542.

Ferrer Montiel, A.V. & Montal, M. (1996) Pentameric subunit stoichiometry of a neuronal glutamate receptor. *Proc Natl Acad Sci U S A*, **93**, 2741-2744.

Fischer, G., Mutel, V., Trube, G., Malherbe, P., Kew, J.N., Mohacsi, E., Heitz, M.P. & Kemp, J.A. (1997) Ro 25-6981, a highly potent and selective blocker of N-methyl-D-aspartate receptors containing the NR2B subunit. Characterization in vitro. *J Pharmacol Exp Ther*, **283**, 1285-1292.

Fletcher, E.J. & Lodge, D. (1988) Glycine reverses antagonism of N-methyl-D-aspartate (NMDA) by 1-hydroxy-3-aminopyrrolidone-2 (HA-966) but not by D-2-amino-5-phosphonovalereate (D-AP5) on rat cortical slices. *Eur J Pharmacol*, **151**, 161-162.

Foster, B. & Richardson, T.L. (1997) Postsynaptic action potentials do not alter short-term potentiation in the dentate gyrus. *Brain Res*, **758**, 59-68.

Fritschy, J.M., Weinmann, O., Wenzel, A. & Benke, D. (1998) Synapse-specific localization of NMDA and GABA(A) receptor subunits revealed by antigen-retrieval immunohistochemistry. *J Comp Neurol*, **390**, 194-210.

Gallagher, M.J., Huang, H., Pritchett, D.B. & Lynch, D.R. (1996) Interactions between ifenprodil and the NR2B subunit of the N-methyl-D-aspartate receptor. *J Biol Chem*, **271**, 9603-9611.

Gao, X.M., Sakai, K., Roberts, R.C., Conley, R.R., Dean, B. & Tamminga, C.A. (2000) Ionotropic glutamate receptors and expression of N-methyl-D-aspartate receptor subunits in subregions of human hippocampus: effects of schizophrenia. *Am J Psychiatry*, **157**, 1141-1149.

Gibb, A.J., Edmonds, B., Silver, R.A., Cull-Candy, S.G. & Colquhoun, D. (1994) Activation of NMDA receptors. In Collingridge, G.L., Watkins, J.C. (eds.) *The NMDA Receptor*. Oxford University Press., New York, pp. 219-242.

Gill, R., Alanine, A., Bourson, A., Buttelmann, B., Fischer, G., Heitz, M.P., Kew, J.N., Levet Trafit, B., Lorez, H.P., Malherbe, P., Miss, M.T., Mutel, V., Pinard, E., Roever, S., Schmitt, M., Trube, G., Wybrecht, R., Wyler, R. & Kemp, J.A. (2002) Pharmacological characterization of Ro 63-1908 (1-[2-(4-hydroxy-phenoxy)-ethyl]-4-(4-methyl-benzyl)-piperidin-4-ol), a novel subtype-selective N-methyl-D-aspartate antagonist. *J Pharmacol Exp Ther*, **302**, 940-948.

Glitsch, M. & Marty, A. (1999) Presynaptic effects of NMDA in cerebellar Purkinje cells and interneurons. *J Neurosci*, **19**, 511-519.

Gotti, B., Duverger, D., Bertin, J., Carter, C., Dupont, R., Frost, J., Gaudilliere, B., MacKenzie, E.T., Rousseau, J. & Scatton, B. (1988) Ifenprodil and SL 82.0715 as cerebral anti-ischemic agents. I.

Evidence for efficacy in models of focal cerebral ischemia. *J Pharmacol Exp Ther*, **247**, 1211-1221.

Greenamyre, J.T. & Young, A.B. (1989) Excitatory amino acids and Alzheimer's disease. *Neurobiol Aging*, **10**, 593-602.

Greene, R.W., and Hass, H.L., (1991) The physiology of adenosine in the mammalian central nervous system. *Prog. Neurobiol.*, **36**, 329-341.

Grimwood, S., Richards, P., Murray, F., Harrison, N., Wingrove, P.B. & Hutson, P.H. (2000) Characterisation of N-methyl-D-aspartate receptor-specific [(3)H]Ifenprodil binding to recombinant human NR1a/NR2B receptors compared with native receptors in rodent brain membranes. *J Neurochem*, **75**, 2455-2463.

Grimwood, S., Slater, P., Deakin, J.F. & Hutson, P.H. (1999) NR2B-containing NMDA receptors are up-regulated in temporal cortex in schizophrenia. *Neuroreport*, **10**, 461-465.

Grimwood, S., Wafford, K.A., Macaulay, A. & Hutson, P.H. (2002) N-Methyl-D-aspartate receptor subtype-selectivity of homoquinolinate: an electrophysiological and radioligand binding study using both native and recombinant receptors. *J Neurochem*, **82**, 794-800.

Groc, L., Heine, M.F., Cognet, L., Brickley, K., Stephenson, F.A., Lounis, B. & Choquet, D. (2004) Differential activity-dependent regulation of the lateral mobilities of AMPA and NMDA receptors. *Nat Neurosci*, **7**, 695-696.

Grollman, A.P. (1967) Inhibitors of protein biosynthesis. II. Mode of action of anisomycin. *J Biol Chem*, **242**, 3226-3233.

Grosshans, D.R., Clayton, D.A., Coultrap, S.J. & Browning, M.D. (2002) LTP leads to rapid surface expression of NMDA but not AMPA receptors in adult rat CA1. *Nat Neurosci*, **5**, 27-33.

Grover, L.M. & Teyler, T.J. (1990) Two components of long-term potentiation induced by different patterns of afferent activation. *Nature*, **347**, 477-479.

Gurd, J.W. (1985) Phosphorylation of the postsynaptic density glycoprotein gp180 by endogenous tyrosine kinase. *Brain Res*, **333**, 385-388.

Gurd, J.W. & Bissoon, N. (1997) The N-methyl-D-aspartate receptor subunits NR2A and NR2B bind to the SH2 domains of phospholipase C-gamma. *J Neurochem*, **69**, 623-630.

Hall, R.A. & Soderling, T.R. (1997) Differential surface expression and phosphorylation of the N-methyl-D-aspartate receptor subunits NR1 and NR2 in cultured hippocampal neurons. *J Biol Chem*, **272**, 4135-4140.

Hamada, T., Sonoda, R., Watanabe, A., Ono, M., Shibata, S. & Watanabe, S. (1998) NMDA induced glutamate release from the suprachiasmatic nucleus: an in vitro study in the rat. *Neurosci Lett*, **256**, 93-96.

Hardingham, G.E., Fukunaga, Y. & Bading, H. (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci*, **5**, 405-414.

Hashimoto, K. & Kano, M. (1998) Presynaptic origin of paired-pulse depression at climbing fibre-Purkinje cell synapses in the rat cerebellum. *J Physiol*, **506 (Pt 2)**, 391-405.

Hayashi, T. (1952) A physiological study of the epileptic seizures following cortical stimulation in animals and its application to human clinincs. *Jap J Physiol*, **3**, 46-64.

Hayashi, T. (1954) Effects of sodium glutamate on the nervous system. *Keio J Med*, **3**, 183-192.

Hebb, D.O. (1946) *The organization of learning*, Wiley, New York.

Hess, G., Kuhnt, U. & Voronin, L.L. (1987) Quantal analysis of paired-pulse facilitation in guinea pig hippocampal slices. *Neurosci Lett*, **77**, 187-192.

Higgins, M.J. & Stone, T.W. (1995) Effect of adenosine on bicuculline-resistant paired-pulse inhibition in the rat hippocampal slice. *Hippocampus*, **5**, 209-216.

Hisatsune, C., Umemori, H., Mishina, M. & Yamamoto, T. (1999) Phosphorylation-dependent interaction of the N-methyl-D-aspartate receptor epsilon 2 subunit with phosphatidylinositol 3-kinase. *Genes Cells*, **4**, 657-666.

Hodgkiss, J.P. & Kelly, J.S. (1995) Only 'de novo' long-term depression (LTD) in the rat hippocampus in vitro is blocked by the same low concentration of FK506 that blocks LTD in the visual cortex. *Brain Res*, **705**, 241-246.

Hollmann, M. & Heinemann, S. (1994) Cloned glutamate receptors. *Annu Rev Neurosci*, **17**, 31-108.

Hrabetova, S., Serrano, P., Blace, N., Tse, H.W., Skifter, D.A., Jane, D.E., Monaghan, D.T. & Sacktor, T.C. (2000) Distinct NMDA receptor subpopulations contribute to long-term potentiation and long-term depression induction. *J Neurosci*, **20**, RC81.

Husi, H., Ward, M., Choudhary, J.S., Blackstock, W.P. & Grant, S.G.N. (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nat Neurosci*, **3**, 661-669.

Ilyin, V.I., Whittemore, E.R., Guastella, J., Weber, E. & Woodward, R.M. (1996) Subtype-selective inhibition of N-methyl-D-aspartate receptors by haloperidol. *Mol Pharmacol*, **50**, 1541-1550.

Isaccson, R.L. (1987) Hippocampus. In Adelman, G. (ed.) *Encyclopedia of Neuroscience*. Birkhauser, Boston, pp. 492-495.

Ito, I., Kawakami, R., Sakimura, K., Mishina, M. & Sugiyama, H. (2000) Input-specific targeting of NMDA receptor subtypes at mouse hippocampal CA3 pyramhidal neuron synapses. *Neuropharmacology*, **39**, 943-951.

Johnson, J.W. & Ascher, P. (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature*, **325**, 529-531.

Johnston, D. & Amaral, D. (1998) Hippocampus. In Shepherd, G.M. (ed.) *Synaptic Organization of the Brain*. Oxford University Press, New York.

Johnston, D., Amaral, D. (1998) Hippocampus. In Shepherd, G.M. (ed.) *Synaptic Organization of the Brain*. Oxford University Press, New York.

Kahle, J.S. & Cotman, C.W. (1993) Adenosine, L-AP4, and baclofen modulation of paired-pulse potentiation in the dentate gyrus: interstimulus interval-dependent pharmacology. *Exp Brain Res*, **94**, 97-104.

Kandel, E.R. (1997) Genes, synapses, and long-term memory. *J Cell Physiol*, **173**, 124-125.

Karachot, L., Shirai, Y., Vigot, R., Yamamori, T. & Ito, M. (2001) Induction of long-term depression in cerebellar Purkinje cells requires a rapidly turned over protein. *J Neurophysiol*, **86**, 280-289.

Katz, B. & Miledi, R. (1968) The role of calcium in neuromuscular facilitation. *J Physiol*, **195**, 481-492.

Kawakami, R., Shinohara, Y., Kato, Y., Sugiyama, H., Shigemoto, R. & Ito, I. (2003) Asymmetrical allocation of NMDA receptor epsilon2 subunits in hippocampal circuitry. *Science*, **300**, 990-994.

Kemp, J.A. & McKernan, R.M. (2002) NMDA receptor pathways as drug targets. *Nat Neurosci*, **5 Suppl**, 1039-1042.

Kemp, J.A., Priestley, T. & Woodruff, G.N. (1988) Differences in the N-methyl-D-aspartate antagonist profiles of two compounds acting at the glycine modulatory site. *Br J Pharmacol*, **95**, 759P.

Kemp, N. & Bashir, Z.I. (2001) Long-term depression: a cascade of induction and expression mechanisms. *Prog Neurobiol*, **65**, 339-365.

Kemp, N., McQueen, J., Faulkes, S. & Bashir, Z.I. (2000) Different forms of LTD in the CA1 region of the hippocampus: role of age and stimulus protocol. *Eur J Neurosci*, **12**, 360-366.

Kerkut, A., and Wheal H., (1981) To slice or not to slice. In: Electrophsiology of isolated mammalian CNS preparations. 15-50.

Kew, J.N., Richards, J.G., Mutel, V. & Kemp, J.A. (1998a) Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons. *J Neurosci*, **18**, 1935-1943.

Kew, J.N., Richards, J.G., Mutel, V. & Kemp, J.A. (1998b) Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons. *J Neurosci*, **18**, 1935-1943.

Kew, J.N., Trube, G. & Kemp, J.A. (1996) A novel mechanism of activity-dependent NMDA receptor antagonism describes the effect of

ifenprodil in rat cultured cortical neurones. *J Physiol*, **497** (**Pt 3**), 761-772.

Kim, W.T., Kuo, M.F., Mishra, O.P. & Delivoria Papadopoulos, M. (1998) Distribution and expression of the subunits of N-methyl-D-aspartate (NMDA) receptors; NR1, NR2A and NR2B in hypoxic newborn piglet brains. *Brain Res*, **799**, 49-54.

Kirkwood, A., Rioult, M.G. & Bear, M.F. (1996) Experience-dependent modification of synaptic plasticity in visual cortex. *Nature*, **381**, 526-528.

Kirson, E.D., Schirra, C., Konnerth, A. & Yaari, Y. (1999) Early postnatal switch in magnesium sensitivity of NMDA receptors in rat CA1 pyramidal cells. *J Physiol*, **521 Pt 1**, 99-111.

Kiyama, Y., Manabe, T., Sakimura, K., Kawakami, F., Mori, H. & Mishina, M. (1998) Increased thresholds for long-term potentiation and contextual learning in mice lacking the NMDA-type glutamate receptor epsilon1 subunit. *J Neurosci*, **18**, 6704-6712.

Kleckner, N.W. & Dingledine, R. (1988) Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. *Science*, **241**, 835-837.

Kleschevnikov, A.M., Sokolov, M.V., Kuhnt, U., Dawe, G.S., Stephenson, J.D. & Voronin, L.L. (1997) Changes in paired-pulse facilitation correlate with induction of long-term potentiation in area CA1 of rat hippocampal slices. *Neuroscience*, **76**, 829-843.

Kohr, G., Jensen, V., Koester, H.J., Mihaljevic, A.L., Utvik, J.K., Kvello, A., Ottersen, O.P., Seeburg, P.H., Sprengel, R. & Hvalby, Ã. (2003) Intracellular domains of NMDA receptor subtypes are determinants for long-term potentiation induction. *J Neurosci*, **23**, 10791-10799.

Kohr, G. & Seeburg, P.H. (1996) Subtype-specific regulation of recombinant NMDA receptor-channels by protein tyrosine kinases of the src family. *J Physiol*, **492 (Pt 2)**, 445-452.

Krogsgaard Larsen, P., Honore, T., Hansen, J.J., Curtis, D.R. & Lodge, D. (1980) New class of glutamate agonist structurally related to ibotenic acid. *Nature*, **284**, 64-66.

Krug, M., Lossner, B. & Ott, T. (1984) Anisomycin blocks the late phase of long-term potentiation in the dentate gyrus of freely moving rats. *Brain Res Bull*, **13**, 39-42.

Krupp, J.J., Vissel, B., Heinemann, S.F. & Westbrook, G.L. (1996) Calcium-dependent inactivation of recombinant N-methyl-D-aspartate receptors is NR2 subunit specific. *Mol Pharmacol*, **50**, 1680-1688.

Kuba, K. & Kumamoto, E. (1990) Long-term potentiations in vertebrate synapses: a variety of cascades with common subprocesses. *Prog Neurobiol*, **34**, 197-269.

Kullmann, D.M. & Nicoll, R.A. (1992) Long-term potentiation is associated with increases in quantal content and quantal amplitude. *Nature*, **357**, 240-244.

Kuryatov, A., Laube, B., Betz, H. & Kuhse, J. (1994) Mutational analysis of the glycine-binding site of the NMDA receptor: structural similarity with bacterial amino acid-binding proteins. *Neuron*, **12**, 1291-1300.

Lambert, N.A. & Wilson, W.A. (1994) Temporally distinct mechanisms of use-dependent depression at inhibitory synapses in the rat hippocampus in vitro. *J Neurophysiol*, **72**, 121-130.

Lau, L.F. & Huganir, R.L. (1995) Differential tyrosine phosphorylation of N-methyl-D-aspartate receptor subunits. *J Biol Chem*, **270**, 20036-20041.

Laube, B., Hirai, H., Sturgess, M., Betz, H. & Kuhse, J. (1997) Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding site on the NR2B subunit. *Neuron*, **18**, 493-503.

Laube, B., Kuhse, J. & Betz, H. (1998) Evidence for a tetrameric structure of recombinant NMDA receptors. *J Neurosci*, **18**, 2954-2961.

Laurie, D.J. & Seeburg, P.H. (1994) Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on subunit composition. *Eur J Pharmacol*, **268**, 335-345.

Lavezzari, G., McCallum, J., Dewey, C.M. & Roche, K.W. (2004) Subunit-specific regulation of NMDA receptor endocytosis. *J. Neurosci*, **24**, 6383-6391.

Law, A.J., Weickert, C.S., Webster, M.J., Herman, M.M., Kleinman, J.E. & Harrison, P.J. (2003) Expression of NMDA receptor NR1, NR2A and NR2B subunit mRNAs during development of the human hippocampal formation. *Eur J Neurosci*, **18**, 1197-1205.

Lee, J.M., Zipfel, G.J. & Choi, D.W. (1999) The changing landscape of ischaemic brain injury mechanisms. *Nature*, **399**, A7-14.

Li, J.H., Wang, Y.H., Wolfe, B.B., Krueger, K.E., Corsi, L., Stocca, G. & Vicini, S. (1998) Developmental changes in localization of NMDA receptor subunits in primary cultures of cortical neurons. *Eur J Neurosci*, **10**, 1704-1715.

Lieberman, D.N. & Mody, I. (1994) Regulation of NMDA channel function by endogenous Ca(2+)-dependent phosphatase. *Nature*, **369**, 235-239.

Lim, R., Hoang, P. & Berger, A. (2004) Blockade of glycine transporter-1 (GLYT-1) potentiates NMDA receptor-mediated synaptic transmission in the hypoglossal motorneurons. *J Neurophysiol*, **92**, 2530-2537.

Linden, J. (1991) Structure and function of A1 adenosine receptors. *FASEB J.*, **5**, 2668-2676.

Lipton, P., Aitken, P.G., Dudek, F.E., Eskessen, K., Espanol, M.T., Ferchmin, P.A., Kelly, J.B., Kreisman, N.R., Landfield, P.W. & Larkman, P.M. (1995) Making the best of brain slices: comparing preparative methods. *J Neurosci Methods*, **59**, 151-156.

Lipton, P. & Whittingham, T.S. (1979) The effect of hypoxia on evoked potentials in the in vitro hippocampus. *J Physiol*, **287**, 427-438.

Lisman, J.E. (1989) A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. *PNAS*, **86**, 9574-9578.

Liu, H., Mantyh, P.W. & Basbaum, A.I. (1997) NMDA-receptor regulation of substance P release from primary afferent nociceptors. *Nature*, **386**, 721-724.

Liu, H., Wang, H., Sheng, M., Jan, L.Y., Jan, Y.N. & Basbaum, A.I. (1994) Evidence for presynaptic N-methyl-D-aspartate autoreceptors in the spinal cord dorsal horn. *Proc Natl Acad Sci U S A*, **91**, 8383-8387.

Liu, L., Wong, T.P., Pozza, M.F., Lingenhoehl, K., Wang, Y., Sheng, M., Auberson, Y.P. & Wang, Y.T. (2004) Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science*, **304**, 1021-1024.

Liu, Z., Stafstrom, C.E., Sarkisian, M., Tandon, P., Yang, Y., Hori, A. & Holmes, G.L. (1996) Age-dependent effects of glutamate toxicity in the hippocampus. *Brain Res Dev Brain Res*, **97**, 178-184.

Loftis, J.M. & Janowsky, A. (2003) The N-methyl-D-aspartate receptor subunit NR2B: localization, functional properties, regulation, and clinical implications. *Pharmacol Ther*, **97**, 55-85.

Lomo, T. (1966) Frequency potentiation of excitatory synaptic activity in the dentate area of the hippocampal formation. *Acta Phys. Scand.*, **68**, 128.

Luo, J., Wang, Y., Yasuda, R.P., Dunah, A.W. & Wolfe, B.B. (1997) The
majority of N-methyl-D-aspartate receptor complexes in adult rat
cerebral cortex contain at least three different subunits
(NR1/NR2A/NR2B). *Mol Pharmacol*, **51**, 79-86.

Lupica, C.R., Proctor, W.R. & Dunwiddie, T.V. (1992) Presynaptic
inhibition of excitatory synaptic transmission by adenosine in rat
hippocampus: analysis of unitary EPSP variance measured by whole-
cell recording. *J Neurosci*, **12**, 3753-3764.

Lynch, D.R. & Gallagher, M.J. (1996) Inhibition of N-methyl-D-aspartate
receptors by haloperidol: developmental and pharmacological
characterization in native and recombinant receptors. *J Pharmacol
Exp Ther*, **279**, 154-161.

Lynch, D.R. & Guttmann, R.P. (2001) NMDA receptor pharmacology:
perspectives from molecular biology. *Curr Drug Targets*, **2**, 215-
231.

Lynch, D.R., Shim, S.S., Seifert, K.M., Kurapathi, S., Mutel, V., Gallagher,
M.J. & Guttmann, R.P. (2001) Pharmacological characterization of
interactions of RO 25-6981 with the NR2B (epsilon2) subunit. *Eur J
Pharmacol*, **416**, 185-195.

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.

MacDermott, A.B., Mayer, M.L., Westbrook, G.L., Smith, S.J. & Barker,
J.L. (1986) NMDA-receptor activation increases cytoplasmic

calcium concentration in cultured spinal cord neurones. *Nature*, **321**, 519-522.

MacDermott, A.B., Role, L.W. & Siegelbaum, S.A. (1999) Presynaptic ionotropic receptors and the control of transmitter release. *Annu Rev Neurosci*, **22**, 443-485.

Magnusson, K.R. (2000) Declines in mRNA expression of different subunits may account for differential effects of aging on agonist and antagonist binding to the NMDA receptor. *J Neurosci*, **20**, 1666-1674.

Magnusson, K.R., Nelson, S.E. & Young, A.B. (2002) Age-related changes in the protein expression of subunits of the NMDA receptor. *Brain Res Mol Brain Res*, **99**, 40-45.

Malenka, R.C., Kauer, J.A., Perkel, D.J., Mauk, M.D., Kelly, P.T., Nicoll, R.A. & Waxham, M.N. (1989) An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature*, **340**, 554-557.

Malinow, R. & Malenka, R.C. (2002) AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci*, **25**, 103-126.

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

Mallon, A.P., Auberson, Y.P. & Stone, T.W. (2005) Selective subunit antagonists suggest an inhibitory relationship between NR2B and

NR2A-subunit containing N-methyl-d-aspartate receptors in hippocampal slices. **162**, 374-383.

Manabe, T., Aiba, A., Yamada, A., Ichise, T., Sakagami, H., Kondo, H. & Katsuki, M. (2000) Regulation of long-term potentiation by H-Ras through NMDA receptor phosphorylation. *J Neurosci*, **20**, 2504-2511.

Manabe, T., Wyllie, D.J., 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. *J Neurophysiol*, **70**, 1451-1459.

Manahan Vaughan, D., Kulla, A. & Frey, J.U. (2000) Requirement of translation but not transcription for the maintenance of long-term depression in the CA1 region of freely moving rats. *J Neurosci*, **20**, 8572-8576.

Mano, I. & Teichberg, V.I. (1998) A tetrameric subunit stoichiometry for a glutamate receptor-channel complex. *Neuroreport*, **9**, 327-331.

Massey, P.V., Johnson, B.E., Moult, P.R., Auberson, Y.P., Brown, M.W., Molnar, E., Collingridge, G.L. & Bashir, Z.I. (2004) Differential Roles of NR2A and NR2B-Containing NMDA Receptors in Cortical Long-Term Potentiation and Long-Term Depression. *J. Neurosci.*, **24**, 7821-7828.

Massey, P.V.J., B.E.;Brown, M.W.;Molnar, E.;Bashir, Z.I. (2003) Extrasynaptic NR2B-containing N-methyl-D-aspartate receptors are

required for LTD in the adult perirhinal cortex in vitro. *The Society for Neuroscience 33rd Annual Meeting*, New Orleans.

Masuko, T., Kashiwagi, K., Kuno, T., Nguyen, N.D., Pahk, A.J., Fukuchi, J., Igarashi, K. & Williams, K. (1999) A regulatory domain (R1-R2) in the amino terminus of the N-methyl-D-aspartate receptor: effects of spermine, protons, ifenprodil, and structural similarity to bacterial leucine/isoleucine/valine binding protein. *Mol Pharmacol*, **55**, 957-969.

Mayer, M.L. & Westbrook, G.L. (1987) Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. *J Physiol*, **394**, 501-527.

Mayer, M.L., Westbrook, G.L. & Guthrie, P.B. (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature*, **309**, 261-263.

McBain, C.J. & Mayer, M.L. (1994) N-methyl-D-aspartic acid receptor structure and function. *Physiol Rev*, **74**, 723-760.

McCarren, M. & Alger, B.E. (1985) Use-dependent depression of IPSPs in rat hippocampal pyramidal cells in vitro. *J Neurophysiol*, **53**, 557-571.

McCool, B.A. & Lovinger, D.M. (1995) Ifenprodil inhibition of the 5-hydroxytryptamine3 receptor. *Neuropharmacology*, **34**, 621-629.

McGehee, D.S. & Role, L.W. (1996) Presynaptic ionotropic receptors. *Curr Opin Neurobiol*, **6**, 342-349.

McLennan, H. (1983) Receptors for the excitatory amino acids in the mammalian central nervous system. *Prog Neurobiol*, **20**, 251-271.

McLennan, H. & Lodge, D. (1979) The antagonism of amino acid-induced excitation of spinal neurones in the cat. *Brain Res*, **169**, 83-90.

Meldrum, B. & Garthwaite, J. (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol Sci*, **11**, 379-387.

Mennerick, S. & Zorumski, C.F. (1995) Paired-pulse modulation of fast excitatory synaptic currents in microcultures of rat hippocampal neurons. *J Physiol*, **488 (Pt 1)**, 85-101.

Monaghan, D.T., Bridges, R.J. & Cotman, C.W. (1989) The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. *Annu Rev Pharmacol Toxicol*, **29**, 365-402.

Monaghan, D.T. & Buller, A.L. (1994) Anatomical, pharmacological, and molecular diversity of native NMDA receptor subtypes. In Collingridge, G.L., Watkins, J.C. (eds.) *The NMDA receptor*. Oxford University Press, NYC, pp. 158-176.

Monaghan, D.T. & Larsen, H. (1997) NR1 and NR2 subunit contributions to N-methyl-D-aspartate receptor channel blocker pharmacology. *J Pharmacol Exp Ther*, **280**, 614-620.

Mori, H. & Mishina, M. (1995) Structure and function of the NMDA receptor channel. *Neuropharmacology*, **34**, 1219-1237.

Morris, R.G., Anderson, E., Lynch, G.S. & Baudry, M. (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature*, **319**, 774-776.

Moser, E.I., Krobert, K.A., Moser, M.B. & Morris, R.G. (1998) Impaired spatial learning after saturation of long-term potentiation. *Science*, **281**, 2038-2042.

Mott, D.D., Doherty, J.J., Zhang, S., Washburn, M.S., Fendley, M.J., Lyuboslavsky, P., Traynelis, S.F. & Dingledine, R. (1998) Phenylethanolamines inhibit NMDA receptors by enhancing proton inhibition. *Nat Neurosci*, **1**, 659-667.

Mulkey, R.M., Endo, S., Shenolikar, S. & Malenka, R.C. (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature*, **369**, 486-488.

Mulkey, R.M., Herron, C.E. & Malenka, R.C. (1993) An essential role for protein phosphatases in hippocampal long-term depression. *Science*, **261**, 1051-1055.

Mulkey, R.M. & Malenka, R.C. (1992) Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of the hippocampus. *Neuron*, **9**, 967-975.

Mutel, V., Buchy, D., Klingelschmidt, A., Messer, J., Bleuel, Z., Kemp, J.A. & Richards, J.G. (1998) In vitro binding properties in rat brain of [3H]Ro 25-6981, a potent and selective antagonist of NMDA receptors containing NR2B subunits. *J Neurochem*, **70**, 2147-2155.

Nakanishi, N., Axel, R. & Shneider, N.A. (1992) Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors. *Proc Natl Acad Sci U S A*, **89**, 8552-8556.

Nakazawa, T., Komai, S., Tezuka, T., Hisatsune, C., Umemori, H., Semba, K., Mishina, M., Manabe, T. & Yamamoto, T. (2001) Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR epsilon 2 (NR2B) subunit of the N-methyl-D-aspartate receptor. *J Biol Chem*, **276**, 693-699.

Nash, J.E., Hill, M.P. & Brotchie, J.M. (1999) Antiparkinsonian actions of blockade of NR2B-containing NMDA receptors in the reserpine-treated rat. *Exp Neurol*, **155**, 42-48.

Nathan, T., Jensen, M.S. & Lambert, J.D. (1990) GABAB receptors play a major role in paired-pulse facilitation in area CA1 of the rat hippocampus. *Brain Res*, **531**, 55-65.

Neveu, D. & Zucker, R.S. (1996) Postsynaptic levels of [Ca2+]i needed to trigger LTD and LTP. *Neuron*, **16**, 619-629.

Nguyen, P.V., Abel, T. & Kandel, E.R. (1994) Requirement of a critical period of transcription for induction of a late phase of LTP. *Science*, **265**, 1104-1107.

Nichols, R.A., Suplick, G.R. & Brown, J.M. (1994) Calcineurin-mediated protein dephosphorylation in brain nerve terminals regulates the release of glutamate. *J Biol Chem*, **269**, 23817-23823.

Nicoletti, F., Meek, J.L., Iadarola, M.J., Chuang, D.M., Roth, B.L. & Costa, E. (1986) Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. *J Neurochem*, **46**, 40-46.

Nikbakht, M.R. & Stone, T.W. (2000) Suramin-sensitive suppression of paired-pulse inhibition by adenine nucleotides in rat hippocampal slices. *Neurosci Lett*, **278**, 45-48.

Nishi, M., Hinds, H., Lu, H.P., Kawata, M. & Hayashi, Y. (2001) Motoneuron-specific expression of NR3B, a novel NMDA-type glutamate receptor subunit that works in a dominant-negative manner. *J Neurosci*, **21**, RC185.

Nowak, L., Bregestovski, P., Ascher, P., Herbet, A. & Prochiantz, A. (1984) Magnesium gates glutamate-activated channels in mouse central neurones. *Nature*, **307**, 462-465.

Obrenovitch, T.P., Hardy, A.M. & Urebjak, J. (1997) High extracellular glycine does not potentiate N-methyl-D-aspartate-evoked depolarization *in vivo*. *Brain Res*, **746**, 190-194.

Oh, J.D., Vaughan, C.L. & Chase, T.N. (1999) Effect of dopamine denervation and dopamine agonist administration on serine phosphorylation of striatal NMDA receptor subunits. *Brain Res*, **821**, 433-442.

Olney, J.W. (1969) Brain lesion, obesity and other disturbances in mice treated with sodium monoglutamate. *Science*, **164**, 719-721.

Olney, J.W. (1986) Excitotoxic amino acids. *News Physiolo. Sci.*, **1**, 19-23.

Olney, J.W. (1990) Excitotoxic amino acids and neuropsychiatric disorders. *Annu Rev Pharmacol Toxicol*, **30**, 47-71.

Otani, S., Marshall, C.J., Tate, W.P., Goddard, G.V. & Abraham, W.C. (1989) Maintenance of long-term potentiation in rat dentate gyrus requires protein synthesis but not messenger RNA synthesis immediately post-tetanization. *Neuroscience*, **28**, 519-526.

Otmakhova, N.A. & Lisman, J.E. (2004) Contribution of I_h and $GABA_B$ to synaptically induced afterhyperpolarizations in CA1: a brake on the NMDA response. *J Neurophysiol*, **92**, 2027-2039.

Otmakhova, N.A., Otmakhov, N. & Lisman, L.E. (2002) Pathway-specific properties of AMPA and NMDA-mediated transmission in CA1 hippocampal pyramidal cells. *J Neurosci*, **22**, 1199-1207.

Paoletti, P., Ascher, P. & Neyton, J. (1997) High-affinity zinc inhibition of NMDA NR1-NR2A receptors. *J Neurosci*, **17**, 5711-5725.

Papatheodoropoulos, D. & Kostopoulos, G.K. (1998) Development of a transient increase in recurrent inhibition and paired-pulse facilitation in hippocampal CA1 region. *Brain Res Dev Brain Res*, **108**, 273-285.

Paquet, M. & Smith, Y. (2000) Presynaptic NMDA receptor subunit immunoreactivity in GABAergic terminals in rat brain. *J Comp Neurol*, **423**, 330-347.

Parpura, V., Basarsky, T.A., Liu, F., Jeftinija, S. & Haydon, P.G. (1994) Glutamate-mediated astrocyte-neurone signalling. *Nature*, **369**, 744-747.

Patneau, D. & Mayer, M.L. (1990) Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. *J. Neurosci*, **10**, 2385-2399.

Perin-Dureau, F., Rachline, J., Neyton, J. & Paoletti, P. (2002) Mapping the binding site of the neuroprotectant ifenprodil on NMDA receptors. *J Neurosci*, **22**, 5955-5965.

Perkins, M.N. & Stone, T.W. (1982) An iontophoretic investigation of the actions of convulsant kynurenines and their interaction with the endogenous excitant quinolinic acid. *Brain Res*, **247**, 184-187.

Petralia, R.S., Yokotani, N. & Wenthold, R.J. (1994) Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody. *J Neurosci*, **14**, 667-696.

Philpott, B.D., Sekhar, A.K., Shouval, H.Z. & Bear, M.F. (2001) Visual experience and deprivation bidirectionally modify the composition and function of NMDA receptors in visual cortex. *Neuron*, **29**, 157-169.

Pina Crespo, J.C. & Gibb, A.J. (2002) Subtypes of NMDA receptors in new-born rat hippocampal granule cells. *J Physiol*, **541**, 41-64.

Pizzi, M., Boroni, F., Bianchetti, K.M., Memo, M. & Spano, P. (1999) Reversal of glutamate excitotoxicity by activation of PKC-associated metabotropic glutamate receptors in cerebellar granule cells relies on NR2C subunit expression. *Eur J Neurosci*, **11**, 2489-2496.

Portera Cailliau, C., Price, D.L. & Martin, L.J. (1996) N-methyl-D-aspartate receptor proteins NR2A and NR2B are differentially distributed in the developing rat central nervous system as revealed by subunit-specific antibodies. *J Neurochem*, **66**, 692-700.

Premkumar, L.S. & Auerbach, A. (1997) Stoichiometry of recombinant N-methyl-D-aspartate receptor channels inferred from single-channel current patterns. *J Gen Physiol*, **110**, 485-502.

Racca, C., Stephenson, F.A., Streit, P., Roberts, J.D. & Somogyi, P. (2000) NMDA receptor content of synapses in stratum radiatum of the hippocampal CA1 area. *J Neurosci*, **20**, 2512-2522.

Revest, P. & Longstaff, A. (1998) *Molecular Neuroscience*. BIOS Scientific publishers Ltd, Oxford.

Reyes Harde, M., Potter, B.V., Galione, A. & Stanton, P.K. (1999) Induction of hippocampal LTD requires nitric-oxide-stimulated PKG activity and Ca2+ release from cyclic ADP-ribose-sensitive stores. *J Neurophysiol*, **82**, 1569-1576.

Reynolds, I.J. & Miller, R.J. (1989) Ifenprodil is a novel type of N-methyl-D-aspartate receptor antagonist: interaction with polyamines. *Mol Pharmacol*, **36**, 758-765.

Riccio, A. & Ginty, D.D. (2002) What a privilege to reside at the synapse: NMDA receptor signaling to CREB. *Nat Neurosci.*

Richard, F., Barroso, S., Martinez, J., Labbe_Jullie, C. & Kitabgi, P. (2001) Agonism, inverse agonism, and neutral antagonism at the constitutively active human neurotensin receptor 2. *Mol Pharmacol*, **60**, 1392-1398.

Rock, D.M. & Macdonald, R.L. (1992) The polyamine spermine has multiple actions on N-methyl-D-aspartate receptor single-channel currents in cultured cortical neurons. *Mol Pharmacol*, **41**, 83-88.

Rosenblum, K., Dudai, Y. & Richter Levin, G. (1996) Long-term potentiation increases tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit 2B in rat dentate gyrus in vivo. *Proc Natl Acad Sci U S A*, **93**, 10457-10460.

Rosenmund, C., Stern Bach, Y. & Stevens, C.F. (1998) The tetrameric structure of a glutamate receptor channel. *Science*, **280**, 1596-1599.

Rossi, D.J., Oshima, T. & Attwell, D. (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature*, **403**, 316-321.

Ruegg, U.T. & Burgess, G.M. (1989) Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends Pharmacol Sci*, **10**, 218-220.

Rusakov, D.A., Scimemi, A., Walker, M.C. & Kullmann, D.M. (2004) Comment on "Role of NMDA Receptor Subtypes in Governing the Direction of Hippocampal Synaptic Plasticity". *Science*, **305**, 1912b-.

Rycroft, B.K. & Gibb, A.J. (2002) Direct effects of calmodulin on NMDA receptor single-channel gating in rat hippocampal granule cells. *J Neurosci*, **22**, 8860-8868.

Sah, P., Hestrin, S. & Nicoll, R.A. (1989) Tonic activation of NMDA receptors by ambient glutamate enhances excitability of neurones. *Science*, **246**, 815-846.

Sattler, R., Xiong, Z., Lu, W.Y., Hafner, M., MacDonald, J.F. & Tymianski, M. (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. *Science*, **284**, 1845-1848.

Scheer, A. & Cotecchia, S. (1997) Constitutively active G protein-coupled receptors: potential mechanisms of receptor activation. *J Recept Signal Transduct Res*, **17**, 57-73.

Scherzer, C.R., Landwehrmeyer, G.B., Kerner, J.A., Standaert, D.G., Hollingsworth, Z.R., Daggett, L.P., Velicelebi, G., Penney, J.B. & Young, A.B. (1997) Cellular distribution of NMDA glutamate receptor subunit mRNAs in the human cerebellum. *Neurobiol Dis*, **4**, 35-46.

Schneggenburger, R., Zhou, Z., Konnerth, A. & Neher, E. (1993) Fractional contribution of calcium to the cation current through glutamate receptor channels. *Neuron*, **11**, 133-143.

Schurr, A., Payne, R.S., Heine, M.F. & Rigor, B.M. (1995) Hypoxia, excitotoxicity, and neuroprotection in the hippocampal slice preparation. *J Neurosci Methods*, **59**, 129-138.

Schwartzkroin, P.A. & Wester, K. (1975) Long-lasting facilitation of a synaptic potential following tetanization in the in vitro hippocampal slice. *Brain Res*, **89**, 107-119.

Seabrook, G.R., Smith, D.W., Bowery, B.J., Easter, A., Reynolds, T., Fitzjohn, S.M., Morton, R.A., Zheng, H., Dawson, G.R., Sirinathsinghji, D.J., Davies, C.H., Collingridge, G.L. & Hill, R.G. (1999) Mechanisms contributing to the deficits in hippocampal synaptic plasticity in mice lacking amyloid precursor protein. *Neuropharmacology*, **38**, 349-359.

Seeburg, P.H., Monyer, H., Sprengel, R. & Burnashev, N. (1994) Molecular biology of NMDA receptors. In Collingridge, G.L., Watkins, J.C. (eds.) *The NMDA receptor*. Oxford University Press, NYC, pp. 147-157.

Seisjo, B.K. (1988) Historical overview. Calcium, ischaemia, and death of brain cells. *Ann. NY. Acad. Sci.*, **522**, 638-661.

Selig, D.K., Lee, H.K., Bear, M.F. & Malenka, R.C. (1995) Reexamination of the effects of MCPG on hippocampal LTP, LTD, and depotentiation. *J Neurophysiol*, **74**, 1075-1082.

Shinohara, Y., Fukazawa, Y. & Shigemoto, R. (2003) Molecular left-right asymmetry in rodent hippocampus. *Abstract Viewer/Itinerary planner. Washington, DC: Society for Neuroscience.*, Program No. 467.462.

Sihra, T.S., Nairn, A.C., Kloppenburg, P., Lin, Z. & Pouzat, C. (1995) A role for calcineurin (protein phosphatase-2B) in the regulation of glutamate release. *Biochem Biophys Res Commun*, **212**, 609-616.

Sjostrom, P.J., Turrigiano, G.G. & Nelson, S.B. (2003) Neocortical LTD via coincident activation of presynaptic NMDA and cannabinoid receptors. *Neuron*, **39**, 641-654.

Sladeczek, F., Pin, J.P., Recasens, M., Bockaert, J. & Weiss, S. (1985) Glutamate stimulates inositol phosphate formation in striatal neurones. *Nature*, **317**, 717-719.

Smart, T.G. (1997) Regulation of excitatory and inhibitory neurotransmitter-gated ion channels by protein phosphorylation. *Curr Opin Neurobiol*, **7**, 358-367.

Smith, K.E., Borden, L.A., Hartig, P.R., Branchek, T. & Weinshank, R.L. (1992) Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. *Neuron*, **8**, 927-935.

Stanford, I.M., Wheal, H.V. & Chad, J.E. (1995) Bicuculline enhances the late $GABA_B$ receptor-mediated paired-pulse inhibition observed in rat hippocampal slices. *Eur J Pharmacol*, **277**, 229-234.

Steece Collier, K., Chambers, L.K., Jaw Tsai, S.S., Menniti, F.S. & Greenamyre, J.T. (2000) Antiparkinsonian actions of CP-101,606, an antagonist of NR2B subunit-containing N-methyl-d-aspartate receptors. *Exp Neurol*, **163**, 239-243.

239

Stevens, C.F. & Wang, Y. (1995) Facilitation and depression at single central synapses. *Neuron*, **14**, 795-802.

Stocca, G. & Vicini, S. (1998) Increased contribution of NR2A subunit to synaptic NMDA receptors in developing rat cortical neurons. *J Physiol*, **507 (Pt 1)**, 13-24.

Stone, T.W. (1984) Excitant activity of methyl derivatives of quinolinic acid on rat cortical neurones. *Br J Pharmacol*, **81**, 175-181.

Stone, T.W. (1993) Neuropharmacology of quinolinic and kynurenic acids. *Pharmacol Rev*, **45**, 309-379.

Stone, T.W. & Burton, N.R. (1988) NMDA receptors and ligands in the vertebrate CNS. *Prog Neurobiol*, **30**, 333-368.

Stone, T.W. & Perkins, M.N. (1981) Quinolinic acid: a potent endogenous excitant at amino acid receptors in CNS. *Eur J Pharmacol*, **72**, 411-412.

Sucher, N.J., Akbarian, S., Chi, C.L., Leclerc, C.L., Awobuluyi, M., Deitcher, D.L., Wu, M.K., Yuan, J.P., Jones, E.G. & Lipton, S.A. (1995) Developmental and regional expression pattern of a novel NMDA receptor-like subunit (NMDAR-L) in the rodent brain. *J Neurosci*, **15**, 6509-6520.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986) Staurosporine, a potent inhibitor of phospholipid/Ca++dependent protein kinase. *Biochem Biophys Res Commun*, **135**, 397-402.

240

Thomas, K.L., Davis, S., Hunt, S.P. & Laroche, S. (1996) Alterations in the expression of specific glutamate receptor subunits following hippocampal LTP in vivo. *Learn Mem*, **3**, 197-208.

Thomas, K.L., Davis, S., Laroche, S. & Hunt, S.P. (1994) Regulation of the expression of NR1 NMDA glutamate receptor subunits during hippocampal LTP. *Neuroreport*, **6**, 119-123.

Thomson, A.M. (2000) Facilitation, augmentation and potentiation at central synapses. *Trends Neurosci*, **23**, 305-312.

Tong, G., Shepherd, D. & Jahr, C.E. (1995) Synaptic desensitization of NMDA receptors by calcineurin. *Science*, **267**, 1510-1512.

Tovar, K.R. & Westbrook, G.L. (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci*, **19**, 4180-4188.

Triller, A. & Choquet, D. (2005) Surface trafficking of receptors between synaptic and extrasynaptic membranes: and yet they do move! *TINS*, **28**, 133-139.

Vazquez, E., Herrero, I., Miras Portugal, M.T. & Sanchez Prieto, J. (1994) Role of arachidonic acid in the facilitation of glutamate release from rat cerebrocortical synaptosomes independent of metabotropic glutamate receptor responses. *Neurosci Lett*, **174**, 9-13.

Vicini, S., Wang, J.F., Li, J.H., Zhu, W.J., Wang, Y.H., Luo, J.H., Wolfe, B.B. & Grayson, D.R. (1998) Functional and pharmacological

differences between recombinant N-methyl-D-aspartate receptors. *J Neurophysiol*, **79**, 555-566.

Victor, R.G., Thomas, G.D., Marban, E. & O'Rourke, B. (1995) Presynaptic modulation of cortical synaptic activity by calcineurin. *Proc Natl Acad Sci U S A*, **92**, 6269-6273.

Watanabe, M., Inoue, Y., Sakimura, K. & Mishina, M. (1993) Distinct distributions of five N-methyl-D-aspartate receptor channel subunit mRNAs in the forebrain. *J Comp Neurol*, **338**, 377-390.

Watt, A.J., Sjostrom, P.J., Hausser, M., Nelson, S.B. & Turrigiano, G.G. (2004) A proportional but slower NMDA potentiation follows AMPA potentiation in LTP. *Nat Neurosci*, **7**, 518-524.

Wenzel, A., Scheurer, L., Kunzi, R., Fritschy, J.M., Mohler, H. & Benke, D. (1995) Distribution of NMDA receptor subunit proteins NR2A, 2B, 2C and 2D in rat brain. *Neuroreport*, **7**, 45-48.

Wigstrom, H., Gustafsson, B., Huang, Y.Y. & Abraham, W.C. (1986) Hippocampal long-term potentiation is induced by pairing single afferent volleys with intracellularly injected depolarizing current pulses. *Acta Physiol Scand*, **126**, 317-319.

Wilcox, K.S. & Dichter, M.A. (1994) Paired pulse depression in cultured hippocampal neurons is due to a presynaptic mechanism independent of GABAB autoreceptor activation. *J Neurosci*, **14**, 1775-1788.

Wilcox, K.S., Fitzsimonds, R.M., Johnson, B. & Dichter, M.A. (1996) Glycine regulation of synaptic NMDA receptors in hippocampal neurones. *J Neurophysiol*, **76**, 3415-3424.

Williams, J.M., Mason Parker, S.E., Abraham, W.C. & Tate, W.P. (1998) Biphasic changes in the levels of N-methyl-D-aspartate receptor-2 subunits correlate with the induction and persistence of long-term potentiation. *Brain Res Mol Brain Res*, **60**, 21-27.

Williams, K. (1997) Interactions of polyamines with ion channels. *Biochem J*, **325 (Pt 2)**, 289-297.

Williams, K., Russell, S.L., Shen, Y.M. & Molinoff, P.B. (1993) Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro. *Neuron*, **10**, 267-278.

Witter, M.P. (1989) Connectivity of rat hippocampus., Chanpalay, V., and Kohler, C., (eds), Newyork. Alan Liss Inc. pp. 53-69.

Wong, T.P., Liu, L., Sheng, M. & Wang, Y.T. (2004) Response to Comment on "Role of NMDA Receptor Subtypes in Governing the Direction of Hippocampal Synaptic Plasticity". *Science*, **305**, 1912c-.

Woodhall, G., Evans, D.I., Cunningham, M.O. & Jones, R.S. (2001) NR2B-containing NMDA autoreceptors at synapses on entorhinal cortical neurons. *J Neurophysiol*, **86**, 1644-1651.

Wu, L.G. & Saggau, P. (1994) Presynaptic calcium is increased during normal synaptic transmission and paired-pulse facilitation, but not in

long-term potentiation in area CA1 of hippocampus. *J Neurosci*, **14**, 645-654.

Yakel, J.L. (1997) Calcineurin regulation of synaptic function: from ion channels to transmitter release and gene transcription. *Trends Pharmacol Sci*, **18**, 124-134.

Yamamoto, C. (1972) Intracellular study of seizure-like afterdischarges elicited in thin hippocampal sections in vitro. *Exp Neurol*, **35**, 154-164.

Yamamoto, C. & McIlwain, H. (1966) Electrical activities in thin sections from the mammalian brain maintained in chemically-defined media in vitro. *J Neurochem*, **13**, 1333-1343.

Yanagihara, N., Tachikawa, E., Izumi, F., Yasugawa, S., Yamamoto, H. & Miyamoto, E. (1991) Staurosporine: an effective inhibitor for Ca2+/calmodulin-dependent protein kinase II. *J Neurochem*, **56**, 294-298.

Yang, S.N., Tang, Y.G. & Zucker, R.S. (1999) Selective induction of LTP and LTD by postsynaptic [Ca2+]i elevation. *J Neurophysiol*, **81**, 781-787.

Yoshimura, Y., Ohmura, T. & Komatsu, Y. (2003) Two Forms of Synaptic Plasticity with Distinct Dependence on Age, Experience, and NMDA Receptor Subtype in Rat Visual Cortex. *J. Neurosci.*, **23**, 6557-6566.

Zeron, M.M., Chen, N., Moshaver, A., Lee, A.T., Wellington, C.L., Hayden, M.R. & Raymond, L.A. (2001) Mutant huntingtin enhances excitotoxic cell death. *Mol Cell Neurosci*, **17**, 41-53.

Zhang, L., Hsu, J.C., Takagi, N., Gurd, J.W., Wallace, M.C. & Eubanks, J.H. (1997) Transient global ischemia alters NMDA receptor expression in rat hippocampus: correlation with decreased immunoreactive protein levels of the NR2A/2B subunits, and an altered NMDA receptor functionality. *J Neurochem*, **69**, 1983-1994.

Zheng, F., Gingrich, M.B., Traynelis, S.F. & Conn, P.J. (1998) Tyrosine kinase potentiates NMDA receptor current by reducing tonic Zn^{2+} inhibition. *Nat Neurosci*, **1**, 185-191.

Zou, J.Y. & Crews, F.T. (2003) Selective NR2B antagonist Ifenprodil potentiates glutamate neurotoxicity in rat brain. *Society for Neuroscience 2003 conference*. Society for Neuroscience, New Orleans.

Zucker, R.S. & Regehr, W.G. (2002) Short-term synaptic plasticity. *Annu Rev Physiol*, **64**, 355-405.