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MODULATION OF NEUROTRANSMISSION IN LOCUS COERULEUS BY METABOTROPIC GLUTAMATE RECEPTORS

by

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Submitted to the School of Graduate Studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiology

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Ever since the age of eight, I had decided that I wanted to do a Ph.D. in microbiology or something related to this. I'm not sure why, but the fact that I lived across from one of the larger Research Institutes in Montréal (Armand Frappier) probably has something to do with it. From the time when I used to go to the back of the Institute to pick up discarded test-tubes and petri dishes, to today, when I am finally submitting my Doctoral thesis, a lot of things have happened. It has been a lengthy and sometimes hard process One has to wonder why one should go through all these years of studying In my case, the answer is simple, **I love it** and I would not do anything else

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To my Science companion, Harman. We got along so well we got married. Need I say more?

Gilles Dubé

25 September 1997

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ABBREVIATIONS:

(R)-CPP ... 3-[(R)-2-carboxypiperazin-4-yl]-propyl-1-phosphonate

(RS)-MCPG (RS)-α-Methyl-4-carboxyphenylglycine

(±)-α-MCPG (±)-α-Methyl-4-carboxyphenylglycine

4-AP 4-aminopyridine

4C, 3H-PG . (R)-4-Carboxy-3-hydroxyphenyl-glycine

5-HT 5-hydroxytryptamine/serotonin

8-Br-cAMP . 8-bromo-cyclic adenosine monophosphate

ADD ..... activity-dependent depression

AC ..... adenylyl cyclase

ACh ..... acetylcholine

AChR ..... acetylcholine receptor

ACSF ..... artificial cerebrospinal fluid

ACTH ..... adrenocorticotrophin hormone

ADH ..... antidiuretic hormone

AHP ..... afterhyperpolarization

AIDA ..... aminoindan dicarboxylic acid

AII ..... angiotensin II

AMPA ..... α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANF ..... atrial natriuretic factor

AP ..... action potential

APDC ..... 2R, 4R-4-aminopyrrolidine -2,4-dicarboxylate

ATP ..... adenosine triphosphate

BHK ..... baby hamster kidney cells

BIC ..... bicuculline

CamKII .... calmodulin kinase II

cAMP ..... cyclic adenosine monophosphate

CCK ..... cholecystokinin

cGMP ..... cyclic guanosine monophosphate

CGRP ..... calcitonin gene-related peptide

CHO ..... Chinese hamster ovary

CNQX 6-cyano-7-nitroquinoxaline-2,3-dione

CNS ..... central nervous system

CRF ..... corticotrophin releasing hormone

DA ..... dopamine

DAG ..... diacylglycetrol

DCG IV ... (2S,1'R,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine

DBH ..... dopamine-beta-hydroxylase

DHP ..... dihydropyridine

DHPG ..... (RS)-3,5-Dihydroxyphenylglycine

DOM ..... domoic acid

DPCPX 8-cyclopentyl-1, 3-dipropylxanthine

DRG ..... dorsal root ganglia

EAA ..... excitatory amino acid

EGLU .... ethyl-glutamate.

Em ..... membrane potential

EPSP ..... excitatory postsynaptic potential

GABA ..... gamma-aminobutyric acid

GABAR ... GABA receptor

GAD ..... glutamic acid decarboxylase

Glu ..... glutamate

GluR ..... glutamate receptor (AMPA/kainate)

GlyR ..... glycine receptor

HEK ..... human embryonic kidney

HRP ..... horseradish peroxidase

IBMX ..... isobutylmethylxantine

IBO ..... ibotenic acid

IP_3 ..... inositol triphosphate

IPSP ..... inhibitory postsynaptic potential

Ka ..... kainate

KO ..... knockout

KYN ..... kynurenic acid

L-AP4 .... L-2-amino-4-phosphonobutyric aci

L-CCG-I ... (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine

LC ..... locus coeruleus

L-SOP ..... L-serine-O-phosphate

LTD ..... long-term depression

LTP ..... long-term potentiation

MAP4 ..... (S)-Amino-2-methyl-4-phosphonobutanoic acid

MCCG .... (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine

MCPG ..... (±)-α-methyl-4-carboxyphenylglycine

mGluR ..... metabotropic glutamate receptor

MSOPPE ... (RS)-α-methylserine-O-phosphate monophenyl ester

mV ..... millivolts

NA ..... noradrenaline

NEM ..... N-methyl-maleimide

NMDA .... N-methyl-D-aspartate

NPY ..... neuropeptide Y

NTS ..... nucleus tractus solitarius

PGi ..... paragigantocellularis nucleus

PKA ..... protein kinase A

PKC ..... protein kinase C

PLA_2 ..... phospholipase A_2

PLC ..... phospholipase C

PLD ..... phospholipase D

PrH ..... nucleus prepositus hypoglossi

PTX ..... pertussis toxin

PVN . . . . . paraventricular nucleus

QUIS . . . . . quisqualic acid

R_{in} . . . . . input resistance

t-ACPD . . . . (±)-1-aminocyclopentane-trans-1,3,dicarboxylic acid

t-PDC . . . . . L-*trans*-pyrolidine-2,4-dicarboxylic acid

TMD . . . . . transmembrane domain

TTX . . . . . tetrodotoxin

VDCC . . . . . voltage-dependent calcium channels

VIP . . . . . vasoactive intestinal peptide

WHA . . . . . wheat-germ agglutinin

Yoh . . . . . yohimbine hydrochloride

WT . . . . . wild-type

ABSTRACT

The locus coeruleus, a small group of pontine neurons, is the most prominent noradrenaline-containing nucleus in the central nervous system. Activity of this nucleus is thought to modulate the function throughout the brain through its widespread afferent network. Yet, the pathways affecting the locus coeruleus are few and the control of the activity of this nucleus is poorly understood. Metabotropic glutamate receptors have been implicated in modulation of synaptic transmission in many different systems. Here, the effects of selective activation of metabotropic glutamate receptors on synaptic transmission in intracellularly recorded locus coeruleus neurons in brain slice preparations are described. Perfusion of either *t*-ACPD (0.1-500 μM) or L-AP4 (0.1-500 μM) caused a depression of excitatory postsynaptic potentials in a dose-dependent fashion to about 70% inhibition. Both agonists exerted their effects with estimated EC_{50}s of 2.6 μM and 11.5 μM for L-AP4 and *t*-ACPD respectively. Additional pharmacological analysis of the *t*-ACPD- and L-AP4-sensitive effects suggested two different receptors belonging to the group II and III metabotropic glutamate receptors respectively. Furthermore, experiments using a mGluR4 deficient mouse indicate that this receptor subtype is not involved.

Both *t*-ACPD and L-AP4 produced an increase in paired-pulse facilitation, and failed to change the response of locus coeruleus neurons to focally-applied glutamate, indicating a presynaptic locus of action. Both group II and III metabotropic glutamate receptors have been shown to be coupled to a $G_{i/o}$-protein and, in certain systems, activation of these

receptors results in a decrease of forskolin-stimulated cAMP levels. However, many all $G_{i/o}$-coupled receptors have also been shown to block calcium channels in different cell types. Thus, several tests were carried out in an attempt to define the signal transduction pathway involved in the *t*-ACPD and L-AP4 effects in locus coeruleus. In a first set of experiments, N-ethylmaleimide, a compound known to disrupt the interaction between $G_{i/o}$ proteins and calcium channels, was tested. Pretreatment of slices with N-ethylmaleimide (50-100 μM, 15 min.) resulted in an almost complete block of the effects of *t*-ACPD and L-AP4. In a second set of experiments, perfusion of a "cAMP cocktail" (200μM 8-bromo-cAMP, 20μM forskolin and 1mM IBMX) was tested on the response of locus coeruleus to *t*-ACPD and L-AP4. Superfusion of slices with the cAMP cocktail resulted in a 67% increase in the amplitude of excitatory postsynaptic potentials (n=7) and paired-pulse facilitation was decreased by more than 70% (n=5), but no significant changes in locus coeruleus responses to focally-applied glutamate or in the input resistance of the cells were observed. Under these conditions, application of *t*-ACPD or L-AP4 produced a similar degree of inhibition of excitatory postsynaptic potential to that observed under control conditions. Together, these results suggest that *t*-ACPD and L-AP4 inhibit excitatory postsynaptic potentials through a $G_{i/o}$ pathway but not through a decrease in cAMP.

In the last part of this study, the functional role of synaptically-released excitatory amino acid on metabotropic glutamate receptors in locus coeruleus was investigated. When single stimuli were applied to the afferents at intervals greater than 200ms, the amplitude of the second [test (T)] excitatory postsynaptic potential was identical in amplitude to the first

[control(C)]. However, when a train of stimulation was delivered prior to T, the amplitude of T was consistently smaller than C. The depression was dependent on the frequency and duration of the train and the interval between the train and T. In most experiments, optimal inhibition was observed with a 300ms, 70 Hz train delivered 600ms prior to the test EPSP. This activity-dependent depression of excitatory postsynaptic potentials was enhanced in the presence of an excitatory amino acid uptake inhibitor L-trans-pyrrolidine-2, 4-dicarboxylic acid (*t*-PDC, 100 μM) from a T/C ratio of 0.80 ± 0.03 (mean ± SEM) in control to 0.68 ± 0.05 in *t*-PDC. It was hypothesized that this inhibition could result from the activation of presynaptic metabotropic glutamate receptors by accumulated excitatory amino acid in the synaptic cleft. To test this, we assessed the effect of selective metabotropic glutamate receptor antagonists on activity-dependent inhibition of excitatory postsynaptic potentials. In the presence of *t*-PDC, bath applied α-methyl-AP4 (MAP4, 500 μM), a metabotropic glutamate receptor group III antagonist which blocked the effect L-AP4 on locus coeruleus, significantly reversed the activity-dependent depression following stimulation volleys [from 0.68±0.07 to 0.86±0.06 (mean ± SEM), n=5]. The T/C ratio in the presence of MAP4 was not different from that of measured without stimulation volleys. Conversely, ethyl glutamate (EGLU, 500 μM), a metabotropic glutamate receptor group II antagonist which blocked the effect of *t*-ACPD on locus coeruleus, failed to significantly change activity-dependent depression. This study demonstrates that group III presynaptic metabotropic glutamate receptor activation may provide a mechanism by which excitatory synaptic transmission can be negatively modulated during high frequency activity of locus coeruleus afferents.

Together, the studies described above provide evidence that activation of either of two different presynaptic metabotropic glutamate receptors belonging to group II and III, respectively, produce a decrease in excitatory synaptic transmission in locus coeruleus through an undetermined mechanism but in a cAMP-independent manner. Furthermore, while both receptors could be activated pharmacologically, only the group III metabotropic glutamate receptor was activated under conditions where endogenous EAA are allowed to accumulate in the cleft. This would suggest that, under the conditions tested, the L-AP4/MAP4-sensitive receptor could play the role of an autoreceptor. The physiological role of the *t*-ACPD/EGLU-sensitive receptor could not be clearly determined under the conditions tested. Together, the work presented here provides clear evidence that metabotropic glutamate receptors can negatively modulate excitatory synaptic transmission to locus coeruleus. In view of the extensive noradrenaline innervation that provides locus coeruleus to the brain, a feedback mechanism such as the one described here would provide an important tool in the control of noradrenaline release throughout the neuraxis.

1. INTRODUCTION

1.1 LOCUS COERULEUS

The locus coeruleus (LC, Latin for blue region), was originally named as such in the last century for its bluish colour in primate resulting from its high melanin content. Although a relatively small pontine structure, LC gives rise to a very divergent and elaborate system of noradrenergic projections throughout the neuraxis. It is the most widely projecting nucleus in the mammalian central nervous system (CNS). Of interest, it provides the entire supply of noradrenergic fibres to the hippocampus (Loy et al., 1980) and the neocortex (Moore and Card, 1984). The discharge characteristics of LC have been investigated both in anaesthetized and awake animals, revealing behaviour during which noradrenaline (NA) would be released from the global efferent network of LC fibbers. These data have led to the formulation of functional hypotheses for LC, including a role in sleep, attention (Robbins, 1984), memory (Sara and Devauges, 1988), and vigilance (Morgane and Stern, 1975; Aston-Jones and Bloom, 1981a; Olpe et al., 1983). The following is a review of LC literature addressing these issues and others. Species differences in LC structure have been reported (i.e., LC is a tightly packed nucleus in rodents and primates but it has a relatively diffuse arrangement in carnivores) so, unless otherwise specified, this review will concentrate on results obtained in rats.

1.1.1. Anatomy of the LC nucleus

In rats, the LC nucleus is limited at its widest extent by the fourth ventricle and the pontine central grey medially, mesencephalic V laterally and the superior cerebellar peduncle dorsolaterally (Russel, 1955; Dahlström and Fuxe, 1964; Foote et al., 1983). It occupies an average volume of 0.8 mm^3, extending for approximately 900 μm rostrocaudally, between 250 and 300 μm mediolaterally, and about 600 μm dorsoventrally. It contains between 1400 and 1800 neurons (Descarries and Saucier, 1972; Ross et al., 1975; Swanson, 1976). Two noradrenaline-containing cell types have been identified within the LC: the dorsal main portion of the nucleus contains densely-packed fusiform cells, and the ventral portion contains primarily large multipolar cells (Swanson, 1976). The somata of the fusiform cells are approximately 15 x 20 μm in size, while those of the multipolar cells are approximately 30 x 40 μm (Shimizu and Imamoto, 1970; Swanson, 1976). The LC neurons, like other central grey and reticular neurons, have long dendrites that branch once or twice and extend outside the limits of the nucleus (Swanson, 1976; Cintra et al., 1982). Most of the extranuclear dendrites are distributed asymmetrically in two perinuclear regions, the rostromedial and the caudal (juxtaependymal) pericoerulear regions (Shipley et al., 1996). In contrast, most of the LC processes found in the dorsolateral region (close to the parabrachial nucleus) are axons (Shipley et al., 1996). In parasagital sections, both the somata and the dendritic fields of the LC neurons appear disk-like in shape with a major axis in the rostrocaudal direction and a minor axis inclined slightly off the dorsoventral direction (Groves and Wilson, 1980a).

Based on formaldehyde-induced fluorescence (resulting from its reaction with catecholamine), Dahlstrom and Fuxe (1964) reported 12 groups of catecholamine-containing neurons in the rat brainstem catalogued as group A1 to A12. A6, one of these cell groups was found to correspond to the LC as earlier localized by Russel (1955), hence providing evidence that the LC neurons were catecholaminergic. It now well established that the prominent catecholamine found in the rat LC is NA, which makes up 90% of the catecholamine synthesized from ^{3}H-tyrosine, whereas the remaining 10% is made up by dopamine (DA) (Kuhar et al., 1972).

1.1.2. Ultrastructure of LC neurons

Ultrastructurally, the overwhelming majority of LC neurons are of a single morphological type, i.e., elongated somata with the cytoplasm concentrated at the poles of the neuron and at the base of the dendritic trunks. The nuclei are large with prominent nucleoli. The cytoplasm contains large amounts of smooth and rough endoplasmic reticulum, a prominent Golgi complex and many lysosomes (Fuxe et al., 1966). Granular and agranular vesicles ranging from 300 to 1200 nm in size are also observed and found not to be associated with NA (Descarries and Droz, 1968). Large areas of the LC neurons, including the dendrites, the soma, the axon, and the synaptic terminals are enwrapped by layers of glial processes (Groves and Wilson, 1980a). These sheet-like processes, 200 to 500 nm thick, appear to arise from astrocytes. Although no precise role for these has been defined, it has been proposed that these could serve a function reminiscent of myelin by isolating the neurons and reducing the electronic distance between the distal thin dendritic region and the soma

(Groves and Wilson, 1980a). The bulk of synapses within the LC proper is found preferentially at small (0.5-2.5 μm of diameter) dendrites and only sparse innervation of the spine-free soma and proximal dendritic trunks was reported (Groves and Wilson, 1980b). No apparent relation between synaptic bouton morphology and the density of the postsynaptic specialization in spines was found, nor is there a segregation of symmetric versus asymmetric synaptic junctions (Groves and Wilson, 1980a). In contrast, pericoerulear dendrites receive numerous synaptic contacts, most of which form asymmetric synapses (Shipley et al., 1996). Morphological evidence of dendro-dendritic synapses and dendro-somatic contacts between LC neurons is well described, and the possibility that these represent means of communication between individual neurons in LC proper has been proposed (Groves and Wilson, 1980a). Communication between neonatal LC neurons has also been shown to occur through low-resistance pathways (possibly gap-junctions) (Christie et al., 1989; Christie and Jelinek, 1993). Two independent sets of evidence support the existence of gap-junctions in neonatal LC. First, multiple LC neurons stained for biocytin (a low molecular weight dye thought to be able to diffuse through gap-junctions) were found following iontophoretic ejection of the dye in a single neuron (Christie and Jelinek, 1993). Secondly, electrotonic-coupling is found between pairs of simultaneously impaled neonatal LC neurons (Christie et al., 1989). This coupling between LC neurons appeared to be developmentally expressed and both dye-coupling and electrotonic-coupling were rarely observed in adult rat LC (Christie et al., 1989; Christie and Jelinek, 1993).

1.1.3. Efferent projections from LC

Early studies of NA cell subgroups [A1-A7; (Swanson and Hartman, 1975; Dahlström and Fuxe, 1964)] efferent connections demonstrated extensive and divergent projections to a myriad of functionally diverse regions of the CNS, raising the question whether the division of NA cells in subgroups was relevant. Later, more detailed analysis (Björklund and Lindvall, 1986) identified 3 groups of NA neuron systems: the LC, the lateral tegmental system, and the dorsal medullary system. The view of NA neurons as a poorly organized projecting system prompted some to hypothesize that the NA systems were likely to exert a global influence on CNS functions rather than acting on specific targets. Recently, however, a growing body of evidence suggests that subgroups of central NA neurons have different, practically non overlapping projections (Jones, 1991; Jones and Yang, 1985). Autoradiographic analysis of the anterograde transport of radiolabelled protein makes it apparent that these projections encompass regions not only divergent in space but also divergent in function (Jones, 1991; Jones and Yang, 1985).

The distribution of efferent projections from LC has been addressed in several comprehensive reviews (Amaral and Sinnamon, 1977; Moore and Bloom, 1979; Foote et al., 1983) (fig. 1) and the following is a partial account of these findings. It is well documented that a large number of LC neurons project to the spinal cord (Swanson and Hartman, 1975; Nagai et al., 1981; Room et al., 1981; Jones and Yang, 1985; Loughlin et al., 1986a, b; Holets et al., 1988; Lyons et al., 1989; Fritschy and Grzanna, 1989). Innervation of spinal cord by LC neurons is primarily located ipsilaterally in the ventral funiculus ending in the

medial part of laminae VII and VIII, and on motoneurons of lamina IX and lamina X. Interestingly, the site of spinal innervation of LC axons was reported to vary in some substrains of rats, in some, ending on the ventral horn while in others the dorsal horn is primarily innervated (Proudfit and Clark, 1991). In the brainstem, LC efferents are largely restricted to the cochlear nuclei, the entire trigeminal sensory complex, nucleus praepositus hypoglossi, inferior olivary complex, the tectum, interpeduncular nucleus, midbrain central grey, superior and inferior colliculi, and the facial nucleus (Cheeseman et al., 1983; Fritschy and Grzanna, 1990). Innervations were found both ipsilaterally and bilaterally. The presence of a cerebellar afferent projection from the LC nucleus has been well documented (Hoffer et al., 1973; Loughlin et al., 1986a; Scatton and Serrano, 1986; Olson and Fuxe, 1972; Pasquier et al., 1980). The cerebellopetal axons from the rostral LC course immediately dorsolateral from the nucleus and join the superior cerebellar peduncle (Hoffer et al., 1973; Swanson and Hartman, 1975). At the caudal level, fibres also travel dorsolaterally and pass medially to the middle cerebellar peduncle (Olson and Fuxe, 1972). In the thalamus, LC efferents are found in the anterior and ventral portions, the nuclear complexes, and the lateral and medial geniculate bodies (Lindvall and Björklund, 1974). LC is the sole source of NA to the lateral geniculate nucleus (Kromer and Moore, 1980). Several hypothalamic structures including among others, the zona incerta, the lateral hypothalamus, the periventricular and arcuate nuclei appear to receive LC efferents (Pickel et al., 1974; Jones, 1991). The paraventricular nucleus (PVN) was also proposed as a target for catecholamine innervation (Pickel et al., 1974; Valentine et al., 1996). The central nucleus of the amygdala and the anterior amygdaloid areas have been shown to receive LC efferents (Mason and Fibiger, 1979;

Cedarbaum and Aghajanian, 1978; Dietrichs, 1985) as have the external plexiform and granular layers of the olfactory bulb (Fallon and Moore, 1978; Solano-Flores et al., 1980). Loy et al., (1980) were the first to demonstrate that LC was the sole source of NA throughout the hippocampus. Similar results have been reported for the neocortex with LC terminals in all six layers (Levitt and Moore, 1978; Artieda and Ullan, 1979; Morrison et al., 1979, 1981).

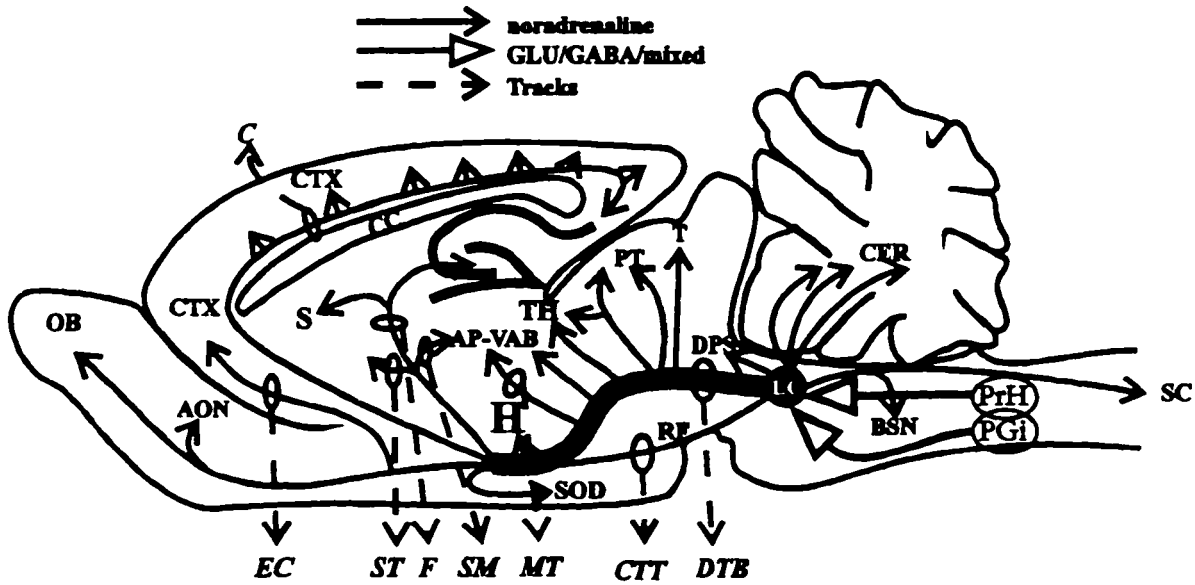


Fig. 1: Diagram of the efferent and afferent projections of the LC viewed in a parasagittal section. Abbreviations: **AON**--anterior olfactory nucleus; **AP-VAB**--ansa peduncularis-ventral amygdaloid bundle; **BSN**--brainstem nuclei; **C**--cingulum; **CC**--corpus collasum; **CER**--cerebellum; **CTT**--central tegmental tract; **CTX**--cerebral cortex; **DPS**--dorsal external capsule; **DTB**--dorsal catecholamine bundle; **EC**--external capsule; **F**--fornix; **H**--hypothalamus; **HF**--hippocampal formation; **LC**--locus coeruleus; **ML**--medial lemniscus; **MT**--mammillothalamic tract; **OB**--olfactory bulb; **PC**--posterior commissure; **PGi**--paragigantocellularis; **PrH**--prepositus hypoglossi; **PT**--pretectal area; **RF**--reticular formation; **S**--septum; **SC**--spinal cord; **SM**--stria medullaris; **SOD**--superior optic decussations; **ST**--stria terminalis; **T**--tectum; **TH**--thalamus (updated from Moore and Bloom, 1979)

1.1.4. Afferent Innervations

In contrast to the widespread efferent projections, LC neurons were found to receive limited afferent innervations. Using wheat-germ agglutinin conjugated to horseradish peroxidase (WGA-HRP), Aston-Jones and collaborators demonstrated that the major inputs to the LC nucleus are the nucleus paragigantocellularis (PGi) and the nucleus prepositus hypoglossi (PrH), both of which lie in the rostral medulla (Aston-Jones et al., 1986; Aston-Jones et al., 1990; Aston-Jones et al., 1991b). Minor inputs from the PVN, midbrain peri-aqueductal gray, spinal lamina X, and the ventromedial pericoerulear region were also reported (Aston-Jones et al., 1991b). This limited distribution contrasted with earlier reports of much broader afferent inputs to LC (amygdala, insular cortex, preoptic area, lateral and dorsomedial hypothalamus, stria terminalis, central grey, nucleus tractus solitarius (NTS), reticular formation, and raphé) using non-conjugated horseradish peroxidase as a retrograde tracer (Cedarbaum and Aghajanian, 1978; Clavier, 1979). These discrepant observations were attributed to the relative ease of non conjugated HRP to diffuse further from the site of injection than the conjugated HRP (Aston-Jones et al., 1990). While these structures were revealed on the basis of their monosynaptic connections to LC, an additional polysynaptic pathway from the frontal cortex has also been reported. This inhibitory pathway involved glutamatergic cortical axons synapsing onto GABAergic interneurons within or proximal to the LC (Sara and Hervé-Minvielle, 1995) which in turn inhibited LC activity.

The restricted profile of afferents to LC was further supported by results from antidromic activation of suspected innervating nuclei. Indeed, direct activation of LC through

a stimulating electrode resulted in the specific antidromic activation of both PGi and PrH nuclei, but little or no antidromic activation of other regions examined (the lateral reticular nucleus, contralateral LC, or NTS) (Ennis and Aston-Jones, 1987; Aston-Jones et al., 1990). Stimulation of suspected afferent nuclei also supported limited output to LC; stimulation of NTS or the central nucleus of the amygdala failed to activate LC neurons (Aston-Jones et al., 1990). However, activation of these structures resulted in the activation of the parabrachial nucleus located next to LC, which may explain some retrograde tracing results using non conjugated HRP (Aston-Jones et al., 1990). Again, under these experimental conditions, LC neurons responded to the stimulation of either the PGi or the PrH nuclei (see below). Interestingly, destruction of the PGi and PrH nuclei did not result in a blockade of the LC response to somatosensory stimuli (see below) (Rasmussen and Aghajanian, 1989) suggesting other afferent pathway(s) must impinge on LC. To date, however, these observations remain unexplained.

1.1.4.1. PGi effects on LC

Activation of PGi exerted a predominantly excitatory influence on LC discharge (Ennis and Aston-Jones, 1986a, 1988) with an onset latency of about 11 ms and was typically followed by a postactivation inhibition lasting between 30 and 600 ms (Ennis and Aston-Jones, 1986b). Only a small fraction of the PGi-evoked responses was inhibitory (16%). Intracerebroventricular (icv) injections or local iontophoretic ejections to LC of the broad spectrum excitatory amino acid (EAA) antagonist kynurenic acid completely blocked PGi-evoked excitation of LC (Ennis and Aston-Jones, 1988). These results support the claim

that PGi innervates LC through EAA afferents. In addition, blockade of PGi-evoked LC excitation with EAA antagonists, revealed an underlying, purely inhibitory response of LC neurons to PGi stimulation (Aston-Jones et al., 1990), which was later identified as an α_2-adrenergic response (Aston-Jones et al., 1992).

1.1.4.2. PrH effects on LC

In contrast to the predominant excitation of LC from PGi, stimulation of PrH resulted in a uniform inhibition of LC activity with a latency of about 20 ms (Ennis and Aston-Jones, 1989a). Application of picrotoxin or bicuculline (Bic) significantly or completely blocked PrH-evoked inhibition of LC neurons supporting a role for gamma-aminobutyric acid (GABA) as the neurotransmitter mediating the PrH effects. Strychnine, a glycine receptor antagonist, or α_2-adrenoceptor antagonists did not produce significant inhibition of the PrH-induced effects (Ennis and Aston-Jones, 1989b; Aston-Jones et al., 1990). Furthermore, immunocytochemical studies of PrH neurons demonstrated the presence of GABA and its synthesizing enzyme glutamic acid decarboxylase (GAD) (Mugnaini and Oertel, 1985).

1.1.5. Synaptic potentials in LC *in vitro*

LC neurons display spontaneous firing activity both *in vivo* and *in vitro*. It was suggested that the intrinsic spontaneous activity of these neurons delivers constant noradrenergic tone to its various targets (Moore and Bloom, 1979). Under basal conditions, neurons were shown to fire asynchronously but synchronized firing patterns have been reported in response to strong synaptic input (Aston-Jones and Bloom, 1981a; Ennis and

Aston-Jones, 1988). LC activity was reported to be increased by a variety of noxious and non noxious stimuli (Foote et al., 1980; Valentino and Aulisi, 1987) (see below) and reduced during rapid eye movement sleep (Aston-Jones and Bloom, 1981b). Alterations of the spontaneous firing activity of LC neurons likely resulted from transient changes in synaptic transmission to this nucleus.

To study synaptic transmission in LC, brain slice preparations were used (Egan et al., 1983; Cherubini et al., 1988). As no visible afferent path can be identified in these preparations, field stimulation was applied within or close to the body of the LC. Under these conditions, electrical stimulation evoked the release of a large array of neurotransmitters, the effects of which were recorded as postsynaptic potentials in LC neurons (Egan et al., 1983; Cherubini et al., 1988; Williams et al., 1991). Several types of synaptic potentials have been described in the cell bodies of LC neurons using brain slice preparations. These can be divided into fast synaptic potentials (onset of 0.5-2 ms and peaked after 20-40 ms) and slow potentials (onset of 40-50 ms and peaked in 200-400 ms) (Williams et al., 1991). While the latter were hyperpolarizing, the former were either excitatory or inhibitory.

1.1.5.1. Fast excitatory synaptic potentials

Excitatory postsynaptic potentials (EPSPs) were characterized using defined receptor antagonists (Cherubini et al., 1988; Williams et al., 1991). These were entirely blocked by EAA antagonists (Cherubini et al., 1988) while antagonists to acetylcholine (ACh) receptors

(either nicotinic or muscarinic) were ineffective (Williams et al., 1991), suggesting that EAA are the primary excitatory neurotransmitters responsible for EPSPs in LC.

1.1.5.2. Fast inhibitory synaptic potentials

Inhibitory postsynaptic potentials (IPSPs) were isolated from EPSPs by application of EAA antagonists and characterized as described above (Cherubini et al., 1988; Williams et al., 1991). IPSPs were dependent on the chloride content of the recording electrode suggesting that, at least in part, those were mediated through changes in chloride conductances. Application of specific GABA antagonists completely and reversibly blocked these synaptic potentials in most tests (Cherubini et al., 1988; Williams et al., 1991). However, occasionally, strychnine-sensitive IPSPs (glycine-mediated) were observed. The time course of glycine-mediated IPSPs was similar to that described for GABA. However, unlike the other synaptic potentials identified in LC, the anatomical origin of the glycine has not been identified (Williams et al., 1991).

1.1.5.3. Slow inhibitory synaptic potentials

Following the fast synaptic potentials, a hyperpolarization phase was observed (Egan et al., 1983; Surprenant and Williams, 1987; Adam-Vizi, 1992). The latter lasted for up to two seconds following the stimulus (Egan et al., 1983) and could be prolonged by application of NA uptake inhibitors (Surprenant and Williams, 1987). Slow IPSPs were blocked by α_2-adrenoceptor antagonists, further supporting a role for adrenergic/noradrenergic involvement (Egan et al., 1983; Williams et al., 1991). The reversal potential of the hyperpolarization was

about 104 mV suggesting that changes in K^+ conductances mediated these IPSPs (Egan et al., 1983). While adrenaline originating from the PGi afferents may be responsible for part of this effect, most of the slow IPSPs has been attributed to the release of NA from LC itself (Surprenant and Williams, 1987; Williams et al., 1991).

1.1.6. Neurochemicals in LC

Many neurotransmitter systems have been found to impinge on the LC (Foote et al., 1983; Marshall and Finlayson, 1988; Aston-Jones et al., 1990). Besides EAA, GABA, glycine and adrenaline/NA systems which have been characterized in synaptic transmission (above), several other neurochemicals (monoamines and peptides) have been associated with LC. A list of these is presented in table 1. Hence more than 20 neurochemicals have been associated with LC based on their localization within LC, localization of synthetic enzyme(s) or receptor(s) associated with these neurochemicals, or based on the responsiveness of LC to these.

1.1.7. Physiology and pathophysiology of the LC-NA system

1.1.7.1. Function of NA in the CNS

NA has both inhibitory and excitatory effects on neuronal function, depending on the postsynaptic receptor subtype expressed (Unnerstall et al., 1984). Iontophoretic application of NA has resulted in inhibitory effects on neuronal spontaneous activity in hippocampus, cerebellum, and auditory cortex (Hoffer et al., 1973; Segal and Bloom, 1974a; Foote et al., 1975; Freedman et al., 1977; Segal, 1981) through the activation of the β-adrenergic receptor

Table 1: Neurochemicals and the locus coeruleus

Neurotransmitter	Presence in LC: Cell body	Terminals	Receptor	Action
5-HT	-	++	+	↘
ACh	-	+	++	↗
ACTH	-	+		↗
Adenosine				↘
ADH	++	+++		↗
Adrenaline		++		↘
AII	?	++	++	↘
ANF	+	+	+	
ATP				
CCK	+	+	++	
CGRP	-	+		
CRF	+	+	+	↗
Dopamine	*	+		
Dynorphin	-	+		
Enkephalin	+++	+++	++	↘
GABA	+	+++		↘
Galanin	++			
Glu	+	+++	+++	↗
Glycine				↘
Histamine		++	++	
NA	+++	+++	α_2 ++	↘
			α_1 ++	
Neurophysin	+	+++		
Neurotensin	++	+		↘
NPY	++	+		
Somatostatin	+	+	++	
Substance P	+	++	++	↗
VIP	+	+		

Adapted from Marshall and Finlayson (1988) and updated with (Caffe et al.1985; Hicks et al.1987; Moore and Gustafson, 1989; Jin et al.1991; Illes et al.1993; Shiekhattar and Aston-Jones, 1993; Engberg and Hajos, 1994; Illes et al.1994; Kunkler et al.1994; Maeda et al.1994; Singewald et al.1994; Xiong and Marshall, 1994; Ivanov and Aston-Jones, 1995; Nieber et al.1995; Pieribone et al.1995; Travagli et al.1995; Holmes and Crawley, 1996, Osborne and Christie, 1996, Schulz and Lehnert, 1996, Shih et al.1996)

(Herrling, 1981; Egawa et al., 1988). Furthermore, electrical stimulation of the LC also resulted in an inhibitory response in the hippocampus (Segal and Bloom, 1974a) and the cerebellum (Hoffer et al., 1973) whereas the destruction of the noradrenergic pathways produced the reverse effect. A ß-receptor-mediated inhibition of neuronal activity has led to the hypothesis that NA could increase the signal-to-noise ratio of neuronal responses by decreasing background activity, possibly potentiating the ability of the individual to attend to relevant stimuli (Woodward et al., 1979; Madison and Nicoll, 1982; Waterhouse et al., 1988; Aston-Jones et al., 1991b). Stimulation of the α_2-adrenoceptor has also been shown to produce an inhibitory response in the cerebral cortex (Lomasney et al., 1991). Alternatively, activation of the α_1-adrenoceptor has been shown to have excitatory effects on neuronal function in many targets such as in deep layers of the cerebral cortex or thalamus (Bevan et al., 1977; Waterhouse et al., 1981; McCormick and Huguenard, 1992; Wang and McCormick, 1993). This change in excitability was found often to result from a change in firing patterns of the target neurons from spontaneously bursting to tonically firing (McCormick, 1992a, b; McCormick and Huguenard, 1992).

1.1.7.2. Roles of the LC in CNS function

Under physiological conditions, the LC-NA system has been proposed to participate in multiple brain functions (noting here that most of the work described below was obtained from experiments done on cats, guinea pigs or monkeys, which have been the models of choice for behavioural studies). Many observations support a role for LC in sleep-wake cycle, through which it was found to discharge tonically during wakefulness, decrease in

activity during slow wave sleep and be virtually quiescent during paradoxical sleep (Aston-Jones and Bloom, 1981b; Darracq et al., 1996). Activity of LC was also altered in association with behavioural state. The highest tonic LC discharge rates were observed with exploratory alertness, presentation of preferred food, or appearance of an unfamiliar person; lower activity occurred during grooming and drinking (Aston-Jones and Bloom, 1981b). In addition, unconditioned auditory, visual, or tactile stimuli phasically activated LC neurons. The extent of this activation was dependent on the behavioural state, with the largest responses observed during high levels of alertness (Aston-Jones and Bloom, 1981a). These observations, with the known effects of NA on target neural activity (see above) suggest a role for the LC-NA system in maintaining vigilance (Aston-Jones and Bloom, 1981a; Aston-Jones et al., 1994; Rajkowski et al., 1994)

A number of investigations have demonstrated a role for LC-NA in learning and memory (Anlezark et al., 1973; Zornetzer et al., 1978; Koob et al., 1978; Velley and Cardo, 1979; Ogren et al., 1980; Archer et al., 1986; Dunn and Everitt, 1987; Pisa et al., 1988; Compton et al., 1995; Cirelli et al., 1996). NA may be critical for memory consolidation, with other NT, by playing a permissive role, and possibly altering the level of expression of specific genes in certain brain areas (Cirelli et al., 1996). Bilateral lesions of LC nuclei resulted in a severe impairment in learning in rats (Compton et al., 1995). This possible role for LC is further supported by the exclusive NA innervation to the neocortex and hippocampus, areas associated with memory formation and consolidation. Several visceral stimuli, such as hypotension, hypercapnia, and distention of the colon, rectum, and bladder (Elam et al., 1984;

Elam et al., 1986; Valentino et al., 1991; Murase et al., 1993; Singewald and Philippu, 1993; Singewald et al., 1994; Page and Valentino, 1994) have been reported to increase LC activity transiently.

The LC-NA system is thought to play an important role in the fear response and anxiety along with other specific brain regions (amygdala, hippocampus, thalamus, hypothalamus, prefrontal cortex, and central grey) [for review, see Bremner et al., (1996a)]. Stress results in a rapid and robust increase in LC activity. This is well exemplified by the observations that LC activity was found to double/triple in association with defensive behaviours such as arched back, piloerection, flattened ears, increased heart rate and blood pressure, and mydriasis following presentation of a stressor (a dog or aggressive cat) to an instrumented cat (Levine et al., 1990). These findings in animal studies relating the LC/NA system to stress have been corroborated in human studies as well. Stressful situations which induce fear in healthy human subjects are associated with increases in heart rate, blood pressure and plasma and urinary NA and adrenaline. Some studies also support a role for LC in the symptomatology associated with panic disorder, and post-traumatic stress disorders in patients [for review, see Bremner et al., (1996b) and Johnson and Lydiard, (1997)].

1.1.7.3. The PGi-LC connection

The PGi is a diffuse, complex, highly integrative nucleus, but its functions are poorly characterized (Andrezik et al., 1981a). Major afferents to the PGi have been reported to originate from the spinal cord, midline pontine and medullary nuclei (Andrezik et al., 1981b).

Besides LC, PGi has been found to innervate the cerebellum (Bishop and Ho, 1985), spinal cord (Brown and Guyenet, 1984; Lovick, 1985; Skagerberg and Bjorklund, 1985), nucleus raphé magnus, and the spinal trigeminal nuclei (Beitz, 1982a, b; Beitz et al., 1983; Lovick and Hilton, 1985). PGi has been functionally implicated in the regulation of peripheral autonomic activity, and the discharge rate of PGi neurons was found to change in relation to various cardiovascular and respiratory parameters (Brown and Guyenet, 1984; Vincent et al., 1985). Many studies have also implicated the PGi region in pain and central analgesia (Kuraishi et al., 1983; Satoh et al., 1983; Gray and Dostrovsky, 1985; Hammond et al., 1985; Heinricher and Rosenfeld, 1985), and noxious stimuli elicited robust discharge of PGi neurons (Azami et al., 1981; Ennis and Aston-Jones, 1988). It is unclear if PGi neurons are uni- or multi-modal and if all or a only few of these neurons project to LC. However, LC neurons respond to multiple modalities suggesting that it is regulated by afferents that integrate sensory information across modalities. As such, PGi neurons are prime candidates to mediate sensory responsiveness of LC neurons. Additional work is required to define more clearly the relationship and circuitry between the two nuclei.

1.1.7.4. The PrH-LC connection

Very little is known about PrH in rat. It was found to be reciprocally interconnected with the contralateral perihypoglossal nuclei, the paramedian pontine and medullary reticular formation, and the cerebellum (Yingcharoen and Rinvik, 1983; McCrea and Baker, 1985). The frontal cortex has also been reported to send projections to the PrH area (Leichnetz, 1985). Besides LC, the PrH also sends afferents to the inferior olive, superior colliculus, and

pretectum and extraocular motor nuclei (Yingcharoen and Rinvik, 1982; Ito et al., 1984; Higo et al., 1990). The overall anatomy and physiology of the PrH have led investigators originally to postulate a role for this nucleus in the control of gaze (Lopez-Barneo et al., 1981; Lannou et al., 1984). However, recent evidence may support a role for PrH in the initiation and coordination of holistic orienting responses (McCrea and Baker, 1985; McCrea, 1988). It has been speculated that this could correlate with the phasic disinhibition of LC observed during orienting behaviours, which could result from an intermittent activation/inactivation of the GABAergic transmission from PrH.

1.2. EXCITATORY AMINO ACID RECEPTORS

EAA are the principal excitatory neurotransmitters in the CNS (Monaghan et al., 1989). L-glutamate (Glu) is the principal endogenous candidate for mediating EAA's actions, but L-aspartate and other amino acids and dipeptides have also been proposed as members of the EAA family (Orrego and Villanueva, 1993). The general features of Glu-mediated synaptic transmission are now well accepted (Mayer and Westbrook, 1987a). Release of Glu at central synapses results in the possible activation of three distinct classes of receptors that can localize at individual synaptic sites (Monaghan et al., 1989; Bekkers and Stevens, 1989; Romano et al., 1995). Despite exposure to the same Glu transient at the synaptic cleft [1 mM for 1 ms (Clements et al., 1992)] these receptors differ markedly in their properties and effect on synaptic transmission. The receptors mediating the various Glu effects can be classified in two different categories: (1) the ionotropic Glu receptors that couple to cation channels, and (2) the metabotropic Glu receptors that couple to G-proteins which, in turn, are coupled

to different signal transduction systems. The ionotropic receptor class contains two subclasses of Glu receptors: (I) those gated by N-methyl-D-aspartate (NMDA) which are characterized by their permeability to calcium ions and their sensitivity to external magnesium blockade, and (II) the non-NMDA receptor-gated channels which can be further divided into two groups named based on their affinity to specific ligands: (a) the high-AMPA (α-Amino-3-hydroxy-5 methyl-4-isoxazolepropionic acid) affinity receptors, and (b) the high-kainic acid (Ka) affinity receptors. Marked differences have been found between the non-NMDA and NMDA receptor. For example, in hippocampal pyramidal neurons, selective activation of AMPA receptors mediated a brief synaptic current (~3ms) resulting from the opening of monovalent-selective cation channels, whereas the kinetics of the Na^+- and Ca^{2+}-permeable NMDA receptors were much slower (about 10 ms rising time and a duration ranging between 200-500 ms) (Mayer and Westbrook, 1987a, b). The NMDA receptor, with its external Mg^{2+} block, is the only ligand-gated channel identified to date for which the probability of opening depends strongly upon membrane potential under physiological conditions. This property of the NMDA receptor makes it an ideal candidate for Hebbian-like processes (Hebb, 1949; Jessel and Kandel, 1993). Attempts to clone the Glu receptors have resulted in the isolation of 30 recombinant receptor cDNAs and many splice variants. As with the pharmacological classification of the Glu receptors, these cDNAs can be divided into four groups with 22 members forming the ionotropic family and eight members forming the metabotropic Glu receptor family [for review, see Hollmann and Heinemann, (1994)].

1.2.1. AMPA-gated glutamate receptors

The AMPA receptor family comprises four highly homologous subunits, termed GluR1-GluR4 (Boulter et al., 1990) [alternatively named: GluRA-GluRD (Keinanen et al., 1990)]. The four subunits are similar in size (900 amino acids), share 68% to 73% homology in their amino acid sequence and contain four distinct hydrophobic domains which are characteristic of transmembrane domains (TMD). The AMPA receptor subunits are highly conserved between mammals. The rat, mouse and human GluR1 are 96-97% conserved at the amino acid level. In spite of some apparent structural similarities, the amino acid sequence homology of the GluR1-4 subunits with other known ligand-gated ion channels (AChR, $GABA_AR$ and GlyR) is very poor (~20%) (Hollmann and Heinemann, 1994). Significant differences exist between the GluR and the better characterized nicotinic receptor superfamily. The GluR are much larger receptors, with 50% of the protein (~500 amino acids) in the N-terminal domain preceding TMD I (Hollmann and Heinemann, 1994). It was initially assumed that the Glu channels might follow the 3+1 TM described for the AChR. However, elegant work by several laboratories now indicates that the Glu receptor channels have only 3 true TM domains and one re-entrant loop which is similar to the S5-6 P loop of voltage-gated channels (Fig. 2) (Hollmann et al., 1994; Bennett and Dingledine, 1995; Wo and Oswald, 1995). This re-entrant loop is thought to be part of the pore region with other parts of the protein. A similar organization and topology was described for Ka and NMDA receptors (Hollmann et al., 1994; Bennett and Dingledine, 1995; Wo and Oswald, 1995).

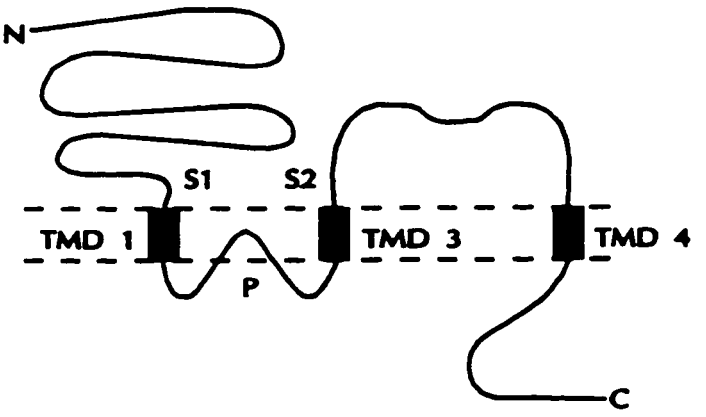


Figure 2: Proposed general topology of ionotropic Glu receptors.
The three transmembrane domains (TMD) model of the Glu-gated channel subunits. The proximal portion of the N-terminus and the extracellular domain between the TMD3 and 4 (S1 and S2) have been involved in agonist binding in AMPA, Ka and NMDA receptors. The P region (formerly TMD 2) appears to be a re-entrant loop similar to that described for voltage-gated channels and contributes to the pore of the channel. The intracellular C-terminal portion of the protein contains multiple consensus sites for different kinases and binding sites for anchoring proteins such as PDS-95, GRIP, α-actinin, or AKAP.

Following transfection of oocytes with individual cDNAs (homomeric), or co-transfection of different combinations of cDNAs (heteromeric), the expressed polypeptides interact together, possibly in a pentameric arrangement (180 KDa) (Wenthold et al., 1992), to form a functional receptor-channel which exhibits high binding and gating affinity to AMPA (in order of potency: QUIS > DOM = AMPA > Glu > Ka), AMPA being the most specific agonist (Boulter et al., 1990; Keinanen et al., 1990). Ka or DOM produced larger currents than that resulting from the activation of the same receptor with AMPA. This is a consequence of lack of desensitization of the receptor when activated by Ka and DOM. When compared to homomeric GluRs, heteromeric channels expressed in oocytes resulted

in a greater effect on current amplitude and displayed properties comparable to native channels (Boulter et al., 1990; Hollmann et al., 1991; Burnashev et al., 1992a, b). These include, in some cases, a selective increase in permeability to divalent ions (calcium and magnesium) caused by a single amino acid change (Q/R site: glutamine 586– arginine) in the re-entrant loop of GluR2 through RNA editing (Hollmann et al., 1991; Burnashev et al., 1992a, b; Sommer et al., 1991; Sommer and Seeburg, 1992). Another identified feature, the Flip/Flop (Monyer et al., 1991; Lambolez et al., 1992), is a result of alternate splicing of a small region (38 amino acids) of every subunit which causes a change in the rate of inactivation when the receptor binds AMPA or Glu. The Flip and the Flop splice variants are expressed in distinct regions of the brain (Lambolez et al., 1992).

1.2.2. Kainate-gated glutamate receptors

Observations of differential, non overlapping distributions of Ka and AMPA binding in certain regions of the CNS have led to the suggestion that distinct groups of non-NMDA receptors were expressed in the brain (Sommer and Seeburg, 1992; Wisden and Seeburg, 1993; Raman and Trussell, 1992). A typical example of this has been characterized in hippocampus where the CA3 region bound Ka preferentially while CA1 bound AMPA. Screening of rat brain cDNA libraries using low stringency hybridization of probes derived from GluR sequences sharing high homology resulted in the cloning of two new groups of receptors, the low-Ka affinity GluR5-7, and the high-Ka affinity Ka1 and Ka2 (Bettler et al., 1990; Sommer et al., 1992; Egebjerg et al., 1991; Bettler et al., 1992). Sequence analysis of the clones demonstrated a high homology ($\approx$70%) within groups but low homology between

the two groups (≈43%) or when compared to the GluR1-4 family (<40%). Both GluR5 and 6 were found to form functional homomeric ligand-gated channels with much higher affinity to Ka than AMPA (GluR6 was not activated by AMPA). GluR7, Ka1 and 2 did not form functional homomeric channels but also had a higher affinity for Ka than for AMPA. However, heteromeric combination of any of the latter ones with GluR5 or 6, but not with GluR 1-4, led to the formation of functional ligand-gated channels with novel properties. As for the GluR1-4 family, GluR5 and 6 properties were found to be altered by RNA editing (Egebjerg and Heinemann, 1993).

1.2.3. NMDA-gated glutamate receptors

Cloning for the NMDA receptor has revealed that this receptor was coded by a family of genes [for review, see Hollmann and Heinemann (1994)]. NR1, the first to be isolated formed functional homomeric ligand-gated channels when analysed in an oocyte expression system (Moriyoshi et al., 1991). However, properties of the resulting channels were different from native channels. Subsequent cloning for NMDA receptors revealed a second family of four highly homologous proteins, NR2A-D. Transfection experiments using only NR2 subunits revealed that these could not form functional ligand-gated channels. However, expression of any NR2 with NR1 produced functional NMDA-gated channels with properties closely resembling those of the native receptors. Thus native NMDA receptors appear to be heteromers of NR1 and NR2 (Meguro et al., 1992; Monyer et al., 1992)

1.2.3.1. Channel properties of NR1

When expressed by itself in an oocyte expression system, NR1, formed a functional channel gated by ligands known to activate NMDA current in native channels, namely, Glu, NMDA, IBO, QUIS, aspartate, N-acetylaspartylglutamate (NAAG) and not AMPA, Ka, or glycine (Moriyoshi et al., 1991). However, omission of glycine substantially reduced the response to agonists (Moriyoshi et al., 1991). As mentioned above, the NMDA receptor-channel is voltage-sensitive. This property is governed by the presence of extracellular Mg^{2+} ions (Mayer and Westbrook, 1987a). This feature of the NMDA receptor was also observed with recombinant homomeric NR1. The current-voltage relationship in the absence of Mg^{2+} was linear with a reversal potential of 0 mV. Physiological concentrations of Mg^{2+} caused a shift in reversal potential to -20 mV and only small or no current at more negative potentials (Moriyoshi et al., 1991). The permeability of the channel to Ca^{2+}, another property of native NMDA channels was also observed with recombinant homomeric NR1 receptor (Moriyoshi et al., 1991). The Ca^{2+} permeability and the Mg^{2+} blockade of the NMDA receptor are governed by the presence of a single asparagine residue in the second putative TMD (Burnashev et al., 1992). Point mutation experiments of this amino acid residue yielded channels with altered Ca^{2+}/Mg^{2+} permeability/blockade (Burnashev et al., 1992). Thus the monomeric NR1 receptor possessed most of the characteristics of the native NMDA receptor, with the exception that the current generated by the opening of the NR1 channel was significantly smaller than that of native channels. Eight splice variants of the NR1 gene, resulting from one N-terminal insertion and two C-terminal deletions, as defined by the original clone NR1-1a (Sugihara et al., 1992), have also been identified, some of which confer

to the NMDA receptor different channel properties than those reported for the originally cloned NR1 (Sugihara et al., 1992).

1.2.3.2. Contribution of NR2 subunits

Homomeric and hetromeric expression of NR2A, B, C, D, failed to generate a functional NMDA receptor channel (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992). However, upon co-transfection of each NR2 subtype with NR1, functional channels with properties different from homomeric NR1 were observed. Most notably, in the presence of glycine, the current amplitude was 5, 20, 40, and 60 fold larger for NR1 co-transfected with NR2D, C, A, and B respectively, when compared to homomeric NR1 (Meguro et al., 1992). Every subtype of NR2 contained a putative asparagine in TMD II and thus, it was not surprising to observe that any combination of NR2 with NR1 produced a channel permeable to Ca^{2+} and blocked by Mg^{2+} (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992). Each recombinant receptor combination displayed reversal potentials between 0 mV and 20 mV in high extracellular Na^{+} and Ca^{2+} solutions, respectively (Monyer et al., 1992). In the absence of external Mg^{2+}, the current-voltage relationship was linear for all combinations as seen for the NR1 homomere. However, in the presence of physiological concentrations of extracellular Mg^{2+}, differences between different subunit combinations were observed. First, the Mg^{2+} blockade was considerably weaker with an NR1/NR2C combination as opposed to NR1/NR2A or B, or as compared to the "characteristic" native NMDA receptor (Monyer et al., 1992). In fact, the NR1/NR2C remained unblocked even at -70 mV membrane potential

in 1 mM external Mg^{2+} (Monyer et al., 1992; Ishii et al., 1993). IC_{50} values for the Mg^{2+} blockade of 10 μM and 130 μM have been reported for NR1/NR2A and NR1/NR2C respectively (Ishii et al., 1993). This property of recombinant NMDA receptors, dictated by the presence of the NR2C subunit, with the restricted distribution of this subunit mainly to the cerebellum (Monyer et al., 1992; Ishii et al., 1993) may represent an important mechanism by which NMDA receptors can support different roles in various regions of the CNS. Significant differences in the Ca^{2+}-dependent inactivation of NMDA currents (Mayer and Westbrook, 1985; Zorumski and Thio, 1992; Vyklicky, 1993; Medina et al., 1995) were also observed between NR1/NR2A and NR1/NR2C (Krupp et al., 1996).

NMDA receptor activation has been shown to be a key step in various neurophysiological phenomena such as long-term potentiation (LTP), and other forms of neural plasticity, cell migration and development, and has been implicated in neurotoxicity and in the development of certain neurodegenerative diseases (Jessel and Kandel, 1993; Lopez-Molina et al., 1993; Kombian and Malenka, 1994; Durand et al., 1992; Stevens and Wang, 1994). As such, understanding of the distribution and function of specific combinations of the NMDA receptor subtypes throughout the brain may be crucial for understanding CNS development and function.

1.2.4. Metabotropic glutamate receptors

As described above, Glu activates ligand-gated ion channels which mediate fast synaptic transmission. However, it became increasingly obvious that Glu produced effects

distinct from those described above. In 1985, Sladeczek et al. (1985), reported that Glu increased inositol triphosphate (IP_3) formation through a phospholipase C (PLC)-sensitive pathway in striatal neurons in culture. Similar observations were soon reported in hippocampal slices (Nicoletti et al., 1986a, b), in cultured cerebellar granule cells (Nicoletti et al., 1986c) and in cultured astrocytes (Pearce et al., 1986). Hence, for the first time, evidence was presented that Glu can act through a G-protein-coupled receptor. This new class of receptors was named metabotropic Glu receptor (mGluR).

Independently, the Glu analogue L-amino-4-phosphonobutyric acid (L-AP4) was identified as a ligand of a yet uncharacterized receptor for which activation resulted in the inhibition of excitatory synaptic transmission (Koerner and Cotman, 1981, 1982; Lanthorn et al., 1984). Furthermore, L-AP4 was without any marked effect described for other EAA agonists (Davies and Watkins, 1982; Davies et al., 1982). It became apparent that L-AP4 acted as an agonist of presynaptic Glu receptors, depressing Glu release, possibly through a G-protein coupled pathway.

To date, eight mGluR subtypes have been cloned as part of what is now accepted to be a new family of receptors, including a Glu unrelated protein, the parathyroid Ca^{2+}-sensing receptor 1 (PCaR1) (Brown et al., 1993). These mGluRs have now been implicated in functions as diverse as neuromodulation, synaptic plasticity, motor control, and neurotoxicity.

1.2.4.1. Molecular biology and structure of the mGluRs

The strategy employed for cloning these presumed G-coupled receptor genes made use of *Xenopus* oocytes' ability to couple G-protein activation with chloride channel currents, which can be detected electrophysiologically. Hence, a cerebellar cDNA library was made and oocytes were screened for oscillatory chloride currents in response to Glu applications. Using this approach, the first cDNA clone was isolated and named mGluR1a (Houamed et al., 1991; Masu et al., 1991). Using the knowledge obtained from the latter's DNA sequence, probes were made to screen cDNA libraries by low stringency hybridization and degenerate primers were designed for polymerase chain reaction (PCR) analysis. Thus far, an additional seven related receptors named mGluR2-8, and splice variants for mGluR1, 4, 5, 7, and 8 have been cloned (Abe et al., 1992; Pin et al., 1992; Tanabe et al., 1992; Minakami et al., 1993; Nakajima et al., 1993; Saugstad et al., 1994, 1997; Duvoisin et al., 1995; Corti et al., 1996). Primary structure comparisons of the proteins indicated that these receptors were all related. They are much larger than other known G-coupled receptors and share negligible homology with those. Based on primary sequence homology, the eight receptors can be divided in three groups: group I comprising mGluRs 1 and 5, group II which contains mGluRs 2, 3, and group III which contains mGluRs 4, 6, 7, and 8. The relative sequence homology within each group is about 70 % while the homology between groups falls to about 45% (Pin and Duvoisin, 1995).

A putative signal peptide was found in the C-terminal amino acid sequence of each receptor suggesting that the amino-terminal portion of the receptor is extracellular. This extracellular portion is markedly larger than for other known G-protein-coupled receptors (>500 amino acid residues) (fig. 3). The mGluRs' sequence also contains seven closely located hydrophobic domains thought to correspond to seven TMD, a characteristic of G-protein coupled receptors. In accord with this topography, the C-terminal tail of the receptor would be intracellular. This topology was further supported by the presence of multiple glycosylation sites on the N-terminal portion of the protein preceding the seven hydrophobic segments and consensus sites for phosphorylation on the C-terminal segment. The C-terminal tail is also the most variable portion among the different receptors. While mGluRs do not share significant homology with other G-coupled receptors, they couple to the same G-proteins. Construction of chimeras between mGluR1 and mGluR3, two receptors with different coupling properties (see below), indicated that the less conserved second intracellular loop and the amino terminal potion of the C-terminus determined the specificity for PLC coupling (Pin et al., 1994). Both segments were rich in basic amino acids, a trademark of G-protein coupled receptors, suggesting that although the primary sequence homology is different from other G-protein coupled receptors, common structural features exist.

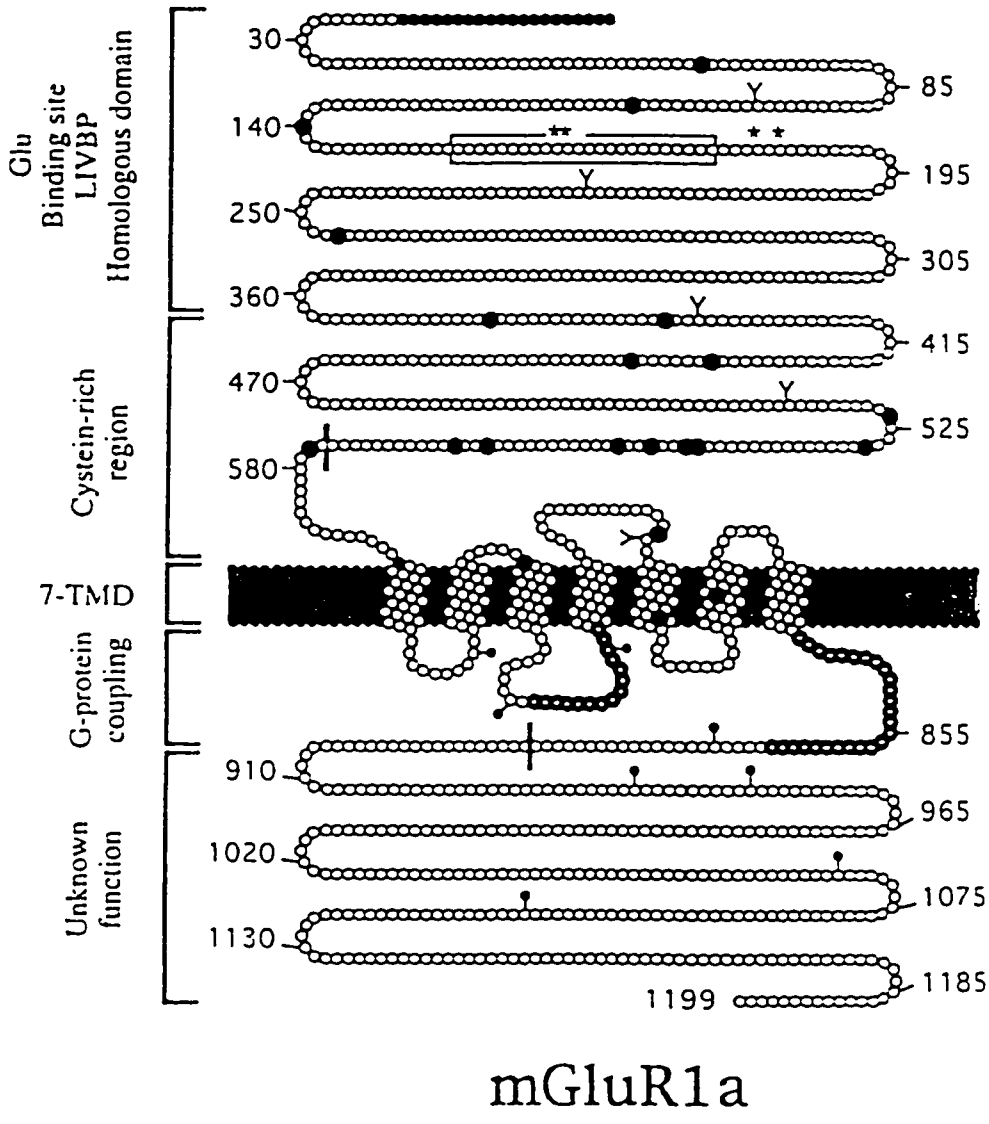


Figure 3: Proposed general topology and structural organization of the mGluR. The dark circles (amino acid residues) at the top (N-terminus) correspond to the signal peptide. The putative binding domain is indicated as the LIVBP homologous domain. The box in this region represents a series of hydrophobic domains thought to form the binding pocket. The larger black circles represent the conserved cysteine residues. The (Y) represent residues which are glycosylated while the small circles with bars associated with residues intracellularly are consensus sites for phosphorylation. Finally, the large open circles depict the residues involved in the G-protein interaction. This topology is derived from mGluR1. (Taken from Pin and Duvoisin, 1995).

1.2.4.2. Pharmacological profile of cloned mGluRs

The pharmacology of mGluRs has developed to be rather complex and confusing, with a plethora of compounds with suggested specific agonistic and antagonistic effects [see Watkins and Collingridge (1994), Pin and Duvoisin (1995) and Roberts (1995), for the most updated accounts of all compounds]. The following is a partial description of some of the most used compounds with relatively well established pharmacology. Overall, the main lines of the pharmacological profile of mGluRs are in accordance with the classification of mGluRs based on their primary structure homology. As such, group I mGluRs are potently activated by QUIS > Glu ≥ IBO > L-CCG-I > *t*-ACPD > DHPG (Houamed et al., 1991; Ito et al., 1992; Schoepp et al., 1991, 1994; Masu et al., 1991; Brabet et al., 1995). QUIS was found to be a more potent agonist on group I mGluRs than on AMPA receptor and had almost no effects on group II and III mGluRs. The obvious complication of this agonist (as for IBO and Glu) has been its agonistic activity on ionotropic GluRs which has limited its usefulness as a metabotropic agonist. *t*-ACPD (1S-3R-ACPD being the active isoform of this racemic compound) and L-CCG-I have been widely used, but have also been good agonists for group II mGluRs. In order of potency, group II mGluRs are activated by DCG-IV > L-CCG-I > Glu ≥ 1S,3R-ACPD ≥ 2R,4R-APDC > 4C, 3H-PG > IBO > QUIS (Tanabe et al., 1992, 1993; Ishida et al., 1993a; Schoepp and Johnson, 1993a; Schoepp et al., 1995a; Laurie et al., 1995). DCG-IV has been by far the most potent agonist for this group with very little effect on group I and III but has some agonistic activity on NMDA receptors. Furthermore, until recently this compound was not available commercially and only a limited number of laboratories had had access to it. The 2R,4R-APDC displayed lower potency than DCG-IV

but was found to be very specific to group II mGluR (Schoepp et al., 1995a) (this compound is not yet available commercially). Finally, group III mGluRs exhibit a very different agonist profile than group II. They are selectively activated by L-AP4 > Glu > L-CCG-I >L-serine-O-phosphate with the exception of mGluR 6 which displayed more affinity for L-serine-O-phosphate than Glu (Nakajima et al., 1993; Tanabe et al., 1993; Okamoto et al., 1994; Saugstad et al., 1994; Duvoisin et al., 1995). For this group, the pharmacology was made easier since L-AP4 is a potent agonist with no reported effects on other mGluRs and *t*-ACPD has no effect of this group (Tanabe et al., 1993; Okamoto et al., 1994; Saugstad et al., 1994; Duvoisin et al., 1995). Like other group III mGluRs, the mGluR 7 receptor was found be agonized preferentially by L-AP4 but the concentrations required for its activation were in the range of hundreds of micromolar, several ten-folds higher than for other receptors in this group. An exception within the group III is the novel mGluR 8 which appears to be activated by *t*-ACPD and L-CCG-I but not by DCG-IV, putting this receptor in a possible class of its own (Saugstad et al., 1997).

The antagonist profiles of mGluRs can also be classified for the different groups. It is noteworthy that, overall, mGluR antagonists display poor potencies and relatively high concentrations (500 μM to 1 mM) had to be used in most of work published. Group I mGluRs have been antagonized by most phenylglycine derivatives (more than 15 compounds characterized to date), the best known and widely used one being α-methyl-4-carboxyphenylglycine (MCPG) (Roberts, 1995). However, these compounds often lacked specificity or displayed agonistic effects on other mGluRs. Recently, a promising antagonist,

1-aminoindan-1,5-dicarboxylic acid (AIDA) was reported as very selective for group I mGluRs (Pellicciari et al., 1995). Until recently, the only antagonist for group II mGluRs has been MCPG, while other phenylglycines like 4C3HPG which were shown to antagonize group I receptors displayed agonistic activity on group II (Hayashi et al., 1994; Thomsen et al., 1994a, b; Roberts, 1995). Ethyl-glutamate (EGLU) and (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG) have been recently reported as a selective group II mGluR antagonists based on electrophysiology recording in motor neurons and other preparations (Jane et al., 1994; Vignes et al., 1995; Thomas et al., 1996). As emphasized earlier, group III have a distinct pharmacology with respect to the other groups. Hence development of antagonists around the structure of the agonist L-AP4 led to compounds with a relatively high selectivity to group III albeit a low potency. The antagonist of choice for group III mGluR has been α-methyl-L-AP4 (MAP4) (Jane et al., 1994; Johansen and Robinson, 1995). With respect to the phenylglycine family of compounds, MCPG has been shown to have no effect on mGluR4 and 7 (Pin and Duvoisin, 1995; Roberts, 1995) but is an antagonist to mGluR8 (Saugstad et al., 1997).

1.2.4.3. mGluR coupling and signal transduction

The classification of mGluR is also respected in the receptors' coupling abilities and transduction mechanisms assessed in *Xenopus* oocytes, Chinese hamster ovary (CHO), baby hamster kidney cells (BHK) and human embryonic kidney 293 (HEK 293) cells. In any of these expression systems, activation of group I mGluRs resulted in an increase in PLC activity as revealed by an increase in phosphoinositide turnover, and transient increases in intracellular

Ca^{2+} concentrations (Houamed et al., 1991; Masu et al., 1991; Pin et al., 1992; Pickering et al., 1993; Gabellini et al., 1994). Different kinetic patterns of intracellular Ca^{2+} release were observed for the different splice-variants of mGluR1 (Pin et al., 1992; Pickering et al., 1993), possibly reflecting a change in the coupling interaction with the G-protein. In all expression systems tested, mGluR1a was found to be partially sensitive to pertussis toxin (PTX) indicating a possible interaction of this receptor with the $G_{i/o}$ family of G-protein (Houamed et al., 1991; Masu et al., 1991; Aramori and Nakanishi, 1992; Pickering et al., 1993). However, a large component of the response was PTX-insensitive suggesting coupling of the receptor to a G_q-like protein. In contrast, mGluR5 and mGluR1c, a splice-variant of this receptor, were insensitive to PTX (Minakami et al., 1993; Pickering et al., 1993) when expressed in mammalian cell lines. Occasionally, mGluR1a has been reported to stimulate cAMP production (Minakami et al., 1993; Aramori and Nakanishi, 1992; Thomsen et al., 1993) an effect which was potentiated by PTX, suggesting a coupling of the receptor to a G_s receptor (Aramori and Nakanishi, 1992).

When transfected into mammalian cells (CHO or BHK), group II and III mGluRs were found to be negatively coupled to the adenylyl cyclase (AC) enzyme. Activation of these receptors resulted in a decrease of forskolin-stimulated cAMP production (Tanabe et al., 1992, 1993; Thomsen et al., 1992; Nakajima et al., 1993; Okamoto et al., 1994; Saugstad et al., 1994; Duvoisin et al., 1995). Group III mGluRs appeared to be less efficient than group II in inhibiting AC. However, every mGluR from these two groups was found to be blocked by PTX, indicating coupling to a $G_{i/o}$ G-protein. Glu-evoked PTX-sensitive K^+

current was observed following co-expression of mGluR8 and the G protein-coupled inwardly rectifying K^+ channels (GIR_K) in oocytes (Saugstad et al., 1997). Finally, functional coupling between mGluR 2 and mGluR 3 has been demonstrated in HEK cells co-transfected with necessary subunits for N-type Ca^{2+} channel expression and resulting in an inhibition of voltage-evoked Ca^{2+} influx (McCool et al., 1996).

How do results obtained with cloned mGluR subtypes tested in expression systems and delineating the signal transduction pathways for each receptor apply to neurons and glial cells? Using the pharmacological profile of different mGluR subtypes combined with, in specific cases, the knowledge that a specific receptor subtype is expressed in the system tested, investigations on preparations as varied as synaptosomes, isolated cells, brain slices, and whole animal, have attempted to clarify this important question. Thus, besides PLC activation, and cAMP inhibition (sometimes stimulation), a collection of different effects has been reported such as activation of cGMP phosphodiesterase, activation of phospholipases A_2 (PLA_2) or D (PLD), modulation of Ca^{2+} and K^+ channels. Furthermore, activation of any of these transduction pathways has caused varied and sometimes multiple modulatory actions in different preparations. For these reasons, mGluRs have been in the centre of an exploding field of research.

Activation of PLC, PKC, and intracellular Ca^{2+} release

Glu-dependent activation of PLC and accumulation of IP_3 and diacylglycerol (DAG) resulting from the hydrolysis of phosphoinositides was the first observation supporting the

existence of mGluRs (Sladeczek et al., 1985). Since then, there have been many reports of similar findings, using Glu and other agonists. The pharmacology of mGluR-induced PLC activity in neurons and glial cells has been well characterized (Manzoni et al., 1991; Guiramand et al., 1991; Patel et al., 1991; Schoepp and Conn, 1993; Llahi et al., 1992). Usually, QUIS is the most potent agonist for this effect, followed by Glu, IBO, and 1S,3R-ACPD, in agreement with the involvement of group I mGluRs. However, subtle differences in the pharmacology of "native" group I mGluRs have been reported. For example, L-SOP, L-AP3, and L-AP4 have been reported as potential antagonists of PLC-coupled mGluR in brain slices (Schoepp et al., 1990), effects not observed with recombinant group I mGluRs. Some of these differences may be explained by the presence of additional uncharacterized PLC-coupled mGluRs. However, the clear lack of specificity of many compounds acting on these receptors may explain many of the differences observed between cloned receptors and native receptors. To date, the G-protein involved in the activation of PLC by group I mGluRs has remained uncharacterized. Activation of PLC by Glu has been shown to be PTX-insensitive (Sladeczek et al., 1985), or PTX-sensitive (Nicoletti et al., 1988; Suzdak et al., 1993) as observed with cloned mGluRs (above). Hence, native receptors may be coupled to $G_{i/o}$ or to G_q. A relatively rapid and persistent tachyphylaxis of the mGluR-dependent PLC activation has been reported in various preparations (Catania et al., 1991; Lonart et al., 1992; Aronica et al., 1993; Herrero et al., 1994). This effect was partly dependent on PKC activity (Catania et al., 1991; Herrero et al., 1994).

Activation of PKC was reported with application of mGluR agonists (Manzoni et al., 1990; Aniksztejn et al., 1992; Schoepp and Conn, 1993). This was a consequence of the activation of PLC, causing the release of DAG, an activating factor of PKC (Weiss et al., 1989). Likewise, mGluR-specific increases in intracellular Ca^{2+} were reported in neurons, synaptosomes, or astrocytes, with a pharmacology identical to that reported for PLC activation (Murphy et al., 1987; Murphy and Miller, 1988, 1989; Bouchelouche et al., 1989; Joels et al., 1989; Courtney et al., 1990; Glaum et al., 1990; Guiramand et al., 1991; Llano et al., 1991; Manzoni et al., 1991). The increase in intracellular Ca^{2+} observed in many instances occurred even in the absence of extracellular Ca^{2+} suggesting release of Ca^{2+} from intracellular stores. Release of intracellular Ca^{2+} likely resulted from the increased production of IP_3 following activation of the PLC pathway (Guiramand et al., 1991). Situations where PLC activation failed to increase intracellular Ca^{2+}, such as in cerebellar Purkinje cells, have been demonstrated (Yuzaki and Mikoshiba, 1992). In cerebellar granule cells, mGluR-mediated intracellular Ca^{2+} release has been associated with a minimal requirement for extracellular Ca^{2+} influx through NMDA activation (Courtney et al., 1990). Additional work is required to establish possible differences between, and exact role(s) of mGluR 1 and 5 and their splice variants.

Stimulation of adenylyl cyclase

Increased basal formation of cAMP following mGluR activation has been reported (Alexander et al., 1992; Casabona et al., 1992; Goh and Ballyk, 1993; Schoepp and Johnson, 1993b; Winder and Conn, 1993; Cartmell et al., 1994; Gereau and Conn, 1994a; Musgrave

et al., 1994; Pilc et al., 1994; Schoepp et al., 1995b; Ogata et al., 1996; Reid et al., 1996). This increase in basal AC activity has been attributed to the activation of an adenosine A_2 receptor, resulting from a group I mGluR-induced adenosine release or potentiation (Alexander et al., 1992; Cartmell et al., 1993; Schoepp and Johnson, 1993b; Winder and Conn, 1993; Schoepp et al., 1995b). However, some results have indicated that the increase in cAMP could be mediated through the activation of an mGluR other than those of group I (Cartmell et al., 1993; Winder and Conn, 1993; Reid et al., 1996; Winder and Conn, 1995). Group III mGluRs have been excluded in these effects (Cartmell et al., 1993; Reid et al., 1996) and no direct activation of AC through a G_s by mGluR has been reported (Pin and Duvoisin, 1995). The potentiating effects of mGluR activation on adenosine-stimulated cAMP have been attributed to the interaction of the βγ-subunit, released following activation of the former, with a type II AC [this cyclase is known to be modulated by G-βγ-subunits (Uezono et al., 1993; Sternweis, 1994; Chen et al., 1995)] (Gereau and Conn, 1994a; Winder and Conn, 1993, 1995). The mGluR-dependent elevation in the basal cAMP formation was found to be developmentally regulated, reaching a peak expression perinatally and decreasing thereafter to reach an apparent minimal level in adult animals (Casabona et al., 1992; Reid et al., 1996).

Inhibition of adenylyl cyclase

Inhibition of AC following activation of mGluRs has been reported in most preparations both in neurons and astrocytes and intimately associated with group II and III mGluRs (Manzoni et al., 1992; Genazzani et al., 1993; Schoepp and Johnson, 1993a; Kemp

et al., 1994; Musgrave et al., 1994; Prezeau et al., 1994; Schoepp et al., 1995a; Wright and Schoepp, 1996). This effect was found to be mediated through the PTX-sensitive G_i G-protein family. Demonstration of direct coupling between Glu-sensitive receptors and inhibition of AC has been directly demonstrated in plasma membrane (Prézeau et al., 1994). All agonists to group II and III mGluRs were shown to decrease forskolin-stimulated cAMP formation, an effect not observed with QUIS (Prézeau et al., 1994). An interesting exception was reported in the cerebellum, where in granule cells which have been found to contain high levels of mGluR4 mRNA, L-AP4 failed to inhibit forskolin-stimulated cAMP accumulation (Tanabe et al., 1993; Prézeau et al., 1994), indicating differences in coupling of this receptor in different tissues.

Activation of cGMP phosphodiesterase

The effect of L-AP4 on ON-bipolar cell in rat retina has been described even before the cloning of mGluR, and in particular mGluR6. In these cells L-AP4 was proposed to activate a cGMP-dependent phosphodiesterase (Nawy and Jahr, 1990; Shiells and Falk, 1990) similar to that described for the transduction cascade following activation of rhodopsin in photoreceptor cells. Furthermore, like the light transduction system, the effects of L-AP4 on ON-cells were PTX-sensitive suggesting a $G_{i/o}$ coupled pathway (Nakajima et al., 1993). Such a transduction system has only been reported for ON-bipolar cells, the only cell type which expressed mGluR6, (Nomura et al., 1994) and whether other mGluRs could exert their effect through a similar mechanism remains to be determined.

Activation of PLA_2

Arachidonic acid release resulting from the activation of the membrane bound enzyme PLA_2 has been implicated in neuromodulation of excitatory synapses and has been a candidate for retrograde signalling between neurons [for review, see Jessel and Kandel (1993)]. Glu-, QUIS-, ACPD-, and NMDA-induced release of arachidonic acid have been reported (Dumuis et al., 1990). While NMDA- and part of the Glu-induced release have been attributed to influx of Ca^{2+} through NMDA channels leading to the activation of PLA_2, QUIS (and Glu) was found to increase arachidonic acid release through a PLC-sensitive pathway (Dumuis et al., 1990). ACPD was found to mimic the effects of QUIS only in the presence of a depolarizing agent like AMPA (Dumuis et al., 1990). Although the signalling pathway linking PLA_2, Ca^{2+}, depolarization and PLC remains obscure, the reversal of the Na^+/Ca^{2+} exchanger has been proposed as a key mechanism linking the different Glu receptors (Dumuis et al., 1993). Direct stimulation of arachidonic acid release from mouse astrocytes following mGluR activation has been reported (Stella et al., 1994), but no clear mechanisms were proposed. The mGluR1a was found to activate PLA_2 in expression systems (Aramori and Nakanishi, 1992).

Activation of phospholipase D

Activation of phospholipase D (PLD) mGluR was first proposed by Boss and Conn (1992). Interestingly, the pharmacology underlying this effect was rather unusual as 1S,3R-ACPD (not 1R,3S-ACPD) stimulated PLD but Glu did not (Boss and Conn, 1992), nor did the group I or III mGluR agonists DHPG and L-AP4. The activation of PLD by ACPD was

independent of PKC (Pellegrini-Giampietro et al., 1996). L-cysteine sulfonate was the only endogenous compound which could potentially mimic the effects of ACPD (Boss et al., 1994). ACPD-induced PLD activity was found to be developmentally regulated with peak activity during the neonatal period (Holler et al., 1993). Taken together, these results suggest that an uncharacterized mGluR may be involved in this effect.

Regulation of Ca^{2+} channels

In neurons, many neurotransmitters can modulate Ca^{2+} currents through a G-protein dependent mechanism, either by direct interaction of the G-protein with the Ca^{2+} channel or via second messengers (Shapiro et al., 1994). The mGluR family is no exception, and since the first demonstration that QUIS could inhibit Ca^{2+} current in hippocampus (Lester and Jahr, 1990), all three groups of mGluR have now been implicated in the modulation of different voltage-dependent Ca^{2+} channels (VDCC) in a variety of neuronal and glial cells. Inhibition has been found to occur at the N-type Ca^{2+} channel (ω-conotoxin GIVA-sensitive) following activation of group I mGluRs (Swartz and Bean, 1992; Rothe et al., 1994; Yoshino and Kamiya, 1995; Choi and Lovinger, 1996), group II (Sayer et al., 1992; Sahara and Westbrook, 1993; Swartz et al., 1993; Hay and Kunze, 1994; Rothe et al., 1994; Stefani et al., 1994; Chavis et al., 1995a; Choi and Lovinger, 1996), and group III (Sahara and Westbrook, 1993; Rothe et al., 1994). Inhibition at the L-type Ca^{2+} channel [nifedipine- or dihydropyridine- (DHP-) sensitive] was also reported with group I (Lachica et al., 1995), group II (Sahara and Westbrook, 1993; Chavis et al., 1994; Chavis et al., 1995b), and possibly group III (Sahara and Westbrook, 1993) [group III mGluRs have been shown to

decrease Ca^{2+} through undetermined Ca^{2+} channels (Trombley and Westbrook, 1992; Herrero et al., 1996; Stefani et al., 1996)]. Modulation of P-type Ca^{2+} channels (ω-Agatoxin IVA- and/or ω-conotoxin MVIIC-sensitive) by mGluRs has also been shown (Glaum and Miller, 1995; Choi and Lovinger, 1996). In addition, functional coupling between recombinantly expressed mGluR2 and native N-type Ca^{2+} channels has been demonstrated in sympathetic neurons (Ikeda et al., 1995). In most of the reports, inhibition of VDCC by mGluR has been shown to result from a membrane-delimited signal transduction system (possibly through a direct interaction between G-proteins and Ca^{2+} channels) and not via diffusible second messenger systems such as cAMP, cGMP, protein kinase, IP_3, or intracellular Ca^{2+} (Lester and Jahr, 1990; Sayer et al., 1992; Swartz and Bean, 1992; Trombley and Westbrook, 1992; Chavis et al., 1994). However, in some cases, mGluR-induced Ca^{2+} channel inhibition was found to be dependent on cAMP levels (Lachica et al., 1995). In some studies, activation of a PKC-dependent pathway was found to reverse the effect of mGluR on Ca^{2+} channels (Swartz et al., 1993; Herrero et al., 1996) although this is thought to be a parallel effect not necessarily related to mGluR.

Potentiation of VDCC following mGluR activation has also been reported (Jaffe and Brown, 1994; Linden et al., 1994; Rothe et al., 1994; Chavis et al., 1995a, b, 1996). Ca^{2+} channels have been shown to be effectively opened by group I mGluR in a PTX-insensitive fashion (Chavis et al., 1995a, b, 1996). This effect was mediated through a DHP-sensitive (L-type) Ca^{2+} channel (Chavis et al., 1995a). This activation of DHP-sensitive Ca^{2+} channels by mGluRs has been proposed to facilitate coupling between the latter and a ryanodine-

sensitive channel in cerebellar granule cells, resulting in an enhanced transmembrane Ca^{2+} current (Chavis et al., 1996). The authors proposed that regulation of neuronal Ca^{2+} homeostasis both through extracellular and intracellular mechanisms by mGluR may be important in the control of neuronal electrical activity, synaptic plasticity or excitotoxicity. These findings may correlate with the observations that activation of mGluR with *t*-ACPD was found to induce Ca^{2+} waves within hippocampal dendrites (Jaffe and Brown, 1994) and in cerebellar Purkinje cells (Linden et al., 1994), both as a result of entry of extracellular Ca^{2+} and Ca^{2+}-induced-Ca^{2+} release mechanisms.

Modulation of K^+ channels

It has been demonstrated that QUIS reduced the afterhyperpolarization (AHP) that follows a burst of action potentials, depolarized neurons and increased input resistance (R_m), and decreased accommodation of cell firing (Stratton et al., 1989; Baskys et al., 1990; Charpak et al., 1990; Baskys, 1992; Glaum and Miller, 1992; Gerber and Gahwiler, 1994; Womble and Moises, 1994; Gereau and Conn, 1995a; Abdul-Ghani et al., 1996). The blockade of I_{AHP} was not affected by TTX, suggesting that it was independent of activity resulting from action potentials (Charpak et al., 1990) and thus, resulted from an inhibition of the slow Ca^{2+}-dependent K^+ current underlying the AHP (Stratton et al., 1989; Baskys et al., 1990; Charpak et al., 1990). It has been suggested that PKC (Baskys et al., 1990) or tyrosine kinase (Abdul-Ghani et al., 1996), but not Ca^{2+}/calmodulin-dependent protein kinase (CamKII) (Pedarzani and Storm, 1996) could mediate the effects of mGluR on K_{AHP}. In addition, the blockade of a non inactivating, voltage-dependent K^+ current known as I_m was

reported in hippocampus (Charpak et al., 1990; Harata et al., 1996), in basolateral amygdala (Womble and Moises, 1994) and in sympathetic neurons transfected with mGluR1a cRNA (Ikeda et al., 1995). This current has been implicated in membrane potential depolarization, and reduced accommodation (Brown, 1988). The pharmacology of the mGluR supports an exclusive role for group I mGluRs in this inhibition (Gereau and Conn, 1995a; Ikeda et al., 1995). However, in NTS neurons, common group I antagonists were ineffective in blocking the effect of ACPD on K^+ currents (Glaum et al., 1993). In visceral sensory neurons, ACPD application resulted in the inhibition of a 4-aminopyridine (4-AP)-sensitive and insensitive K^+ current (Hay and Lindsley, 1995). The authors suggested that in these neurons, mGluR activation could result in an increase in transmitter release through the inhibition of presynaptic K^+ channels, an action opposing that observed in central synapses (Hay and Lindsley, 1995). Indeed, in rat neonatal hippocampus, presynaptic inhibition following activation of mGluRs was found to result from the activation of a 4-AP-sensitive K^+ channel resulting in a decreased presynaptic depolarization and a reduction in Ca^{2+} influx (Yoshino and Kamiya, 1995). Activation of outward currents or hyperpolarizations induced by mGluR agonists have been reported in Purkinje neurons of the cerebellum, NTS, hippocampus, basolateral amygdala neurons (Glaum and Miller, 1992; Vranesic et al., 1993; Gerber and Gahwiler, 1994; Rainnie et al., 1994; Premkumar and Chung, 1995; Holmes et al., 1996a). In the latter neurons, this effect was mediated through a PTX-sensitive group II mGluR.

Modulation of receptor-coupled ion channels

Glu-specific (Aniksztejn et al., 1992; Bleakman et al., 1992; Kinney and Slater, 1992, 1993; Glaum and Miller, 1993a; Harvey and Collingridge, 1993; Fitzjohn et al., 1996) and GABA-specific(Glaum and Miller, 1993a) ligand gated channels have been shown to be modulated by mGluRs. These effects were all mediated through a receptor with the pharmacology and coupling associated with group I mGluRs. In NTS neurons, $GABA_A$-receptor-mediated current was inhibited by ACPD (Glaum and Miller, 1993a, 1994), an effect blocked by group I mGluR antagonists. This inhibition was mimicked by cGMP or by activation of guanylate cyclase by natriuretic peptides (Glaum and Miller, 1993a), and could be blocked by application of phosphoprotein phosphatase inhibitors (Glaum and Miller, 1994). Even more interesting was the observation that while $GABA_A$-mediated current was inhibited, AMPA-mediated current was potentiated in an identical fashion in this preparation (Glaum and Miller, 1993a, 1994). To date, this has been the only report supporting a role for increased cGMP in the signal transduction underlying a mGluR effect, despite the fact that this signal transduction pathway appears to be extensively involved in many neuromodulatory processes.

Modulation of NMDA-gated ion channels has been studied more extensively. Always, activation of mGluRs with QUIS or ACPD produced a PTX-insensitive potentiation of NMDA currents when tested in normal extracellular Mg^{2+} (Aniksztejn et al., 1992; Bleakman et al., 1992; Harvey and Collingridge, 1993; O'Connor et al., 1994). This effect was reproduced in a dose-dependent fashion by the group I-specific agonist DHPG, but not with

the group II or III agonists DCG-IV and L-AP4, respectively (Fitzjohn et al., 1996). The ACPD-mediated potentiation of NMDA was blocked in the presence of PKC inhibitors in CA1 hippocampal neurons (Aniksztejn et al., 1992), although results to the contrary have also been reported in the same preparation (Harvey and Collingridge, 1993) and in turtle cerebellar granule cells (Kinney and Slater, 1993).

1.2.4.4. Physiological roles of mGluRs

Excitatory effects

Activation of mGluRs with ACPD (or QUIS in the presence of ionotropic GluR antagonists) has often been reported to cause slowly developing depolarization, inward currents and increase in firing rates in many neuronal types such as in the hippocampus (Desai and Conn, 1991; Hu and Storm, 1991; Caeser et al., 1993; Gereau and Conn, 1994b; Abdul-Ghani et al., 1996), the neocortex (Greene et al., 1992, 1994), the ventrobasal thalamus (Salt and Eaton, 1995), the basolateral amygdala (Womble and Moises, 1994; Salt and Eaton, 1996), the cerebellum (Yool et al., 1992) or the NTS (Glaum and Miller, 1992; Glaum et al., 1993). A shift in the firing mode from rhythmic burst to single spikes of dorsolateral geniculate thalamic neurons (McCormick and von Krosigk, 1992) and layer V pyramidal neurons (Wang and McCormick, 1993) has also been reported in a fashion reminiscent of some actions of NA (see section 1.1.7). Most of these effects likely resulted from the inhibition of K^+ channels described above, i.e., negative modulation of IK_{Leak} and IK_M which control the membrane potential, and IK_{AHP} which participates in the repolarisation and afterhyperpolarisation phases of action potentials. Modulation of spike accommodation,

which is thought to depend on IK_{AHP} and IK_{M}, by ACPD has also been reported in the hippocampus (Stratton et al., 1989; Charpak et al., 1990), the septum (Zheng and Gallagher, 1992), or the thalamus (Salt and Eaton, 1996). Most of the effects described above appeared to be mediated through group I mGluRs although some of the pharmacology remains disputed. For example, MCPG has failed to block the effects of ACPD in some preparations (Chinestra et al., 1993; Glaum et al., 1993).

Occasionally, ACPD-dependent depolarization occurred without significant changes in K^+ fluxes. In cerebellar Purkinje neurons, a combination of intracellular recordings and intracellular Ca^{2+} measurements revealed a depolarizing effect of ACPD accompanied by a large increase in Ca^{2+} in the cell soma (Vranesic et al., 1991). This increase in Ca^{2+} was later demonstrated to result from the activation of an electrogenic Na^+/Ca^{2+} exchanger (Glaum and Miller, 1992). This depolarization was also blocked by group I mGluR antagonists (Glaum and Miller, 1992).

Only a few studies have demonstrated activation of mGluRs following endogenous release of EAA. Activation of the parallel fibres which impinge on Purkinje neurons resulted in a stimulus frequency-dependent slow and relatively long-lasting depolarization that was independent of ionotropic GluRs (Batchelor and Garthwaite, 1993, 1997; Batchelor et al., 1994; Vranesic et al., 1993). This effect, termed mGluR-EPSP or slow EPSP, was blocked by MCPG and, with other findings, implicated a group I mGluR-dependent effect (Batchelor et al., 1994; Batchelor and Garthwaite, 1997). Similar activation of the climbing fibre input

did not cause a depolarization. However, a single stimulation of the climbing fibres markedly potentiated the mGluR-mediated excitation via the parallel fibres at stimuli strength that otherwise would have failed to cause a depolarization (Batchelor and Garthwaite, 1997). This dramatic potentiation was long lasting, and could be observed even 90 seconds after activation of the climbing fibres. The climbing fibre-mediated potentiation was dependent on the entry of Ca^{2+} through a Ca^{2+} spike, which is a physiological response of Purkinje cells to climbing fibres (Batchelor and Garthwaite, 1997). The effect was mimicked by increasing intracellular Ca^{2+} concentration through photolysis of caged Ca^{2+}. This process could seriously impact on current understanding of integration of incoming synaptic information by allowing not only spatially distant synaptic signals but also temporally dispersed (up to two minute apart) signals to be summated, with all the possible implications regarding synaptic plasticity and memory formation.

Inhibitory effects:

Inhibitory effects of Glu in the retina have been observed for some time. Glu was found to depolarize OFF-bipolar cells and hyperpolarize ON-bipolar cells. While the depolarization has been ascribed to the action of AMPA receptors (Nawy and Copenhagen, 1987), hyperpolarization in ON-bipolar cells was shown to be mimicked by the application of L-AP4 and L-SOP (Slaughter and Miller, 1981, 1985). The hyperpolarization was shown to result from the closure of the cGMP-gated cation channels, through the activation of cGMP phosphodiesterase (Nawy and Jahr, 1990; Shiells and Falk, 1990; Scott and Jahr, 1991; Yamashita and Wässle, 1991; Thoreson and Miller, 1993). The pharmacology of the

L-AP4-mediated hyperpolarization and the PTX sensitivity are reminiscent of a group III mGluR (Tian and Slaughter, 1994). In conjunction with the restricted localization of mGluR6 to these cells (Ueda et al., 1997), this receptor has been the prime candidate involved in mediating the effect of L-AP4 on ON-bipolar cells. This was recently confirmed by target-specific disruption of mGluR6 expression in retina resulting in a specific failure of ON-bipolar cells to participate in visual transmission (Masu et al., 1995). In salamander bipolar cells the hyperpolarizing effect of L-AP4 was a consequence of the opening of a K^+ channel (Hirano and MacLeish, 1991).

Hyperpolarization resulting from mGluR activation was also reported in cerebellar Purkinje cells, besides the depolarizing effects described above. The hyperpolarization followed the depolarization induced by ACPD (Glaum et al., 1992; Burke and Hablitz, 1996; Vranesic et al., 1993). Similar effects were reported with QUIS, but not with AMPA (Takagi et al., 1992), suggesting the involvement of group I mGluRs. This hyperpolarization is thought to have resulted from the rise of intracellular Ca^{2+} described earlier (section 1.2.4.3.), activating Ca^{2+}-sensitive K channels (Glaum et al., 1992; Takagi et al., 1992) or through the inhibition of a tonic Ca^{2+} channel (Vranesic et al., 1993). A similar scenario has been described for cerebellar granule cells following ACPD or QUIS applications (Fagni et al., 1991).

Presynaptic inhibition of synaptic transmission:

One of the most commonly described effects of mGluR activation that is consistent throughout the CNS is the reduction of synaptic transmission at glutamatergic synapses. All major pathways of the hippocampus (Ishida et al., 1993b; Baskys and Malenka, 1991; Kroona et al., 1991; Peterson et al., 1992; Swartz et al., 1993; Maki et al., 1994, 1995; Gereau and Conn, 1995b; Johansen et al., 1995; Jouvenceau et al., 1995; Manzoni et al., 1995; Ohno-Shosaku and Yamamoto, 1995; Vignes et al., 1995; Kamiya et al., 1996; Holmes et al., 1996a), the neocortex (Sheardown, 1992; Sladeczek et al., 1993; Swartz et al., 1993; Burke and Hablitz, 1994), the striatum (Lovinger, 1991; Calabresi et al., 1993; Lovinger et al., 1993; Lovinger and McCool, 1995; Tyler and Lovinger, 1995), the cerebellum (Takagi et al., 1992; Glitsch et al., 1996; Pekhletski et al., 1996), the spinal motoneurons (Pook et al., 1992; Jane et al., 1994; Kemp et al., 1994; King and Liu, 1996), the NTS (Glaum and Miller, 1993a, b, 1995), and others (Jacobson et al., 1986; Rainnie and Shinnick-Gallagher, 1992; Trombley and Westbrook, 1992; Devau et al., 1993; East et al., 1995; Schrader and Tasker, 1997) have displayed similar behaviour in the presence of mGluR agonists. Furthermore, GABAergic synaptic transmission in different regions of the CNS has been shown to be inhibited following activation of mGluR (Glaum and Miller, 1993a; Burke and Hablitz, 1994; Stefani et al., 1994; Jouvenceau et al., 1995; Llano and Marty, 1995; Ohno-Shosaku and Yamamoto, 1995; Di Iorio et al., 1996; Fitzsimonds and Dichter, 1996; Salt and Turner, 1996). There is overwhelming evidence to suggest that more than one group of mGluRs, and more than one subtype in each group, can mediate presynaptic inhibition.

Reports of EAA-mediated presynaptic inhibition have long preceded the identification and cloning of mGluRs. In 1981, Koerner and Cotman demonstrated that superfusion of hippocampal slice with L-AP4, a new L-Glu analogue at the time, potently blocked lateral perforant path-evoked excitation of dentate gyrus granule cells in a stereospecific manner. These results were soon reported in many systems and the receptor mediating the effect was named the "L-AP4 receptor" to distinguish it from other pharmacologically characterized Glu receptors [for review, see Monaghan et al. (1989)]. The lack of effect of L-AP4 on neuron responses to exogenously applied Glu, and the amplitude fluctuations of spontaneous EPSPs suggested that L-AP4 acted presynaptically (Cotman et al., 1986b). Although no specific mechanisms for the actions of L-AP4 were proposed, results obtained with synaptosomes suggested that L-AP4 blocked presynaptic Ca^{2+} influxes induced by QUIS (Adamson et al., 1990).

As other potential mGluR agonists were developed, the possibility that receptors other than L-AP4 could also cause presynaptic inhibition arose. Baskys and Malenka (1991) demonstrated that presynaptic inhibition could be observed in Schaffer collateral-CA1 hippocampal synapses using *t*-ACPD, in addition to L-AP4. Both *t*-ACPD and L-AP4 effects were dose-dependent and developmentally regulated. The effect of *t*-ACPD could be mimicked by application of QUIS. The effects of L-AP4 were practically gone in adult animals (>80 days) while *t*-ACPD effects, although reduced, remained. The possibility that the two agonists acted on the same receptor could not be excluded, but this study was the first demonstration that there could be presynaptic mGluR other than the "L-AP4 receptor".

Effects of *t*-ACPD, but not L-AP4, on this pathway could be antagonized by MCPG supporting the existence of multiple distinct presynaptic mGluRs (Watkins and Collingridge, 1994). It was later demonstrated in a similar preparation that a group I and a group III mGluR were involved in the inhibition at the CA1 synapses (Gereau and Conn, 1995b) and that the group II specific agonist DCG-IV was without effects (Kamiya et al., 1996). Interestingly, in the former report, the miniature EPSPs (mEPSPs; residual synaptic activity after the blockade of action potentials with TTX) amplitude and frequency were not affected by group mGluR I (DHPG) activation while L-AP4 significantly reduced the frequency of the mEPSPs (Gereau and Conn, 1995b). These results suggested different mechanisms of inhibition by different mGluRs at the same presynaptic terminals. L-AP4 seemingly inhibited synaptic transmission in a manner similar to that observed following blockade of VDCC (with cadmium for example) while the effects of DHPG appeared to modulate the action potential prior to the activation of presynaptic VDCC (Gereau and Conn, 1995b). As high concentrations of L-AP4 were required for its effects, mGluR7 was suggested as the receptor involved. This is in sharp contrast with the results reported by Vigne et al. (1995) which demonstrated that group II and group III mGluRs were responsible for presynaptic inhibition at the same synapse. Furthermore, besides being insensitive to MCPG, the actions of L-AP4 were observed at low micromolar concentrations ruling out the primary involvement of mGluR7 in this effect. The obvious discrepancy between this study and that of Gereau and Conn (1995b) and Kamina et al.(1996) could possibly be explained by the age of the animal., Vigne et al. (1995) used much younger animals (two weeks old) as opposed to four-five weeks old used in the two other studies. This hypothesis is further supported by the report

that DCG-IV could potently antagonize synaptic transmission at this synapse in neonatal rats (Yoshino and Kamiya, 1995). Interestingly, mGluR1-deficient mice continued to respond to *t*-ACPD postsynaptically and presynaptically in adult animal brain slice preparations (Aiba et al., 1994a), likely ruling out mGluR1 and leaving mGluR5 as a candidate for the group I-mediated effects described in Schaffer collateral CA1 synapses.

Desai and Conn (1991) reported *t*-ACPD-mediated presynaptic inhibition at the perforant path-dentate gyrus synapse similar to that reported with L-AP4 (Ishida et al., 1993b). However, in contrast to the Schaffer-collateral-CA1 path, further discrimination between lateral and medial perforant paths suggested that the presynaptic effects of L-AP4 were restricted to the lateral path while *t*-ACPD, and the specific group II agonist DCG-IV, exerted their presynaptic effects exclusively on the medial path (Macek et al., 1996). On this pathway, only micromolar concentrations of L-AP4 were required for the presynaptic inhibition (Bushell et al., 1995; Pérez and Ruiz, 1995), suggesting a group III receptor other than mGluR7, thus further contrasting these results to those obtained at the CA1 synapses (Macek et al., 1996).

In the mossy fibre-CA3 synaptic circuit, *t*-ACPD and L-AP4 both inhibited synaptic transmission at micromolar concentrations (Manzoni et al., 1995). The effects of *t*-ACPD were mimicked by specific group II agonists (L-CCG1 and 4C3HPG) (Manzoni et al., 1995) and DCG-IV (Kamiya et al., 1996) suggesting a group II involvement. Interestingly, the effects of L-AP4 on this synapse were antagonized by MCPG, indicating that group III

receptor subtypes 4 or 7 are not involved and leaving mGluR8, which best fits this pharmacological profile, as the prime candidate involved in the inhibition. The *t*-ACPD inhibition of synaptic transmission appeared to be independent of VDCC, but possibly caused its effects through a direct interaction at a site distal to the Ca^{2+} influx (Scanziani et al., 1995). Recently, Yokoi et al. (1996) reported that functional deletion of mGluR2 receptors resulted in a partial impairment of group II mGluR-dependent synaptic inhibition at the mossy fibre-CA3 synapse. These results would suggest that both mGluR2 and mGluR3, the two known mGluRs activated by DCG-IV participated in the inhibition at this synapse.

Taken together, the results observed at major synapses of the hippocampal trisynaptic circuit clearly emphasize the pronounced heterogeneity of mGluR groups and subtypes involved in presynaptic inhibition. From these results all but one mGluR receptor (mGluR6 is not expressed in hippocampus) could potentially be involved in presynaptic inhibition in hippocampus.

While many reports have indicated mGluR-mediated presynaptic inhibition in hippocampus, this process has also been described in other regions of the CNS, with results which are as diverse as described above. In striatal brain slice preparations, Lovinger's laboratory reported that *t*-ACPD, but not L-AP4, caused a dose-dependent inhibition of synaptic transmission at glutamatergic cortical afferents (Lovinger, 1991), an effect mimicked by group II specific agonists and blocked by group II-specific antagonists (Lovinger et al., 1993; Lovinger and McCool, 1995). However, others have reported that, besides *t*-ACPD,

low doses of L-AP4 inhibited this pathway in similar preparations (Calabresi et al., 1993) or in synaptosomal preparations (East and Garthwaite, 1992). When investigated in corticostriatal co-cultures, the effects of *t*-ACPD were PTX-sensitive and appeared to be mediated via mechanisms distal to the activation of VDCC (Tyler and Lovinger, 1995). Inhibition of excitatory synaptic transmission to spinal cord motor neurons, following stimulation of dorsal neurons, was observed with both *t*-ACPD and L-AP4 at micromolar concentrations (Pook et al., 1992). All pharmacological evidence suggests that both a group II and a MCPG-insensitive group III are involved (Ishida et al., 1993a; Jane et al., 1994; Kemp et al., 1994). Short latency (presumably mediated by low threshold myelinated fibre like Aß-afferents) or long latency EPSPs (possibly from C-fibres) to motoneurons were both depressed by group II mGluRs (King and Liu, 1996). Inhibition of excitatory synaptic transmission by group II and III mGluRs has also been reported in basolateral amygdala (Rainnie and Shinnick-Gallagher, 1992), hypothalamic magnocellular neurons (Schrader and Tasker, 1997), NTS (Glaum and Miller, 1993b, 1995). Other synapses, such as olfactory bulb neurons (Trombley and Westbrook, 1992) and barrel cortex neurons (Wan and Cahusac, 1995), have been characterized for L-AP4 effects and whether other groups of mGluRs could also modulate synaptic transmission to these neurons was not determined. In the cerebellum, the parallel fibre-Purkinje synapse was inhibited by *t*-ACPD and L-AP4, an effect which was cancelled when expression of mGluR1 or mGluR4 receptors respectively for *t*-ACPD and L-AP4 was compromised (Conquet et al., 1994; Pekhletski et al., 1996).

The endogenous activation of presynaptic mGluR has been recently demonstrated. Glaum and Miller reported that low frequency stimulation of the afferent projections to the NTS was sufficient to release enough Glu to depress EPSCs presynaptically (Glaum and Miller, 1993b). Intermittent stimulations of 1 Hz at the mossy fibre-CA3 synapses resulted in a MCPG-sensitive presynaptically-mediated decrease in EPSP amplitude, an effect which was blocked by MCPG and potentiated by the application of the Glu uptake inhibitor L- *t*-pyrrolidine-2,4-dicarboxylic acid (*t*-PDC) (Scanziani et al., 1997). Similar results with *t*-PDC were reported in low density cultures of hippocampal neurons, an effect which was mimicked by *t*-ACPD and blocked by MCPG (Maki et al., 1994).

Presynaptic potentiation of synaptic transmission:

Activation of mGluRs by ACPD has been occasionally shown to potentiate synaptic transmission in the presence of arachidonic acid, an effect mimicked by QUIS, suggesting a PLC pathway-mediated mechanism (Herrero et al., 1992, 1994). This effect was antagonized by 4-AP. While the mechanism is unclear, it appeared that 4-AP-sensitive K^+ currents and an increase in intracellular free Ca^{2+} were involved (Adamson et al., 1990). In other cases, *t*-ACPD was found to potentiate spontaneous unitary EPSP frequency in hypothalamic magnocellular neurons in brain slice preparations in a TTX-sensitive manner, an effect mimicked by group I-specific agonists (Schrader and Tasker, 1997). This increase was attributed to the stimulation of somata/dendritic spike generation of the presynaptic cell (Schrader and Tasker, 1997). Similar effects of *t*-ACPD were found on GABAergic cells (Schrader and Tasker, 1997).

mGluRs and synaptic plasticity

Plasticity of glutamatergic synapses is characterized by long-term changes in synaptic efficacy. Both long-term potentiation (LTP) and long-term depression (LTD) of glutamatergic synapses are observed (Bliss and Collingridge, 1993; Jessel and Kandel, 1993; Plewako and Kostowski, 1984; Izquierdo, 1994; Malenka, 1994; Barnes, 1995). Since they last for hours *in vitro* and for weeks in freely moving animals, they are taken as models to study the mechanisms involved in memory formation. For example, spatial learning is thought to involve hippocampal LTP, and the LTD of parallel fibres to the Purkinje neurons may be implicated in the plasticity of the vestibulo-ocular reflex. The following is a summary of some evidence supporting a role for mGluRs in these two phenomena. The mechanisms involved in the induction of LTP in the CA1 region of the hippocampus have been extensively studied (Bliss and Collingridge, 1993). High frequency stimulation of the Schaffer collaterals, or concomitant activation of this neuronal pathway with the depolarization of the pyramidal neurons leads to an association between pyramidal NMDA receptor activation and cell depolarization resulting in the influx of Ca^{2+} in the postsynaptic cells. This Ca^{2+} entry is one key element which triggers a Ca^{2+}-sensitive enzymatic reaction resulting in postsynaptic modification. Thus, the NMDA receptor, through its voltage-dependent activation, serves as a conditional element critical for the induction of LTP in CA1. As described above, some of the effects of mGluR activation have been the modulation of the NMDA receptor and/or transient depolarizations and/or altered intracellular Ca^{2+} concentrations (see above) and thus, mGluRs could play a role in LTP.

Accordingly, changes in LTP establishment following tetanic stimulation were observed in the presence of *t*-ACPD (McGuinness et al., 1991). Application of QUIS and subthreshold tetanic stimulations also resulted in LTP (Aniksztejn et al., 1992; Otani et al., 1993), as did coapplication of mGluR agonists and NMDA (Musgrave et al., 1993). Antagonism of mGluRs in an attempt to prevent LTP has yielded conflicting data. Collingridge and colleagues first reported that MCPG blocked the induction of both NMDA-dependent LTP induction in CA1 and NMDA-independent LTP induction in CA3 in rat brain slice preparations (Bashir et al., 1993). Similar findings were soon reported by others (Riedel and Reymann, 1993; Sergueeva et al., 1993). The latter studies also demonstrated that MCPG could disrupt spatial learning in water maze and Y-maze tasks, tests used to measure hippocampus-dependent learning. However, these results are in contradiction with findings that MCPG did not affect the establishment or maintenance of LTP in similar preparations (Chinestra et al., 1993; Manzoni et al., 1994; Breakwell et al., 1996). Discrepancies between different results obtained with MCPG remain to be clarified. However, it was later reported that a blockade of mGluRs by MCPG was only effective if no prior LTP had been induced at the cell, i.e., the process was only observed with "naive" cells (or slices) (Bortolotto et al., 1994). NMDA receptor activation was essential in all circumstances (Bortolotto et al., 1994). Thus, it was suggested that mGluR could act as a molecular switch for LTP, and its activation would only be required when switching the cell or cells from an unconditioned mode to a conditioned mode (Bortolotto et al., 1994). No effects of MCPG on short-term potentiation were observed (Bashir et al., 1993).

The exact mechanism underlying LTP induction by mGluRs is poorly understood, but likely involves the release of intracellular Ca^{2+}. Supporting this mechanism are observations that thapsigargin, which prevents release from IP3-sensitive Ca^{2+} stores, and BAPTA, a Ca^{2+} chelator, both blocked mGluR involvement in LTP (Bortolotto and Collingridge, 1993, 1995; Collins, 1994). Activation of PKC also appeared to play an important role in mGluR-dependent LTP (Bortolotto and Collingridge, 1993; Otani et al., 1993; O'Connor et al., 1995). Together, these results would support a role for a group I mGluR. Perhaps the best evidence supporting a role for a PLC-coupled mGluR, however, came with the results demonstrating that LTP was significantly reduced in CA1 of mice with functionally deleted mGluR1 (Aiba et al., 1994b). Conversely, no differences between mutant and wild-type mice in LTP induction at the mossy fibre-CA3 synapse were found (Hsia et al., 1995).

In the cerebellum, conjunctive activation of parallel fibre and climbing fiber inputs to Purkinje cells results in the induction of LTD at the parallel fibre-Purkinje cell synapse (Bear and Malenka, 1994; Linden, 1994). Induction of LTD required Ca^{2+} entry to Purkinje neurons through voltage-gated Ca^{2+} channels that are activated by climbing fibre activity (Llano et al., 1991; Schneggenburger et al., 1993). In addition, LTD requires factors that result from stimulation of parallel fibres that use Glu as a transmitter. Parallel fibre stimulation can be mimicked by exogenous application of Glu, QUIS and *t*-ACPD but not Ka, or aspartate, suggesting that mGluR(s) activation was required (Linden et al., 1991, 1994; Daniel et al., 1992). Later, it was reported that both AMPA receptors and mGluR activation were required for LTD (Ito and Karachot, 1990; Linden et al., 1991; Daniel et al., 1992). An

involvement for PLC-coupled mGluR in LTD was supported by the presence of high levels of mGluR1 in Purkinje neurons. This was soon unequivocally confirmed as LTD expression in the cerebellum was impaired in mice lacking mGluR1 (Aiba et al., 1994b) or in cultures treated with antibodies against this receptor (Shigemoto et al., 1994). Furthermore, the phenotype of mice lacking mGluR1 was consistent with cerebellar-dependent motor impairment such as ataxia, intense tremor, and impaired eye blink conditioning (Aiba et al., 1994b). Recent studies have also demonstrated a role for mGluR in homosynaptic LTD in hippocampus and other tissues induced by low frequency stimulation (Bashir et al., 1993; Malenka, 1993; O'Mara et al., 1995; Malenka and Nicoll, 1993). MCPG blocked LTD in hippocampus (Bashir and Collingridge, 1994; O'Mara et al., 1995) although others failed to demonstrate any effect of this antagonist (Selig et al., 1995). An elevation in intracellular Ca^{2+} was essential for the expression of LTD in hippocampus (Cummings et al., 1996; Neveu and Zucker, 1996) but the source of the Ca^{2+} remained an element of controversy, although it was shown to be independent of the NMDA receptor activation (Bashir and Collingridge, 1994; Bolshakov and Siegelbaum, 1994; O'Mara et al., 1995).

***mGluR and neurotoxicity*:**

Sustained and diffuse increases in Glu kill neurons (Choi, 1992). The mechanism of cell death occurs primarily by the persistent action of Glu on NMDA receptors resulting in an excessive influx of Ca^{2+}. This is one of the key initial steps in the cascade leading to cell death. This is further supported by the observation that NMDA antagonists can prevent neuronal damage *in vitro* (Choi, 1992). Hence, Glu excitotoxicity is thought to be one

leading cause of cell damage and death following acute brain injury such as in stroke, seizures and trauma. According to the previously described effects of mGluRs on glutamatergic synaptic transmission, two opposing results from the activation of these receptors could be expected: 1) an increase in toxicity due to the cell depolarization and NMDA potentiation, effects attributable to the activation of group I mGluRs, 2) a protective effect resulting from group II and III-dependent presynaptic inhibition of Glu release (Holmes et al., 1996b; Nicoletti et al., 1996). The group II and III mGluR agonist DCG-IV and L-AP4 respectively prevented in part NMDA- or Ka-induced neuronal cell death in culture (Bruno et al., 1994, 1995a, 1996, 1997; Buisson et al., 1996). The mechanism involved in this effect was not clear but did not likely involve presynaptic inhibition. Inhibition of cAMP production, or inhibition of Ca^{2+} influx through VDCC has been proposed (Nicoletti et al., 1996). Conversely, activation of group I mGluRs with QUIS or DHPG enhanced neuronal damage induced by NMDA application (Bruno et al., 1995b; Buisson et al., 1996). Thus, mGluR agonists and antagonists represent a possible alternative approach in the prevention and treatment of acute or chronic neuronal cell death.

1.3. RESEARCH PROPOSAL

Background:

As outlined in the pages above, the LC, although a relatively small pontine structure, is the most prominent noradrenergic nucleus of the mammalian CNS, and can influence the activity of wide areas of the neuraxis. In view of this extensive noradrenergic innervation provided by LC, including the entire NA input to the neocortex and the hippocampus, the

control of the activity of LC neurons is highly relevant. The control of LC neuron activity has been found to be influenced by many neurotransmitters and neuromodulators, even though afferent innervation to the LC is very limited. The main excitatory neurotransmitters impinging on LC neurons appear to be EAA. Modulation of EAA actions on LC by other substances has been reported (Aston-Jones et al., 1991a; Marshall and Xiong, 1991; Charléty et al., 1993; Oleskevich et al., 1993; Xiong and Marshall, 1994; Ivanov and Aston-Jones, 1995).

Increasing evidence suggests that in most synapses, in many different brain structures, EAA themselves can modulate their own actions through their G-protein coupled mGluRs. These modulatory effects can be mediated through postsynaptic and/or presynaptic pathways, result in potentiating or inhibitory effects on glutamatergic synaptic transmission, and be readily reversible or long lasting. To date, there are no reports on mGluR action on LC neurons. However, mGluR subtypes have been localized to LC: mGluR7 mRNA is highly and specifically expressed in this structure (Kinzie et al., 1995; Ohishi et al., 1995), in addition to very low levels of mGluR3 mRNA (Ohishi et al., 1993), although no mGluR3 (or mGluR2) immunoreactivity was reported in this nucleus (Petralia et al., 1996a). Moderate levels of mGluR5 immunoreactivity were reported (Romano et al., 1995). Besides LC, low to moderate levels of immunoreactivity for mGluR2/3 were associated with PGi and PrH nuclei (Petralia et al., 1996a), sources of afferent to LC, and low levels of mRNA of mGluR3 have also been found in PrH (Ohishi et al., 1995). Moderate levels of mGluR7 mRNA were localized in PrH (Ohishi et al., 1995). Finally, mGluR4 mRNA was undetectable in both LC

and PrH nuclei (Ohishi et al., 1995). Therefore, members of the three groups of mGluRs are expressed by LC neurons and many of these receptors are also expressed by the two main structures which innervate LC.

Hypothesis to test:

Selective activation of different groups of mGluRs produces modulatory actions on excitatory synaptic transmission to LC and/or on electrophysiological behaviour of the neurons from this important central noradrenergic nucleus in rat brain slice preparation

Specific objectives:

1) To investigate the effects of specific mGluR agonists on evoked excitatory synaptic transmission.
2) To investigate the effect mGluR activation on LC responses to focally applied Glu.
3) To discriminate between potential presynaptic and postsynaptic actions of mGluR on excitatory synaptic transmission.
4) To investigate the effects of selective activation of mGluR subtypes on LC electrophysiology.
4) To investigate the identity of the mGluR subtype(s) responsible for the observed effects.
5) To investigate the cellular mechanisms involved in the mGluR effects.
6) To characterize the conditions leading to endogenous activation of mGluRs and compare these effects to those obtained with selective pharmacological activation of these receptors.

2. MATERIALS AND METHODS

2.1. SURGERY

Brain slices were prepared from young adult (4-8 weeks; 50 -150g) male Sprague-Dawley rats (Charles River, St. Constant, Québec) as described elsewhere (Dubé and Marshall, 1997) or from adult CD1 black mice (about 15g). In this procedure, every effort was made to minimize animal suffering. Briefly,following oxygen-rich halothane (2%) anaesthesia for three to five min., the animals were surgically decapitated at the atlanto-occipital joint following a rostrocaudal cut over the scalp and neck. The skull of the animal was removed using surgical forceps. The dura mater was carefully cut and removed and a coronal cut through the brain was made close to the bregma line (rostral to the pons). The brain was gently lifted-up and the cranial nerves attached to the pons were sectioned. The brain was lifted-out of the cranial cavity and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) NaCl 118.0, KCl 3.0, CaCl 2.5, $MgSO_4$ 0.8, NaH_2PO_4 1.0, D-glucose 10.0, and $NaHCO_3$ 19.0, equilibrated to pH 7.4 by continuously bubbling with 95% O_2/5% CO_2.

2.2. BRAIN SLICE PREPARATION

Brain tissue was transferred onto an iced petri-dish cover covered with a piece of filter paper moistened with ACSF. Using razor blades, the pons with part of the cerebellum was separated from the rest of the brain tissue by two coronal cuts: one at the extreme caudal limit of the pons, eliminating part of the brain stem, and one at the extreme rostral limit

(rostral to the colliculi). The block of tissue was then rotated on its rostral side and further trimmed-down, removing the cranial nerve roots and the pial tissue at the ventral surface, the excess cerebrum and cerebellum at the dorsal surface (dorsal to the aqueduct) and lateral sides leaving a block of tissue of approximately 6 x 5 x 3 mm. The tissue was glued onto a glass stage using cyanoacrylate glue (Crazy Glue™) with the dorsal side facing up. The caudal side rested against a plexiglass block cushioned with a slab of agar. Horizontal slices (350 μM in thickness) were cut in ice-cold ACSF using a Vibroslice (Campden Instruments, England). The blade vibration was set on the highest frequency and advance of the blade was set on the lowest speed. Appropriate slices were selected by naked eye, placed in the perfusion chamber and continuously superfused with warm ACSF (32.0 ± 0.5°C) at a rate of 1.5 ml/min. The total time from surgical decapitation to the transfer of the slice to the perfusion chamber was usually less then ten min.

2.3. PERFUSION CHAMBER AND HEATING

The perfusion chamber used throughout this study contains two compartments. The perfusate enters at the top of the first chamber which contains the nylon mesh on which the slice is placed and is sucked away through a needle connected to a vacuum from the small separated chamber which is connected to the slice chamber through a tunnel. The fluid level in the chambers can be adjusted by varying the vertical position of the suction needle. The total volume of the chamber is 0.8 ml, but the working volume is approximately 0.5ml once the fluid level is adjusted. Inside the slice chamber near the bottom, a circular step supports a plastic O-ring to which is attach a nylon mesh made from wedding veil. For increased

support a small piece of lens paper (the size of the slice) was placed between the slice and the nylon mesh.

The ACSF was contained in a 1 L jacketed reservoir and heated through a water circulation heating system (Heto, Denmark). The temperature of the circulating water was set so that the ACSF was maintained at 32 ± 0.5°C. The ACSF was continuously equilibrated with 95% O_2 / 5% CO_2 and pH maintained at 7. To assure that the ACSF was 32°C upon entering the perfusion chamber, the flow line entered another jacketed component, just before entering the chamber. This component was heated using a separate water circulation heating system so that the ACSF entering the perfusion chamber is 32 ± 0.5°C and pH 7.4.

2.4. ELECTRODES

2.4.1. Intracellular recording electrodes

Microelectrodes for intracellular recording were pulled from single-barrelled borosilicate glass capillaries with a filament [1.2 O.D./0.68 I.D., World Precision Instruments (WPI), Fl, USA] using a Brown-Flaming puller (Sutter Instruments Co., Model P-87, Ca, USA). Microelectrodes, filled with 2M potassium acetate, had DC tip resistances ranging from 60 to 120 MΩ.

2.4.2. Pressure ejection electrodes

The electrodes for pressure ejection were pulled from single-barrelled borosilicate glass capillaries as described above but using four steps which were preprogrammed on the Brown-Flaming puller. Each pull produced two usable pipettes with a tip diameter of 8-12 μm.

2.4.3. Stimulating electrodes

The stimulating electrode used to evoke postsynaptic potentials (see below) was made from two pieces of teflon-coated platinum wire twisted together. The Teflon insulation at the electrode tip was carefully scraped-off to form a low electrical resistance tip (≈ 10 KΩ).

2.5. DRUG APPLICATIONS

2.5.1. Bath application

For bath application, drugs were dissolved and diluted in ACSF to the desired final concentration, heated to 32°C and equilibrated with 95% O_2 / 5% CO_2 oxygenated in a 100 ml jacketed reservoir. The pH was adjusted to 7.4, if necessary. Drugs were introduced to the perfusion line using a three-way valve. Drugs used were: CNQX, *t*-ACPD, L-AP4, MAP4, DHPG, MCCG, MSOPPE, AIDA, EGLU, 4C-3H-PG, *t*-PDC (purchased from Tocris Cookson, USA), (R)-CPP, (±)-α-MCPG, mianserin, DPCPX, phaclofen, saclofen, 8-phenyltheophylline, aminophylline [purchased from Research Biochemical International (RBI), MA, USA], IBMX, (Calbiochem, CA, USA), 8-Br-cAMP (Biomol, PA, USA). APDC (2R,

4R-4-aminopyrrolidine -2,4-dicarboxylate), was a generous gift from Dr D. Schoepp at Eli Lilly (Indianapolis, USA) and CGP 35348 was a generous gift from Novartis Pharma Canada Inc. (Québec, Can.). Exceptions to this are (-)-Bic methiodide and, picrotoxin, NEM, Forskolin, Yoh (Sigma Chemical Co., MI, USA) which were added to the main ACSF reservoir and were always present.

2.5.2. Pressure Ejection

For pressure application, drugs were dissolved in ACSF and the pH was adjusted to 7.4 if necessary. Pressure electrode tips were placed over the recording area, 50-100 μm away from the surface of the slice. Compressed nitrogen gas was used as a pressure source. The pressure [3 to 100 pounds per square inch (psi)] applied to the electrode was adjusted through the pneumatic valve of the Picospritzer™ II (General Valve Corp., N.J.). Regular application of drugs was controlled by computer through the pClamp™ program (Axon Instruments Inc., Ca, USA). Drugs were prepared daily from stock solutions to a final concentration in ACSF (pH=7.4). Drugs used for pressure ejection were: Glu (20 mM), AMPA (20 μM), Ka (0.1-1 μM), NMDA (1-10 μM)

2.6. ELECTROPHYSIOLOGICAL RECORDINGS

2.6.1. Intracellular Recording

Intracellular recording electrodes were connected to an HS-2 (gain: 0.1L) head stage of an Axoclamp 2A™ amplifier (Axon Instruments Inc., Ca, USA) by an electrode holder

(EH-2MSW, 1.2 mm, E.W. Wright, Guilford, Connecticut, USA) through a silver chloride coated silver wire (0.25 mm in diameter). The electrodes were placed over the LC nucleus. Current pulses, converted to constant current pulses by the amplifier were applied to the electrode to detect increases in resistance when the electrode contacted the cell surface.

Intracellular potentials measured under current clamp were amplified, displayed by conventional methods and recorded on chart paper through a chart recorder (Gould 2200s, USA). During some experiments, potential was also recorded with a magnetic tape recorder (Racal Recorders, LTD, UK), for further analysis. Spike frequency was monitored by a frequency counter (model RD-1, Vancouver, BC, Canada) and displayed on the chart paper on a separate channel. Action potentials were captured either by a digital storage oscilloscope (model 1421, Gould, USA) or by computer through the pClamp 6.0 software. Small hyperpolarizing currents (-20 to -70 pA) were generally injected to the impaled cell by an active bridge circuit in an attempt to increase cell stability following penetration of the electrode. The bridge was balanced before impalement of the cell and corrected for any deviation in balance which sometimes occurred during the experiments.

Membrane input resistance was measured for all impaled cells by passing hyperpolarizing and depolarizing current pulses in successive steps through the recording electrode and recording the change in membrane potential through a computer with the pClamp 6.0 acquisition software. Current steps were generated through the amplifier under the control of a PC computer (pClamp 6.0) following a preprogrammed protocol. The

duration of the current pulse, 200 ms, was long enough to charge the membrane capacitance and reach a steady-state voltage response. Voltage-current relationships were constructed off-line by measuring steady-state voltage changes resulting from current steps and the slope input resistance of the cell was measured.

2.6.2. Recording of Synaptic Potential

Synaptic potentials were evoked by applying single rectangular voltage pulses (4-60V, 0.25ms, 1 Hz) to the bipolar stimulating electrode (see above) placed within 1mm rostrolateral to the recording site. The stimulus was generated from a Grass S-88 unit (under pClamp 6.0 control), through a constant-current stimulus isolation unit. For paired-pulse facilitation, the second pulse was delivered 20-40 ms following the first one depending on the size and shape of the first synaptic potential. Synaptic potentials were amplified, displayed by conventional methods, digitized and stored on the hard drive of the computer for further analysis (below).

2.7. IDENTIFICATION OF LC NEURONS

In horizontal slices, LC neurons can be identified by their location and their electrophysiological properties. In brain slice preparations, the LC nuclei are clearly discernable when viewed under a dissecting microscope with transillumination at a magnification of 10x to 40x. In horizontal slices, LC nuclei are localized to the ventrolateral edge of the 4th ventricle (fig. 4). Electrophysiologically, impaled LC neurons display distinctive traits such as a regular pattern of spontaneous action potentials (APs), a relatively

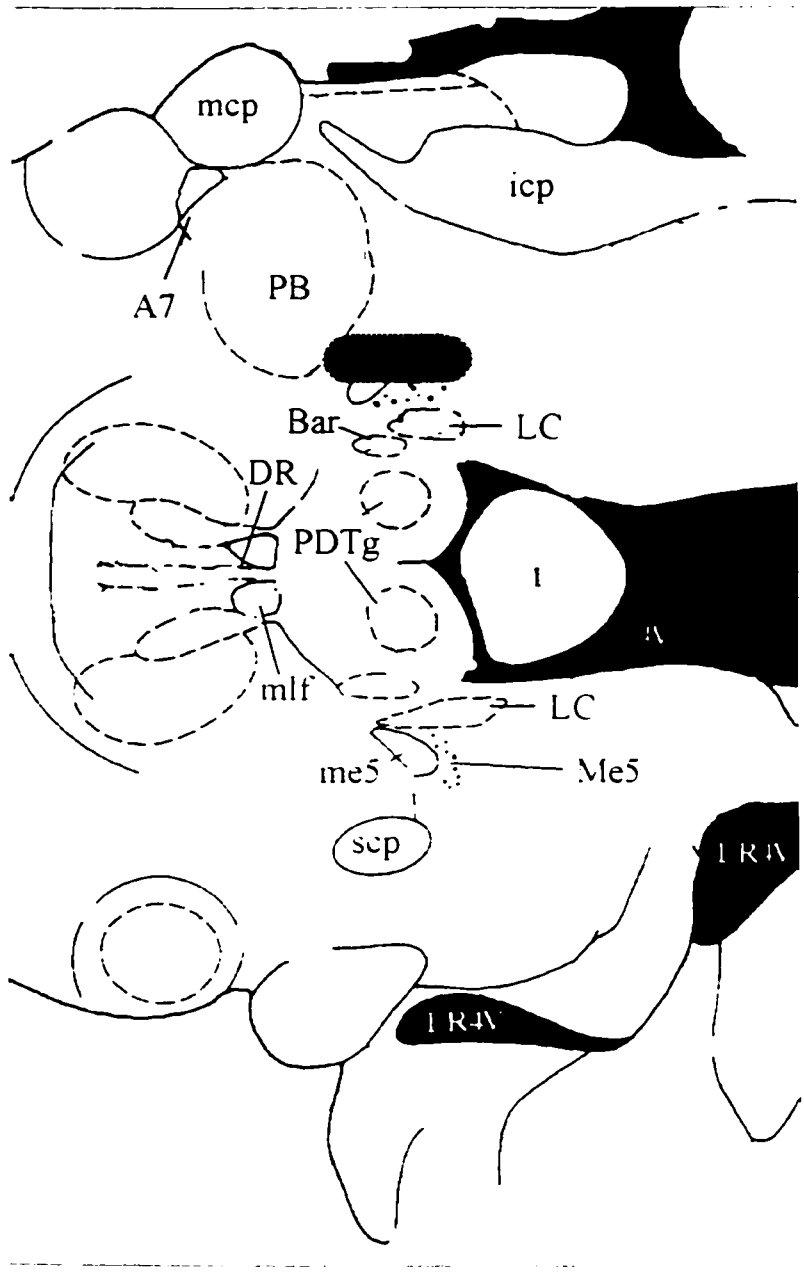


Figure 4: Schematic representation of a rat horizontal brainstem slice. Abbreviations: 1-cerebellar lobule 1, 4V-Fourth ventricle, Bar-Barrington's nu., DR-Dorsal Raphé, icp-inferior cerebellar peduncle, LC-locus coeruleus, mcp-middle cerebellar peduncle, me5-mesencephalic trigeminal tract, Me5-mesencephalic trigeminal nu., mlf-medial longitudinal fasciculus, PB-parabrachial nu., PDTg-postdorsal tegmental nu., scp-superior cerebellar peduncle. The shaded xxx-region for placement of the stimulating electrode. (slice taken at Bregma -7.34 mm, interaural 2.66 mm)

long duration AP with a characteristic 'shoulder ' on the falling phase and an intrinsic membrane potential oscillation (see results).

2.8. EXPERIMENTAL PROCEDURE

Once the appropriate slice for recording was selected and transferred to the perfusion chamber, gold electron microscope grids or slots were placed over the areas of the LC on each side of the slice to stabilize the tissue in the areas where the microelectrode will be probing. Small platinum bars (approximately 0.3 mm in diameter and 1 mm to 1.5 mm in length) were placed on top of the grids to increase the stability of the slice further. Intracellular recordings were conducted following a minimum of one hour equilibration in the recording chamber. When a cell was penetrated, hyperpolarizing current (-70 pA) was routinely injected to the intracellular recording electrode to help the cell to stabilize. Impaled cells were considered acceptable when the membrane potential was more negative than -50 mV and the amplitude of the action potential greater than 60 mV, following which the hyperpolarizing current was reduced (to about -30 pA).

Once a stable recording had been established and the identity of the LC neuron confirmed, single rectangular voltage pulses of increasing amplitude (5V to 60V) were applied to the bipolar electrode and evoked postsynaptic potentials were monitored. If no synaptic potentials were observed, the bipolar electrode was moved to another position. The amplitude of the stimulus applied to the bipolar electrode was chosen to produce a postsynaptic potential with amplitude about 70-80% of the threshold for action potentials.

When required, paired-pulse stimuli were also applied and the interval (20-40 ms) between the two pulses was adjusted to produce an optimal facilitation (see results). At that time, Bic (and sometimes Yoh) was added to the main ACSF reservoir.

To test responses to the different EAA agonists (glu, AMPA, Ka, NMDA), pressure ejection was conducted by placing the pipet over the pertinent area and applying pulses with increasing pressure (3-30 PSI) and/or duration (5-60 ms) until the response was judged arbitrarily satisfactory, whether a subthreshold or a larger response was desired. Drug application was regularly repeated every two to three min. Once the response to the application was reproducible (minimum three successive applications), bath application of a second agent was started. Membrane potential changes measured under current clamp were amplified, displayed by conventional methods and recorded on chart paper through a chart recorder (Gould 2200s, USA). Finally, potential changes following pressure application were digitized, acquired through pClamp 6.0 and stored on the computer hard drive until further analysis.

2.9. DATA ANALYSIS

All acquisition and measurements were made using pClamp 6.0 software. Data for EPSPs were averaged off-line (using the clampfit software) with eight individual sweeps per averaged waveform. The amplitude of the averaged waveform was then measured with respect to its baseline a few ms before the stimulus artifact. For paired-pulse facilitation, paired recording of eight single EPSP sweeps and eight paired-pulse sweeps were recorded

consecutively. These were later individually averaged as described above. The single EPSP waveform was then subtracted from the averaged paired-pulse EPSP waveform and the amplitude of the second EPSP was measured. Data from voltage-current protocols were averaged on-line with four individual sweeps per averaged waveform. Exponential fit convergence was determined by the sum-of-squared errors and the best fit was determined on the basis of the lowest standard deviation (for the membrane time constant, Clampfit module), or based on F-tests. Averaged data are presented as mean ± SEM unless otherwise stated. Data were statistically analysed using two-tail Student's t test when comparing 2 conditions, or analysis of variance (ANOVA) when testing for differences between multiple conditions followed by tukey post-hoc analysis when overall means where significantly different ($p<0.05$). Differences were considered significant at $p<0.05$.

3. RESULTS

3.1. PROPERTIES OF INTRACELLULARLY RECORDED RAT LC NEURONS

A total of 383 LC neurons were recorded intracellularly from 288 brain slices (287 rats). Rats' body weights ranged from 48 to 150 g (mean ± SEM=85.6 g ± 1.9 g, n=160). Frequently upon impalement, LC neurons displayed high frequency discharges lasting from one to several minutes and had membrane potentials between -25 and -45 mV. When impalement was successful and the cell membrane sealed around the glass electrode, the membrane potential slowly decreased to return to resting membrane potential (Em). Often, upon seal formation, Em decreased to about 20 mV below resting Em (resting Em≈-55 mV) and slowly returned to the latter. Upon returning to resting Em, intrinsic Em oscillations often appeared. The time for stabilization of neurons was decreased when hyperpolarizing current (-50 pA to -80 pA) was injected through the recording electrode. Figure 5 shows a digital reconstruction of a rat LC neuron injected with neurobiotin and stained with CY3 and scanned with a confocal microscope. The cell soma can clearly be delineated and an abundant dendritic arborization can be seen. In this example a second neuron can be seen in the backgroung, indicative of dye-coupling between the cells as only one neuron was impaled.

3.1.1. Action potentials and spontaneous activity

In the majority of cases, when hyperpolarizing current was reduced (to -30 pA) or removed, the stabilized LC neuron displayed regular spontaneous discharge (fig. 6A) with

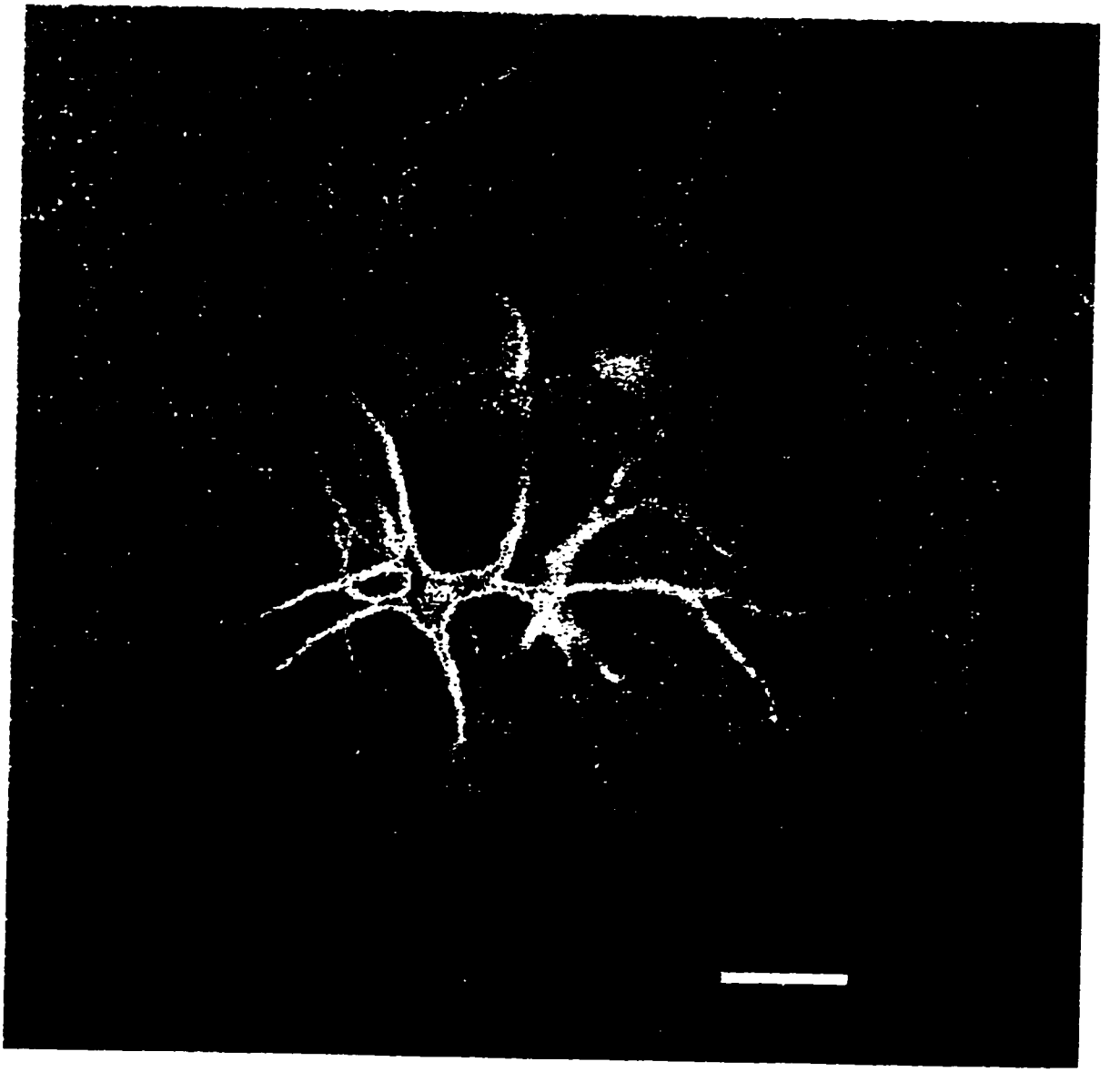


Figure 5: Computer reconstruction of a confocal image of a locus coeruleus neuron in rat brainstem slice. Neuron was impaled and, following stabilization, was injected with neurobiotin (in 2mM KCl) for 20 min by iontophoresis (2 nA, 3.3 Hz, 150 ms pulses). Following injection the dye was allowed to equilibrate into the cell for 1 hour before fixing. Post-fixation staining was carried out with using CY3-conjugated streptavidin following standard protocols in use in the lab (A. Doja and K. Marshall, personal communication). Scale bar = 32 μm.

frequencies ranging between 0.5 and 5 Hz. The action potential (AP) threshold was about -50 mV and possessed distinctive characteristics. The rising phase of the AP was typically fast and TTX-sensitive (fig. 6B). The amplitude of the AP ranged between 60 and 80 mV (measured from the threshold). The falling phase of the AP displayed three distinctive phases, an initial rapid repolarisation followed by a relatively slow repolarisation and another fast repolarisation phase, giving the appearance of a shoulder on the falling phase of the AP (fig. 6B). In TTX, the component responsible for the shoulder phase could be visualized as a slower AP with a threshold around -40 mV (fig. 6B) and was due to a transient increase in calcium permeability. Following the AP, a large afterhyperpolarisation was observed, which slowly returned to resting Em, merging with the slow spontaneous depolarisation underlying the spontaneous firing activity, or dissipated in the Em oscillations (see below). These features of the LC AP were useful trademarks to confirm the identity of the neuron impaled.

LC neuron depolarization above the -50 mV through positive current injection through the intracellular recording electrode resulted in an increase in firing rates with a small accommodation during the pulse (fig. 7). Upon ending the depolarizing pulse a relatively long lasting hyperpolarization period followed the spike cluster.

3.1.2. Intrinsic oscillations of LC neurons

In LC neurons recorded from horizontal slices, intrinsic Em oscillations, underlying the spontaneous firing activity could be observed, the frequency of which (1 Hz-10 Hz) was equal to or greater than that of the firing rate (fig. 6A). The amplitude of the oscillations

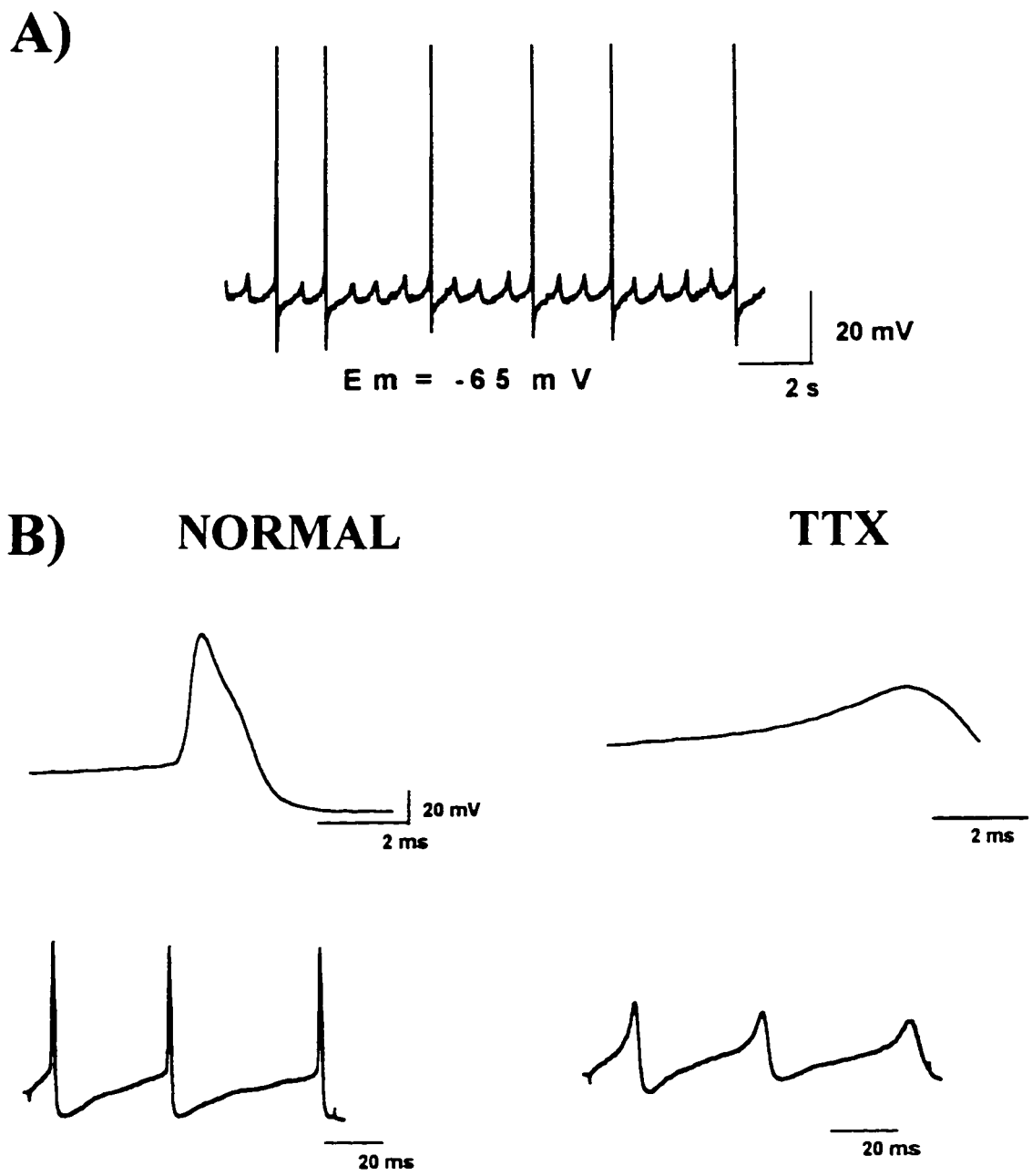


Figure 6: Electrophysiological basal membrane and action potential properties of impaled LC neurons in horizontal brainstem slice preparation. Representative examples of A) Typical LC spontaneously firing activity and underlying intrinsic membrane oscillations. B) Typical action potentials (left) displayed with a fast and a slow sweep speed recording. The shoulder on the falling phase of the action potential can be seen (fast sweep) while the large afterhyperpolarization can be better appreciated with the slow sweep (bottom). On the right, the effects of TTX (0.1μM) are seen. The fast rising phase of the action potential is missing in TTX (fast sweep), and is replaced with slower a and broader action potential with a more positive threshold. The latter corresponds to the shoulder observed under basal conditions. The afterhyperpolarization was not affected by TTX (bottom right).

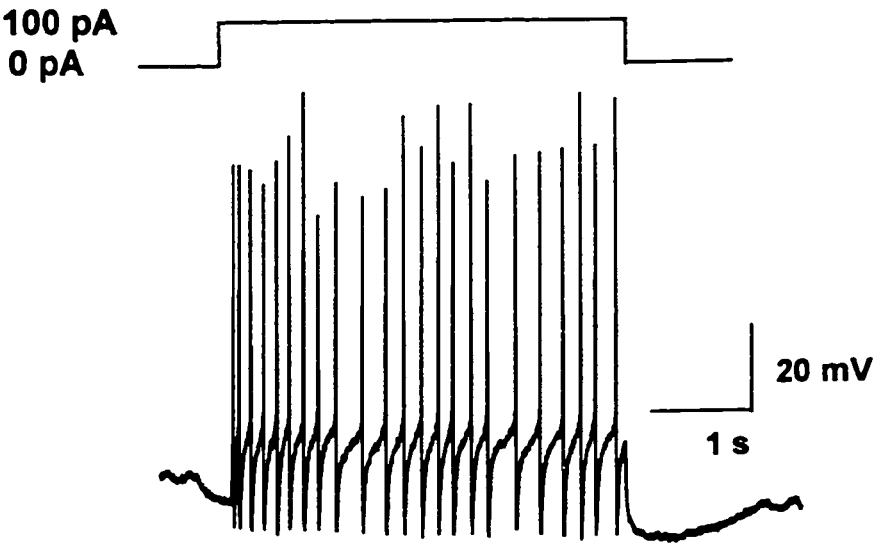


Figure 7: Discharge induced by current injection in an LC neuron. A representative example of the electrophysiological response of an LC neuron to a depolarizing current injected though the intracellular recording electrode. A small accommodation of the firing frequency, following the initial depolarization can be observed (resting Em=-60 mV).

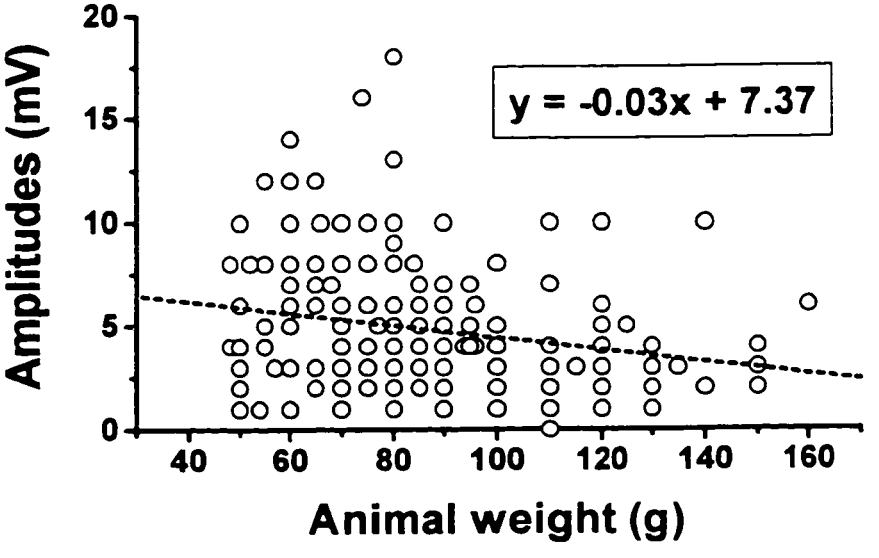


Figure 8: Negative correlation between rat weight and amplitude of intrinsic membrane oscillations. Scattered plot displaying the distribution of oscillation amplitudes with respect to rat body weights (used as an index for age). A body weight of 50g roughly corresponds to a 30-day-old Sprague-Dawley rat while a body weight of 150 corresponds to a 50-day-old rat. Each points in the plot represents one animal. Oscillation amplitudes were measured in the first cell impaled in that experiment, immediately following recovery from impalement. Slope of the linear fit was -0.029 ± 0.009 (n=188) and was significantly different from zero ($p<0.001$, assessed by F-test).

ranged between 1 mV and 18 mV (Mean ± SEM: 4.71 mV ± 0.24 mV, n=160). A small but significant correlation between the age of the animals and the amplitude of the oscillations was found (slope =-0.029 ± 0.009, $p<0.001$, n=188) (fig. 8).

3.1.3. Voltage-current relationship

Passage of current pulses of varied amplitude through the intracellular recording electrode (200 ms duration, which was sufficient to charge the cell membrane capacitance) caused voltage deflections which were proportional to the current in amplitude and in direction (negative and positive) (fig. 9A). When the change in Em was plotted against the amplitude of current injected (V-I relationship), a linear relationship was observed (fig. 9B). Rectification often, but not always, occurred at more hyperpolarized potentials (> 100 mV) causing a deviation from linearity in the voltage-current relationship. From Ohm's law, input resistance of the cells could be calculated from the slope (slope resistance) of the linear portion of the V-I relationship. Alternatively the input resistance of the cell was also determined by measuring the change in Em following a single current pulse (chord resistance). As long as the Em change was linear with respect to its V-I relationship, the value of the chord resistance approximated that obtained with the slope resistance (slope=0.87 ± 0.07, R=0.94, n=29; fig. 9C). Rat LC neurons recorded from horizontal slices had apparent input resistances ranging from 45.72 MΩ to 135.1 MΩ (mean slope resistance ± SEM: 75.44 ± 3.53, n=29).

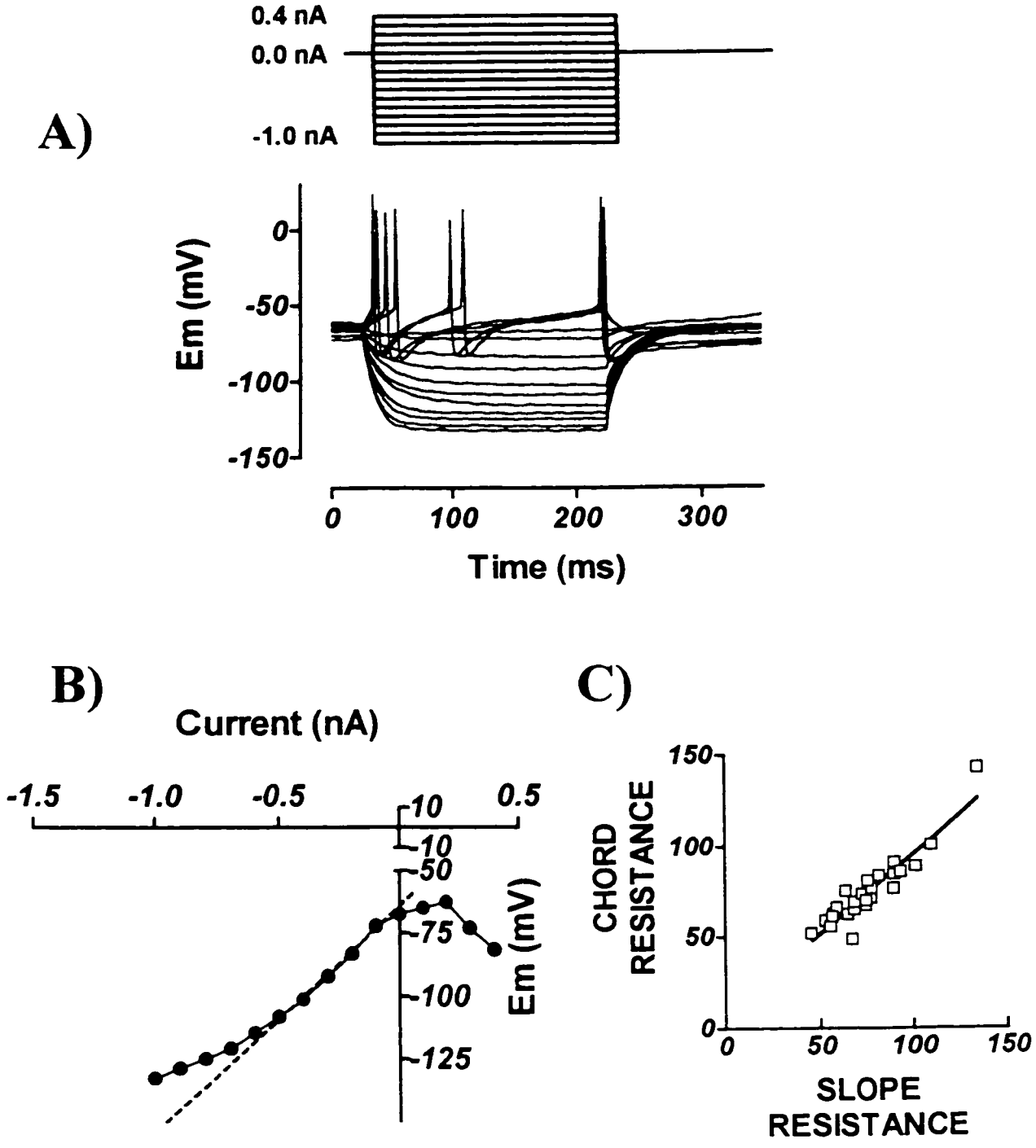


Figure 9: Apparent membrane input resistance in an LC neuron. A) Representative example of the membrane potential change (Em or V) of an LC neuron in response to 200 ms current injections (I). B) The V-I plot of the graph A) showing a linear relationship between V and I over a discrete range. C) Relationship between the slope resistance of neurons determined from the slope of the linear fit in B) and the chord resistance determined from the change in V associated with a single current pulse and calculated from Ohm's law: V=IR (R=resistance). See text for additional details.

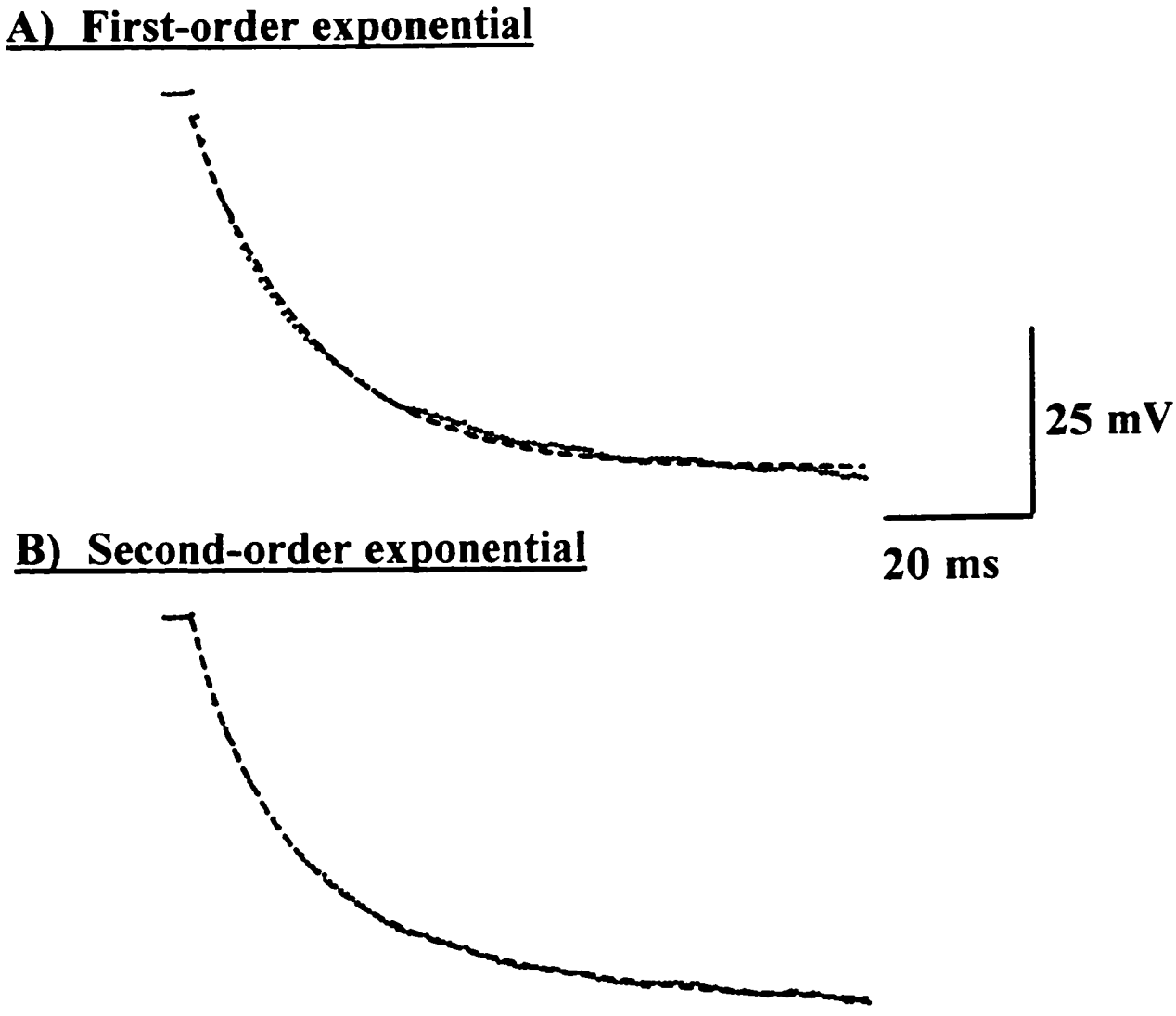


Figure 10: Membrane time-constant of LC neurons. Representative example of the time course of change in electrotonic potentials following a 100 ms current pulse (-0.6 nA). A) Portion of the potential waveform (dashed-line) fitted with a first-order exponential (dotted-line) ($y=A_1*\exp[-(t-t_0)/tau_1]+C$; where C is a constant and tau is the membrane time-constant) and B) is the same waveform fitted with a second-order exponential ($y=A_2*\exp[-(t-t_0)/tau_2] + A_1*\exp[-(t-t_0)/tau_1]+C$). In this example, the waveform is obviously better fitted with double exponential.

Using a single current pulse (-600 pA was arbitrarily chosen) the time constant (τ) of the cell membrane was measured. This τ was used to describe the time course of the potential change following the step in current injection and was estimated from an exponential fit of this time course. In most of cells used for measurements (15 out of 16) the time course of the potential change was best fitted with a 2^{nd} order exponential as opposed to a single exponential (fig. 10). The value for $\tau 2$, which represents the time constant for the initial portion of the fit, ranged from 0.041 ms to 5.6 ms (mean ± SEM: 2.3 ms ± 0.48 ms, n=15) and while $\tau 1$ ranged from 10.7 ms to 35.3 ms (mean ± SEM: 17.1 ms ± 1.6 ms, n=15).

3.1.4. Postsynaptic potentials

In horizontal slices, single electrical pulses 100-400 μs in duration, applied to the bipolar stimulating electrode (positioned rostrolateral to the recording electrode) evoked EPSPs in all neurons tested. To prevent the EPSPs from reaching threshold, especially with cells displaying large membrane potential oscillations, a small hyperpolarizing pulse (-0.2 to -0.7 nA) was applied for 300 ms, temporarily setting the cell to lower potentials. EPSPs were evoked midway through the hyperpolarization pulse (fig. 11A). The hyperpolarizing protocol also allowed us to measure larger postsynaptic potentials which would otherwise reach the threshold.

3.1.4.1. Properties of single EPSPs

The latency from the end of the stimulus artifact to the onset of an EPSP ranged from 0.4 ms to 3.6 ms (1.77 ± 0.14 ms, n=36 experiments randomly selected). All EPSPs had a

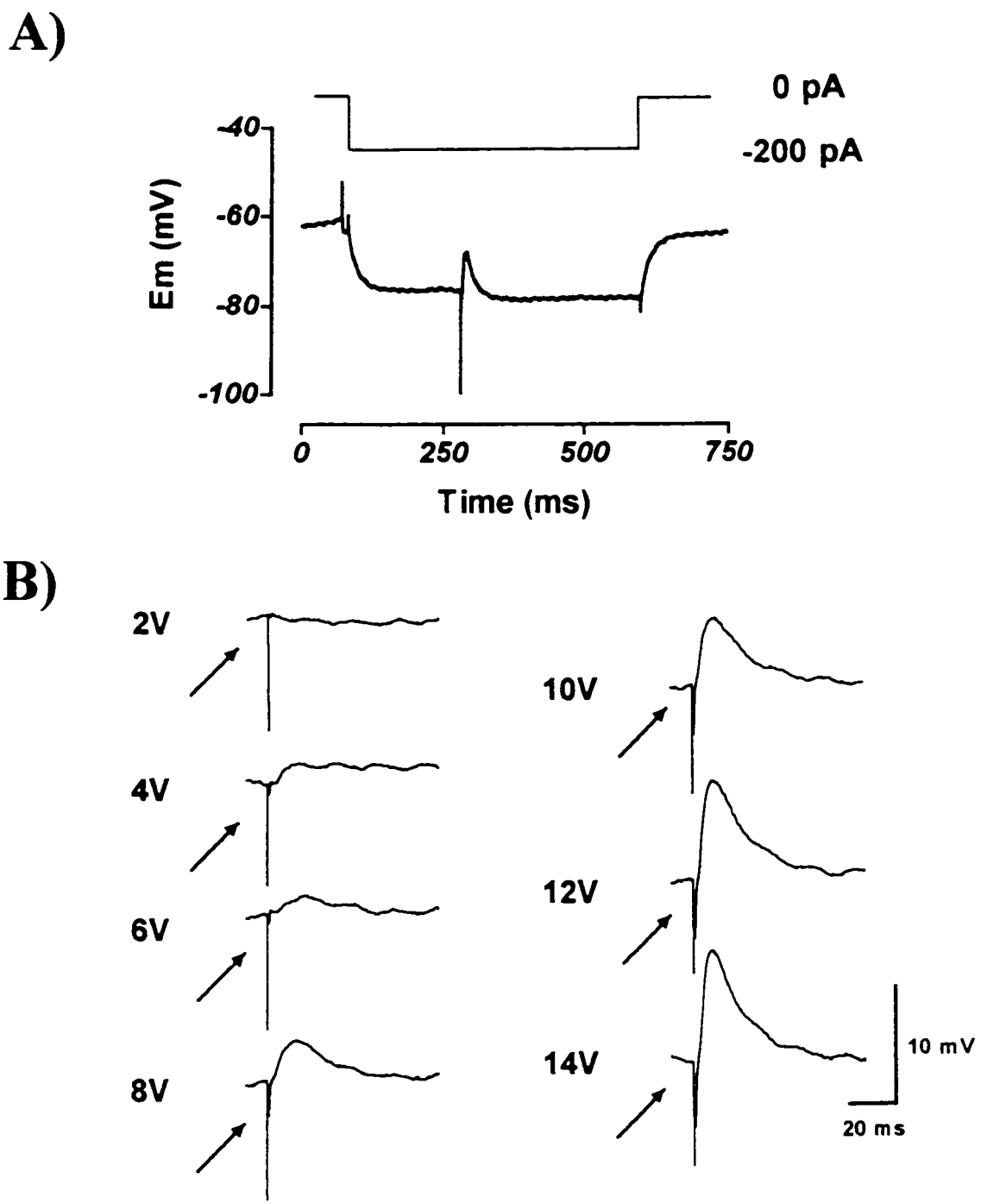


Figure 11: Evoked EPSPs in a rat LC neuron in a horizontal brainstem slice preparation. A) Representative example showing the hyperpolarizing protocol used to set Em temporarily to lower potentials before evoking the EPSP. B) Variation in EPSP amplitudes with respect to the strength of the stimulus delivered to the slice. Arrows point to the rapid downward deflection representing the stimulus artifact, i.e., when the stimulus was applied. The variation in amplitude is a reflection of the recruitment of afferents with increasing stimulus strength.

fast rising inital phase with a slower decay phase phase. EPSP amplitudes ranged from 4.6 mV to 27.2 mV (13.2 ± 0.49 mV, n=100). This was largely dependent on the intensity of the stimulus (fig. 11B) and was limited at higher amplitudes by threshold for dendritic and/or full AP. The EPSP amplitude depended also on Em (fig. 12) and varied linearly in amplitude between about 60mV and 100mV (fig. 12B).

Characterisation of the subtype of Glu receptors activated following a single stimulation was carried out in the presence of 10 μM Bic (to eliminate fast IPSPs), using selective antagonists to non-NMDA (CNQX) and NMDA [(R)-CPP] receptors (fig. 13). CNQX caused a dose-dependent reduction of the Bic-resistant evoked EPSP amplitudes to about 15% of control at the highest dose tested (20 μM) (percent of control: 15.8% ± 0.8%, n=5), indicating that most of the voltage change measured during EPSPs in LC neurons is mediated through the activation of non-NMDA Glu receptor subtypes. Supporting this observation, (R)-CPP had little or no effect on the EPSP amplitude (percent of control for 50 μM: 95.2% ± 6.3%, n=3).

3.1.4.2. Properties of paired-pulse EPSPs

Paired-pulse EPSPs could be observed when a double stimulus was applied to the bipolar stimulating electrode. Under conditions where the two stimuli are delivered at relatively short intervals (<100 ms), the amplitude of the EPSP resulting from the second stimulus was consistently larger than for the first (fig. 14A). This phenomenon, known as paired-pulse facilitation (PPF), was quantified by comparing the amplitudes of the second

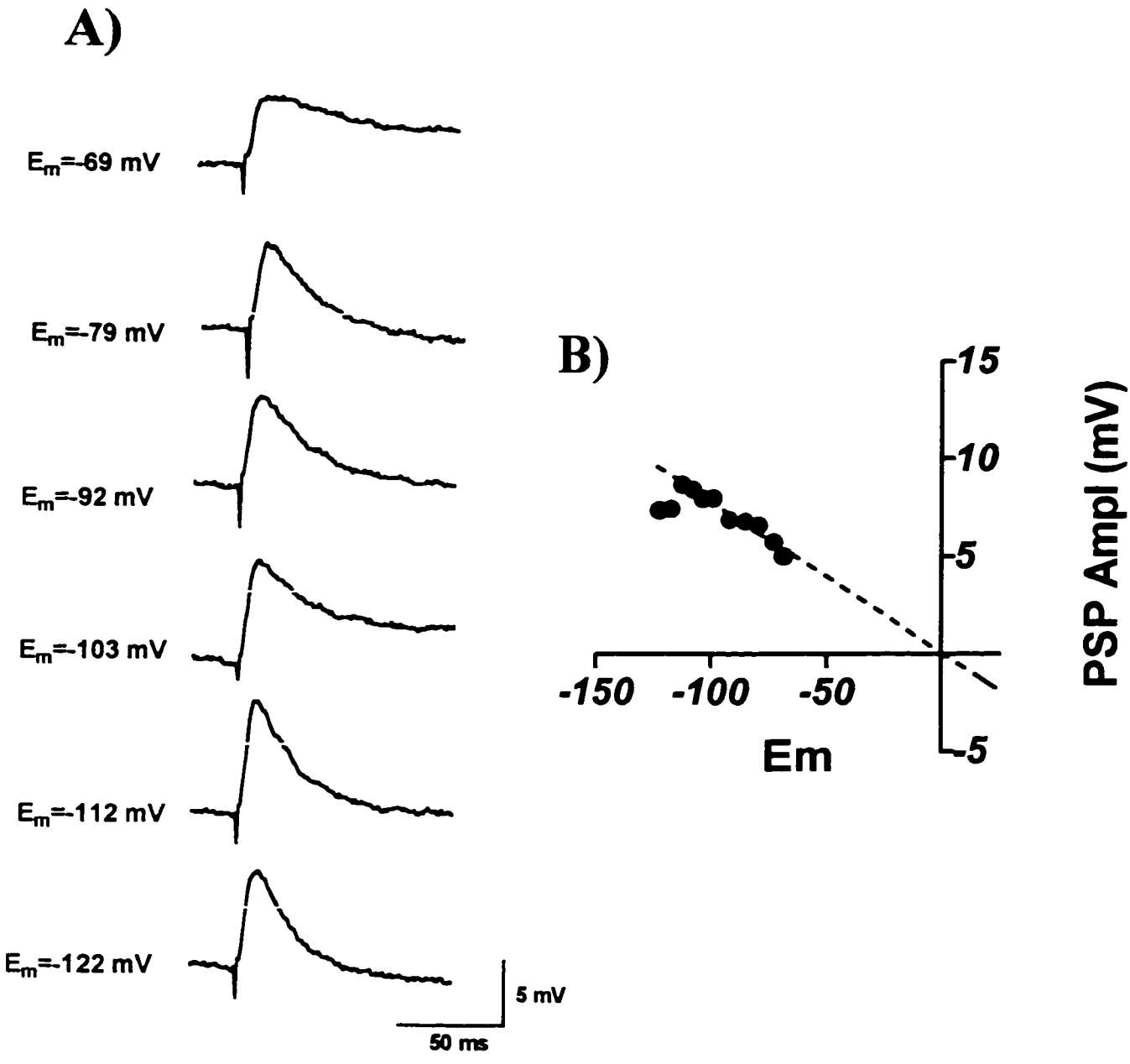


Figure 12: EPSPs are dependent on membrane potentials. A) Representative examples of EPSPs evoked at times when the membrane potential was set to different values. B) Plot showing a representative relationship between the membrane potential and the amplitude of the EPSP for traces in A). For this neuron, and many others, the relationship is linear and the extrapolated value for the reversal potential of EPSPs is close to zero mV. Note the chage in the repolarization kinetics. This was often observed in LC and did not appear to correlate with membrane potential or NMDA activation. The implication of other transmitters (other than GABA) or neuropeptides in shaping the falling phase of the EPSPs cannot be excluded.

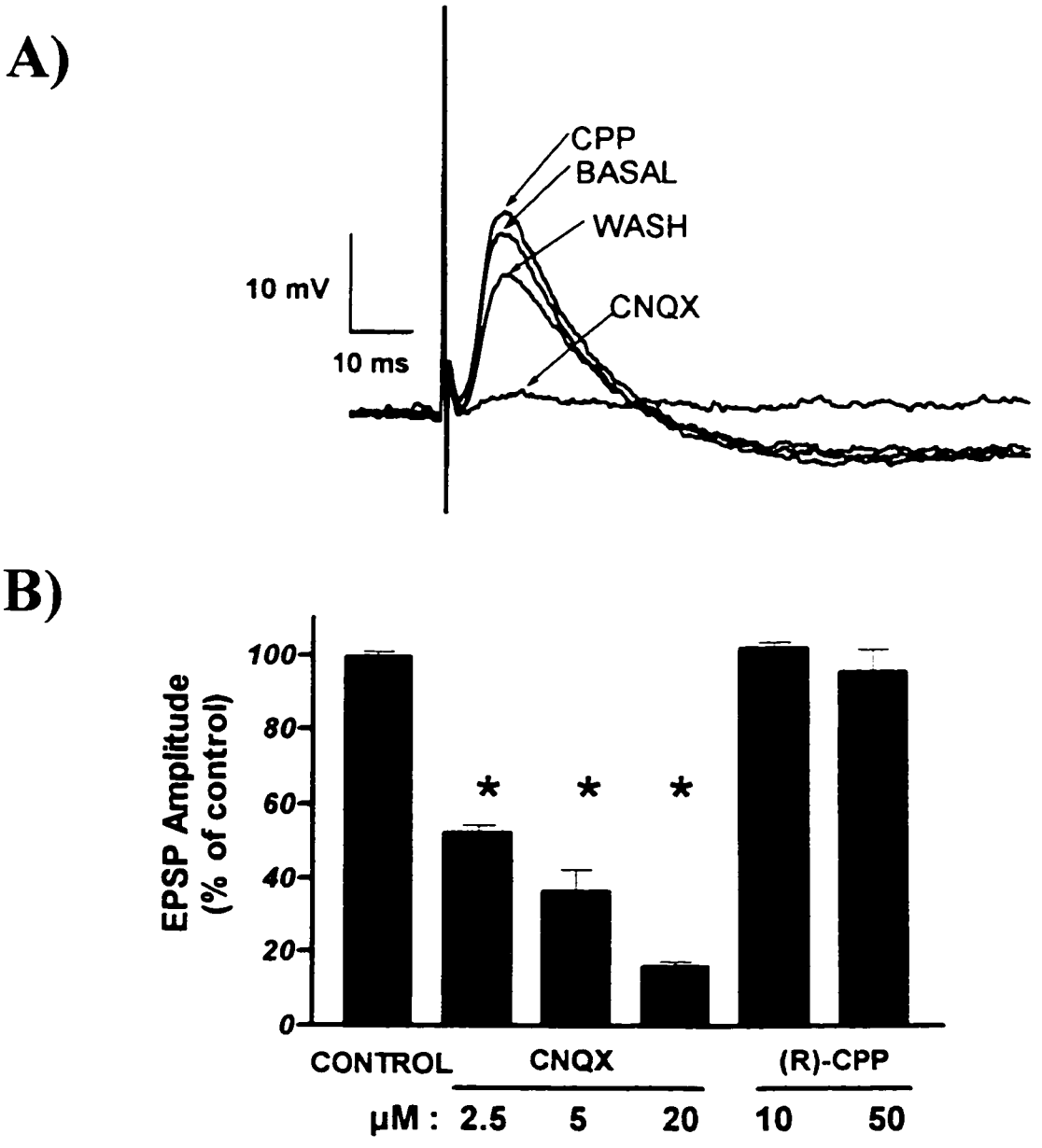


Figure 13: Effect of specific ionotropic glutamate receptor antagonists on evoked EPSPs in rat LC neurons. A) A representative example of EPSPs evoked in an LC neuron under the following conditions (in the order tested): 1-Basal, 2-in the presence of 50 μM CPP (NMDA receptor antagonist, 3-in the presence of 20 μM CNQX (a non-NMDA receptor antagonist), and wash of CNQX (20 min. post antagonist). The wash following CPP (20 min) was not shown for the sake of clarity but was the same as basal. B)Bar graph illustrating the mean antagonism activity of CNQX and CPP at various doses on EPSP amplitude (% of control EPSP amplitude). At all doses tested, CNQX produced a significant inhibition of EPSPs. (n=7, 8, 4, 5, 3, 3, respectively for each bar; * $p<0.001$).

EPSP to the first. The amplitude of the second EPSP varied in size (compared with the first) depending on the interval between the two stimuli. The decay in the facilitation with increasing inter-stimulus intervals (fig. 14B) could be fitted with a first order exponential (when compared, the first order exponential fitted data better than a second order exponential, $p<0.05$) and had a half life decay time (τ) of 71 ms. During PPF experiments, it was common to observe more prolonged hyperpolarization phases following the application of the stimuli (not shown). These have been previously characterized as resulting from the of activation of α_2-adrenergic receptors, through the release of noradrenaline from LC neurons. Therefore, to control for the possibility that activation of α_2-adrenergic receptors could affect the amplitude of the second EPSP, PPF experiments were also tested in the presence of Yoh (1 μM applied by bath), an α_2-adrenergic receptor antagonist. Results indicate that Yoh did not significantly change paired-pulse facilitation. However, when the entire waveforms from control and Yoh were compared, a Yoh-sensitive component was often observed (fig. 14C). This was not investigated further.

3.2. PROPERTIES OF INTRACELLULARLY RECORDED MOUSE LC NEURONS

In total, 43 mouse LC neurons were recorded intracellularly from 18 brain slices (18 CD1 black mice weighing between 15-18 g.), eight of which were wild type (WT) and ten which were deficient (knockout, KO) for mGluR4 (see below). Data presented in this section were extracted only from experiments using WT mice. As for rats, LC neurons from mice

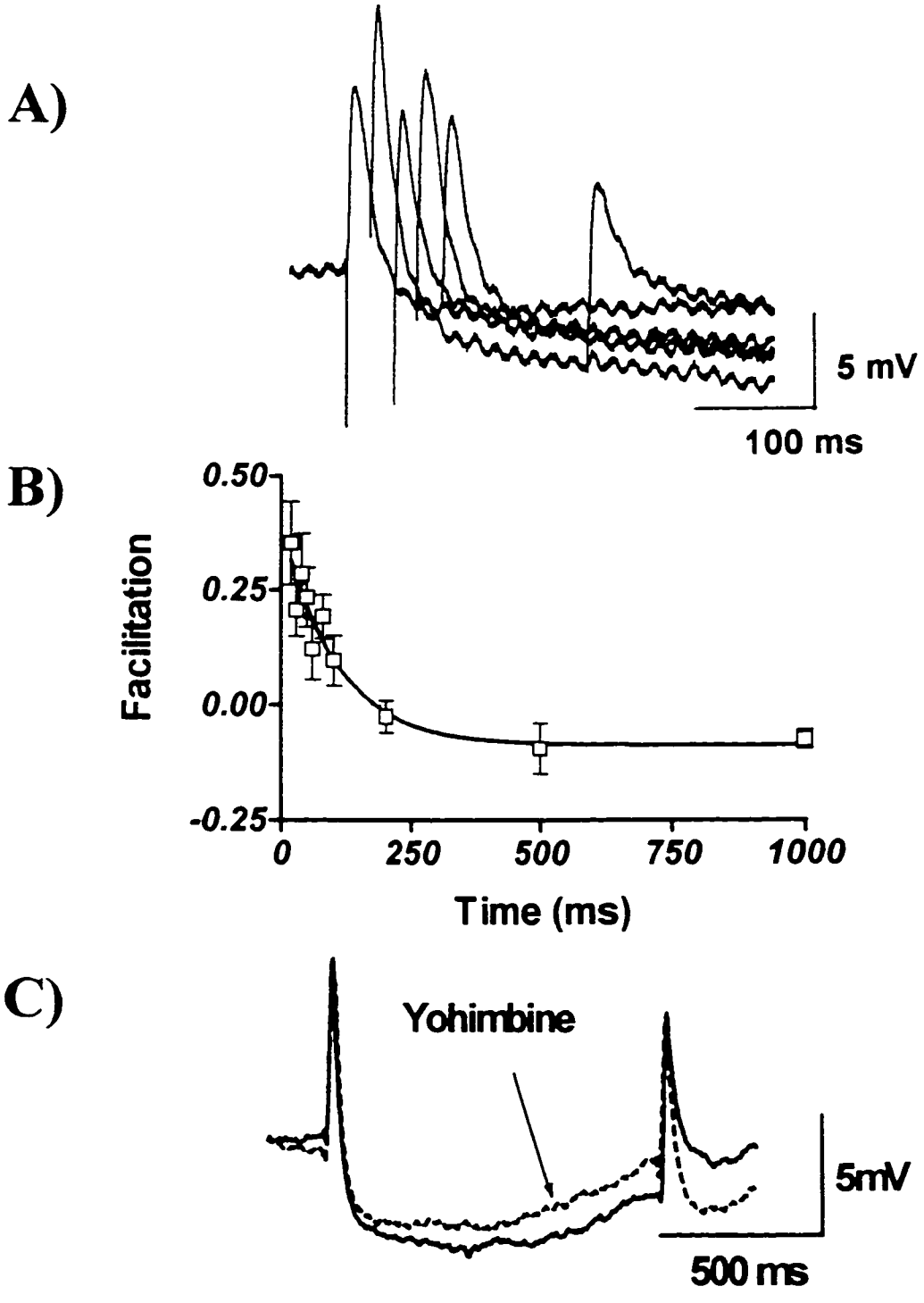


Figure 14: Paired-pulse facilitation in rat LC neurons. A) Representative examples of EPSPs evoked in pairs with increasing inter-stimulus intervals. The First EPSP on the left is a control resulting from a single stimulation. B) Averaged time-course of facilitation [F=(EPSP2-EPSP1)/EPSP1] from 9 independent experiments as in A). The rate of decay of the EPSP was best fitted with a first order exponential. C) Superimposed waveforms showing the effect of Yoh (1 μM, dashed-line) on the baseline potential following the first EPSP but lack of effect on the amplitude of the second EPSP.

displayed a high frequency discharge, upon impalement, lasting from one to several minutes and displayed membrane potentials between -25 and -45 mV. When cells recovered from impalement, the membrane potential slowly decreased to return to resting membrane potential (Em). Mice LC neurons were markedly more difficult to find, and the recovery from impalement was also less reliable.

3.2.1. Action potentials and spontaneous activity

As for rats, when hyperpolarizing current was reduced (-30 pA) or removed, most of the stabilized mouse LC neurons displayed spontaneous discharges with frequencies ranging between 0.5 and 5 Hz. The threshold for firing APs was about -50 mV. The rising phase of the AP was typically fast (fig. 15), and the amplitude of the AP ranged between 55 and 70 mV (mean ± SEM: 63 ± 1, n=15 WT mice). The falling phase of the AP also displayed the distinctive shoulder, followed by a large afterhyperpolarisation that slowly returned to resting Em (fig. 15). Depolarization of mouse LC neurons to potentials more positive than -50 mV resulted in an increase in firing rates, with a small accommodation during the pulse (fig. 15). Upon ending the depolarizing pulse a relatively long lasting hyperpolarization period followed the spike cluster. Together, these observations are similar to those made in rats (section 3.1.).

3.2.2. LC Intrinsic Oscillation

A striking difference between rat and mouse LC neurons recorded from horizontal slices was the lack of Em oscillations in all mouse LC neurons recorded from (15 of 15 cells,

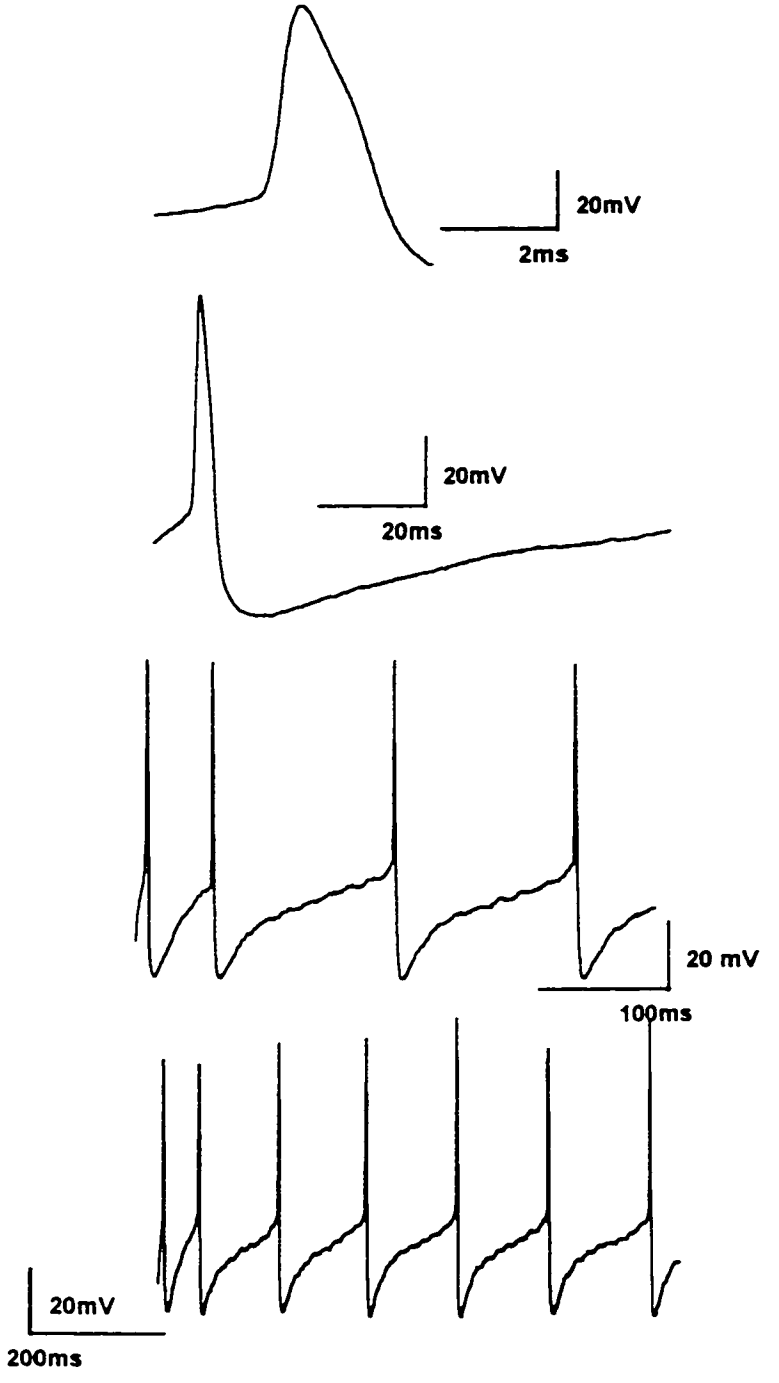
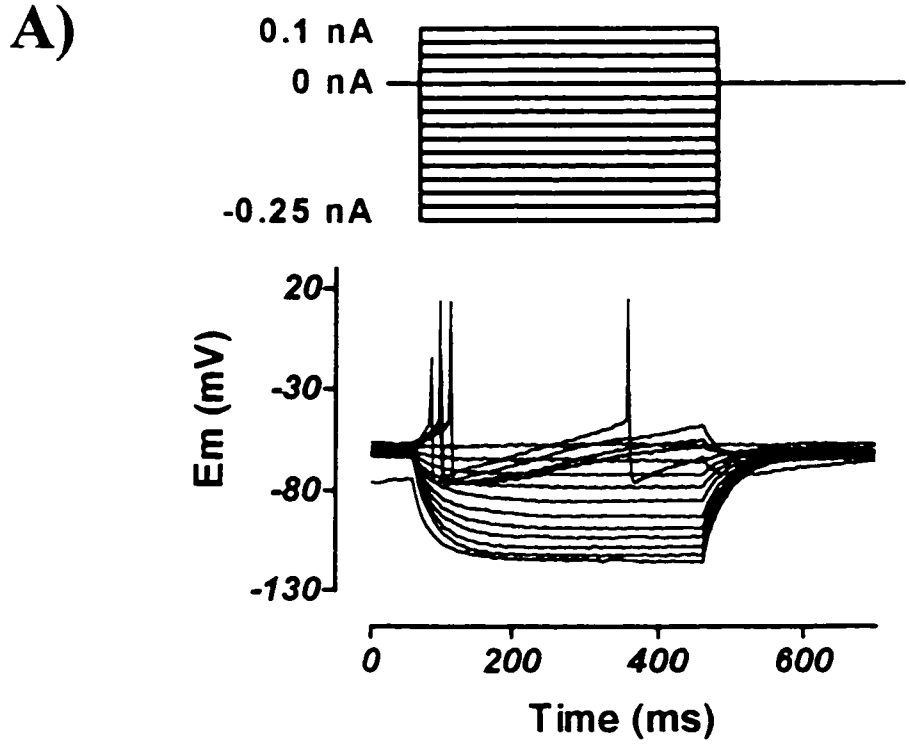


Figure 15: Properties of action potentials recorded from mouse LC neurons. Action potential recorded at different sweep speeds. At fast-speed sweeps, (top waveforms), the action potential (spontaneous) displays kinetics similar to those described in rats, i.e., a fast rising depolarization phase and a shoulder on the falling phase. Lower speed sweep waveforms display the pronounced afterhyperpolarization typical of LC neurons. The bottom two sweeps also display some level of accommodation in the spike frequency (those traces result from a 0.1 nA depolarizing pulse).

eight WT mice). However, this did not prevent the spontaneous firing activity of mouse LC neurons that resulted from a slow and steady depolarization, which brought the cell membrane to the threshold.

3.2.3. Voltage-Current Relationship

Another marked difference between rat and mouse LC is the input resistance of the mouse neurons. Figure 16 contains a representative example of a V-I relationship measured from a WT mouse LC. As for rats, mouse LC responded to transient current injections with a proportional deflection in Em. When the change in Em was plotted against the amplitude of current injected (V-I relationship), a linear relationship was observed (Ohm's law). Some rectification was also seen at hyperpolarized potentials (> 100 mV), causing a deviation from linearity in the current-voltage relationship. Input resistance of the cell was calculated from the slope (slope resistance) of the linear portion of the V-I relationship. WT mouse LC neurons recorded from horizontal slices had input resistances ranging from 75 MΩ to 345 MΩ (mean ± SEM: 193 ± 20, n=17). Using a single current pulse (-300 pA was arbitrarily chosen) the time constant (τ) of the cell membrane was measured. As for rats, in most of the mouse cells used for measurements (9 out of 10), the time course of the potential change was best fitted with a second-order exponential as opposed to a first-order exponential. The value for $\tau 2$, ranged from 1.3 ms to 17.8 ms (mean ± SEM: 8.1 ms ± 1.6 ms, n=9) and while $\tau 1$ ranged from 10.3 ms to 41.4 ms (mean ± SEM: 25.74 ms ± 3.0 ms, n=9).



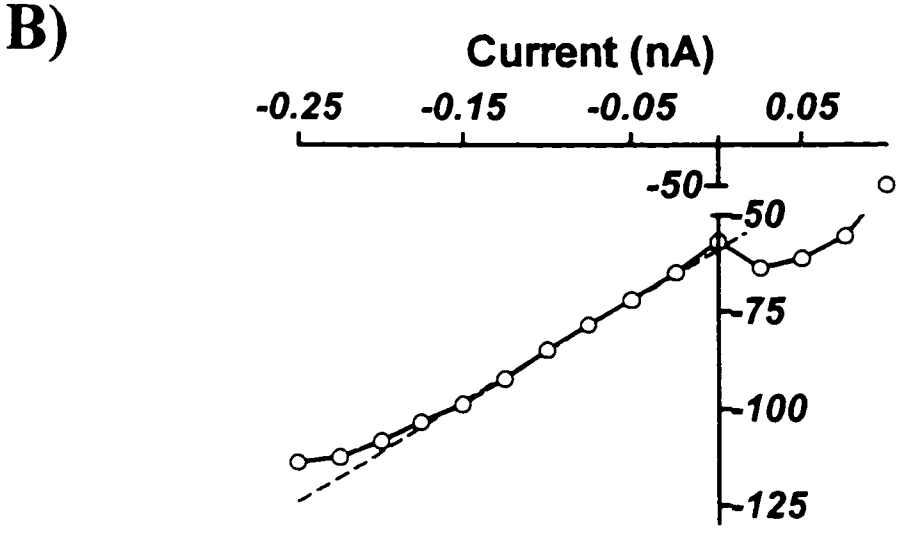


Figure 16: Apparent membrane input resistance in a mouse LC neuron. A) A representative example of the membrane potential change (Em or V) of a mouse LC neuron in response to 400 ms current injections (I). B) The V-I plot of the graph (top) showing a linear relationship between V and I over a discrete range.

3.2.4. Basic properties of single EPSPs

LC EPSPs in mouse horizontal slices were evoked as described for rats. Single electrical stimuli of 100–400 μs in duration were delivered through the bipolar stimulating electrode, positioned within 1mm rostrolateral to the recording electrode. Mouse LC neurons were also transiently hyperpolarized to about -75 mV (-0.2 to -0.7 nA was given for 400 ms). EPSPs were evoked midway through the hyperpolarization pulse. EPSP amplitudes in WT mice LC ranged from 4.5 mV to 16.2 mV (9.4 ± 0.8 mV, n=14). As for rats, amplitude was mainly determined by the strength of the stimulus applied to the stimulating electrode (fig. 17A), and limited at higher amplitude by threshold for dendritic and/or full action potentials. Mouse LC EPSPs were almost entirely inhibited by CNQX (25 μM, n=2) (fig. 17B) indicating that most of the voltage change measured during EPSPs in mouse LC neurons is mediated through the activation of non-NMDA Glu receptor subtypes.

3.3. mGluRs AND SYNAPTIC TRANSMISSION TO RAT LC

3.3.1. Action of mGluR agonists on EPSPs

3.3.1.1. *t*-ACPD

To test the possibility that activation of mGluRs may modulate glutamatergic transmission in LC, the effects of bath-applied mGluR agonists on EPSP amplitudes were investigated. Other parameters such as LC membrane potential, input resistance, firing frequency, and intrinsic oscillation, were also examined. The first agonist tested, *t*-ACPD, was chosen for its specificity to both group I and II mGluRs and its relative lack of effect for

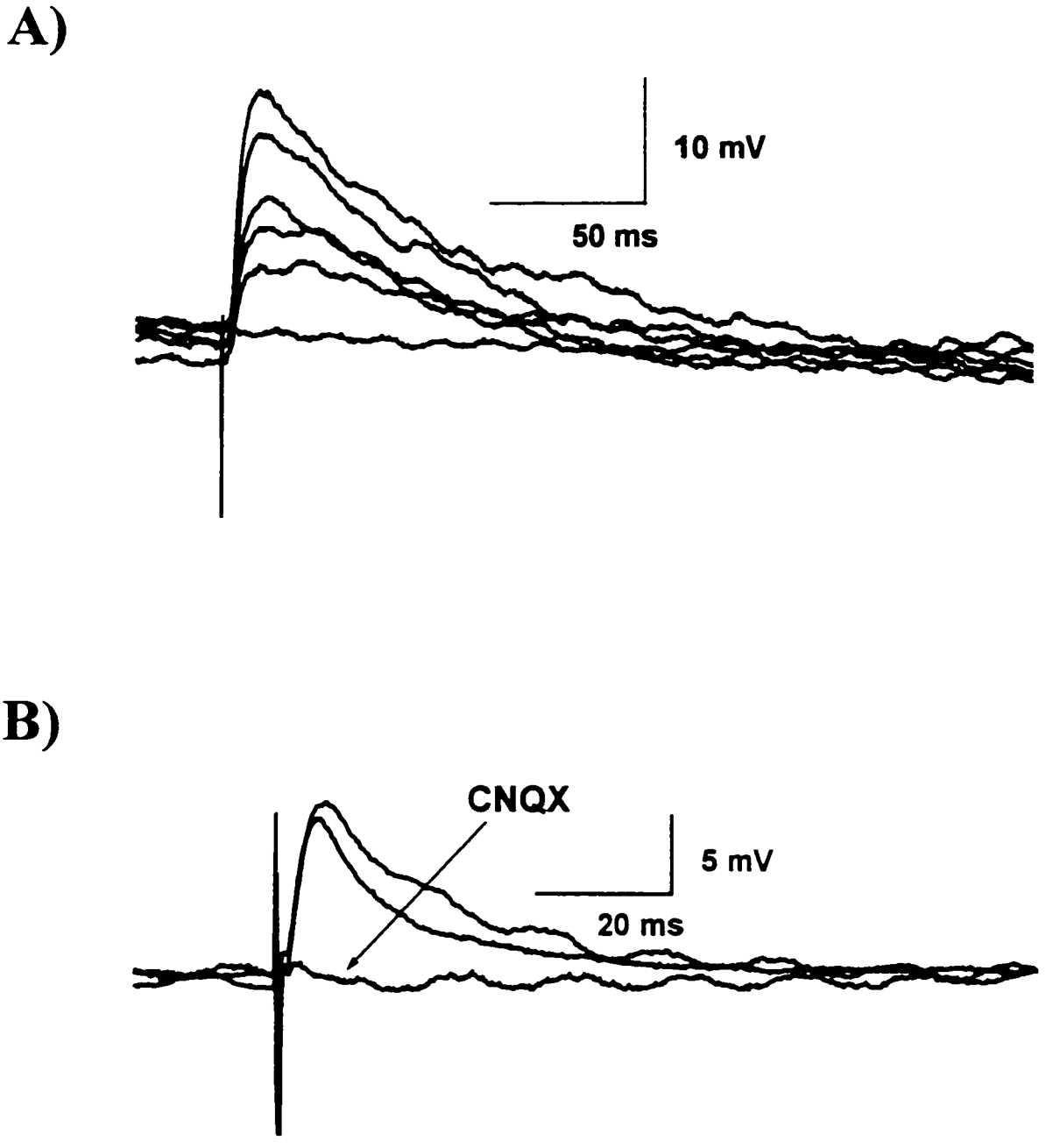


Figure 17: Evoked EPSPs in a mouse LC neuron. A) Variation in EPSP amplitudes with respect to the strength of the stimulus delivered to the slice (0V-10V, by increment of two V). The rapid downward deflection represents the stimulus artifact. B) A representative example of the CNQX (20 μM) inhibition of EPSPs (compare to basal and wash).

group III mGluRs. Bath application of *t*-ACPD resulted in a reproducible inhibition of EPSP amplitudes (fig. 18 and 19). Figure 18A compares the EPSP waveforms (average of eight consecutive EPSPs) recorded under control conditions, after 15 min of perfusion with *t*-ACPD (30 μM), and 15 min after removal of *t*-ACPD (Wash). The latency of onset of EPSPs was not affected by *t*-ACPD (fig. 18B) and EPSP amplitude recovered fully from the inhibition following the removal of the agonist. Figure 19A displays the average time-course of action of *t*-ACPD (n=8). The time required for the agonist to produce its maximal effect was approximately 10 min, as was the time required for full recovery following withdrawal of the agonist. The effect of *t*-ACPD was dose-dependent, causing an extrapolated maximal inhibition of 66.1% ± 4.5% at a concentration of 500 μM. This dose-response relationship could be best fitted with a single binding site model sigmoidal curve (i.e., Hill coefficient of 1) ($R=0.89$, $\upsilon=61$) and the estimated EC_{50} was 11.5 μM ± 1.4 (fig. 19B).

In most tests, *t*-ACPD failed to change significantly the LC input resistance (fig. 18C, table 2), membrane potential (table 2) or firing rates. When changes did occur, either a small depolarization or hyperpolarization was observed and no trends were apparent. One exception to this was during application of higher concentrations of *t*-ACPD (500 μM) where in three of the four cells tested, a transient depolarization was observed, accompanied with an increase in firing rates. However, this only lasted a short time (about five min following the beginning of the drug application) and membrane potential and the firing rate had returned to the baseline after ten min. In such cases, measurements of EPSP amplitudes were taken

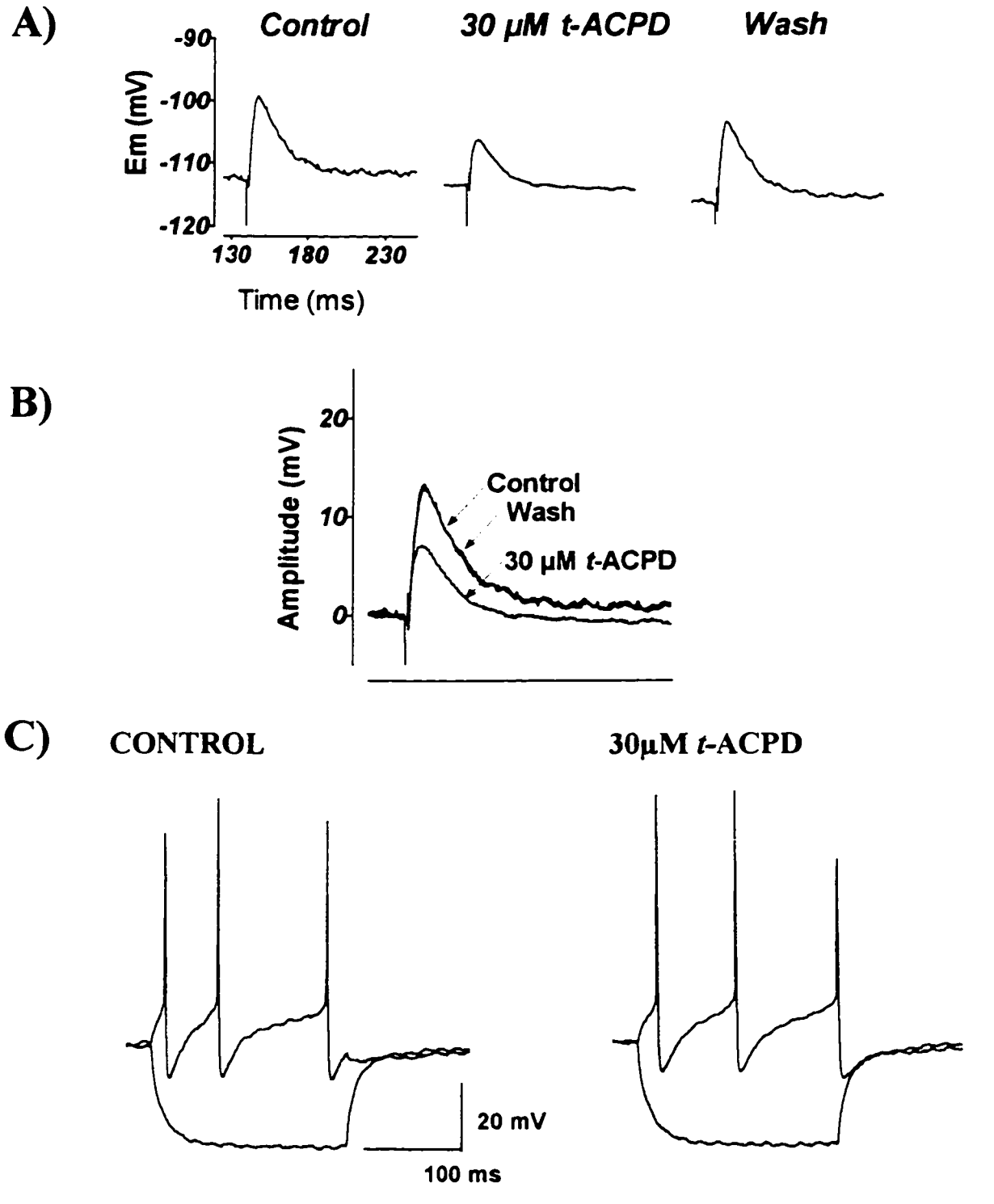


Figure 18: Inhibition of EPSPs in rat LC neurons by *t*-ACPD. A) Average of eight consecutive EPSPs evoked before, during and after perfusion with *t*-ACPD. B) Same as A) but superimposed to compare time courses. C) Representative examples of electrotonic potential before and during perfusion with 30 µM *t*-ACPD.

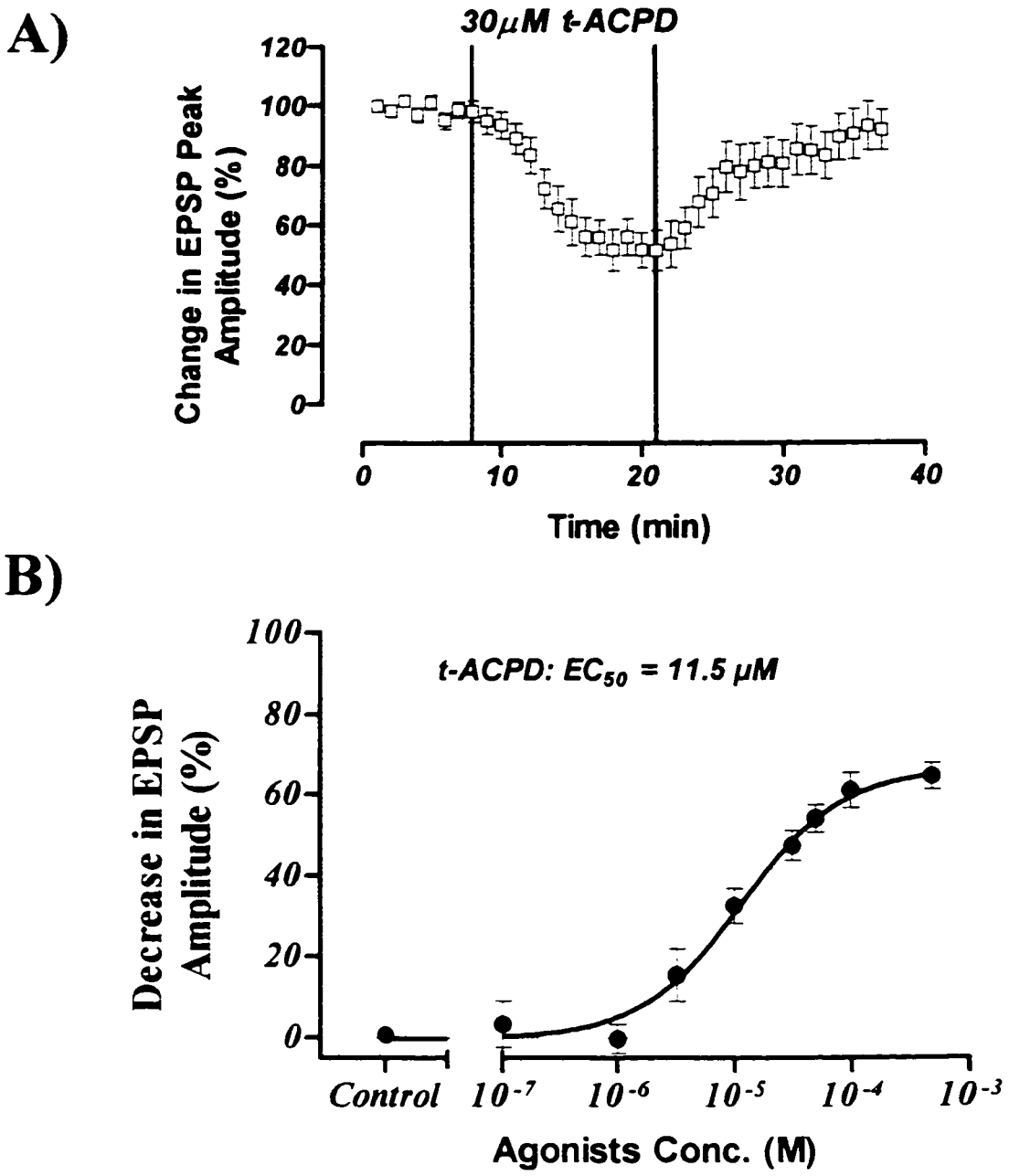


Figure 19: Time course and dose-response profile of the effects of *t*-ACPD on amplitude of EPSPs in rat LC neurons. A) Averaged (± SEM) time course for inhibition of EPSPs by *t*-ACPD (n=8 slices, only one cell per slice). The period of drug application is indicated on the graph by vertical lines. B) Dose-response relationship for the inhibition of EPSPs by *t*-ACPD. The estimated EC50 value (as extrapolated by computer with a single binding site, i.e., Hill slope=1) for the inhibitory effect was 11.5 μM (R=0.89). The control point represents the average of seven experiments where no drugs were added for the first 45 min, a period that represents the time necessary to record a baseline, test, and washout periods. Each point represents an average of 3-12 independent experiments ± SEM.

only after 10-15 min following the beginning of the application, when cell excitability was back to the baseline.

3.3.1.2. L-AP4

The second agonist tested, L-AP4, has been characterized as a selective agonist of group III, with no reported effect on group I or II mGluRs. As for *t*-ACPD, bath application of L-AP4 resulted in a reproducible inhibition of EPSP amplitude (fig. 20 and 21). Figure 20A compares the EPSP waveforms (average of eight consecutive EPSPs) recorded under control conditions, after 15 min of perfusion with L-AP4 (3 μM), and 15 min after removal of L-AP4 (wash). The latency of onset of EPSPs was not affected by L-AP4 (fig. 20B) and EPSP amplitude recovered fully from the inhibition following the removal of the agonist. Figure 21A displays the average time-course of action of L-AP4 (n=4). The time required for the agonist to produce its maximal effect and for washout of the effect was approximately 10 min. The effect of L-AP4 was dose-dependent, causing a maximal inhibition of 69.4% ± 2.6% at a concentration of 500 μM. This dose-response relationship could be best fitted with a single binding site model sigmoidal curve (i.e., Hill coefficient of 1) ($R=0.89$, $\upsilon=72$) and the estimated EC_{50} was 2.6 μM ± 1.2 (fig. 21B). In most tests, L-AP4 failed to change the LC input resistance significantly (fig. 20C, table 2), membrane potential (table 2) or firing rates. When changes did occur, either a small depolarization or hyperpolarization was observed and no trends were apparent.

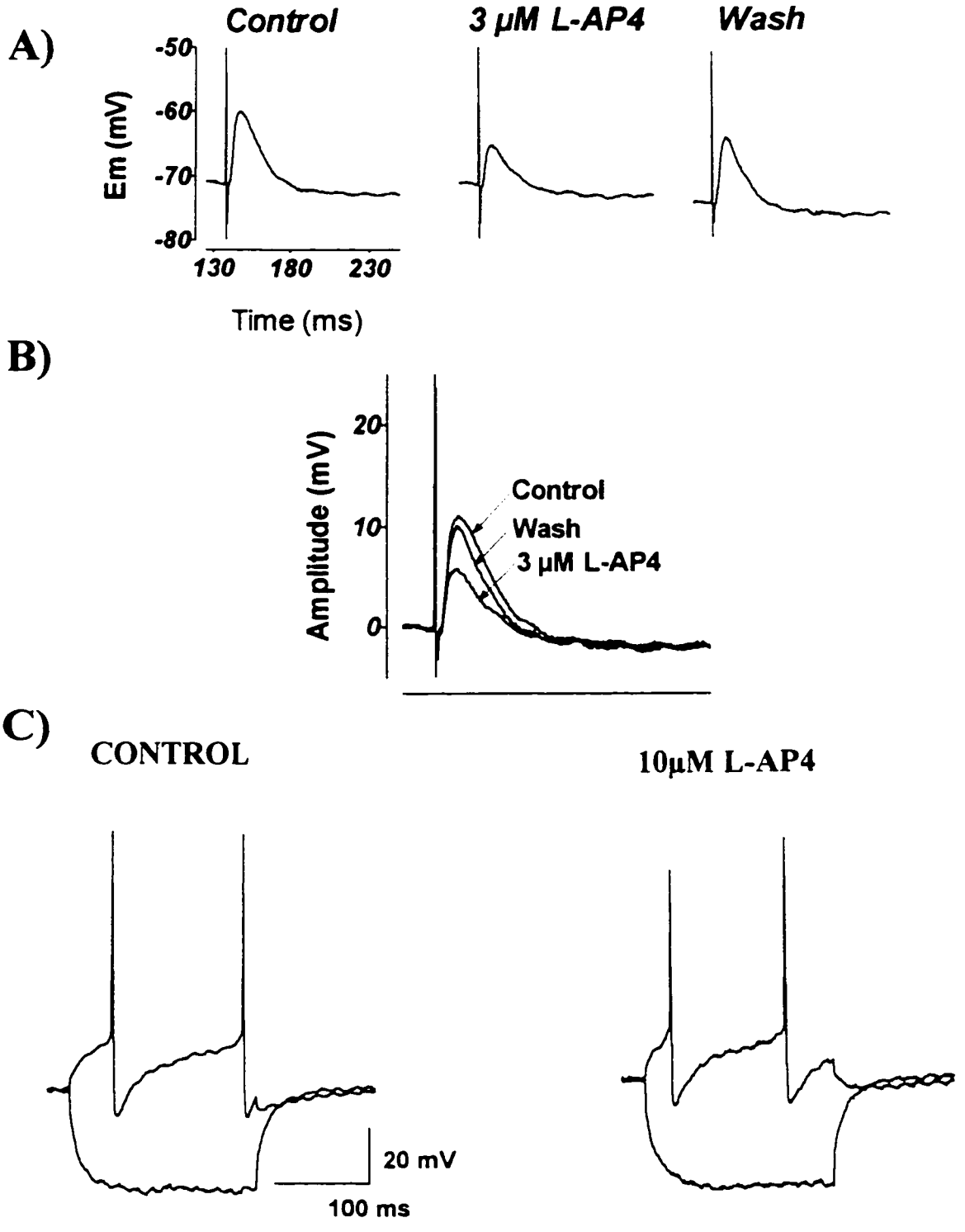


Figure 20: Inhibition of EPSPs in rat LC neurons by L-AP4. A) Average of eight consecutive EPSPs evoked before, during and after perfusion with L-AP4. B) Same as A) but superimposed to compare time courses. C) Representative examples of electrotonic potential before and during perfusion with 10 µM L-AP4.

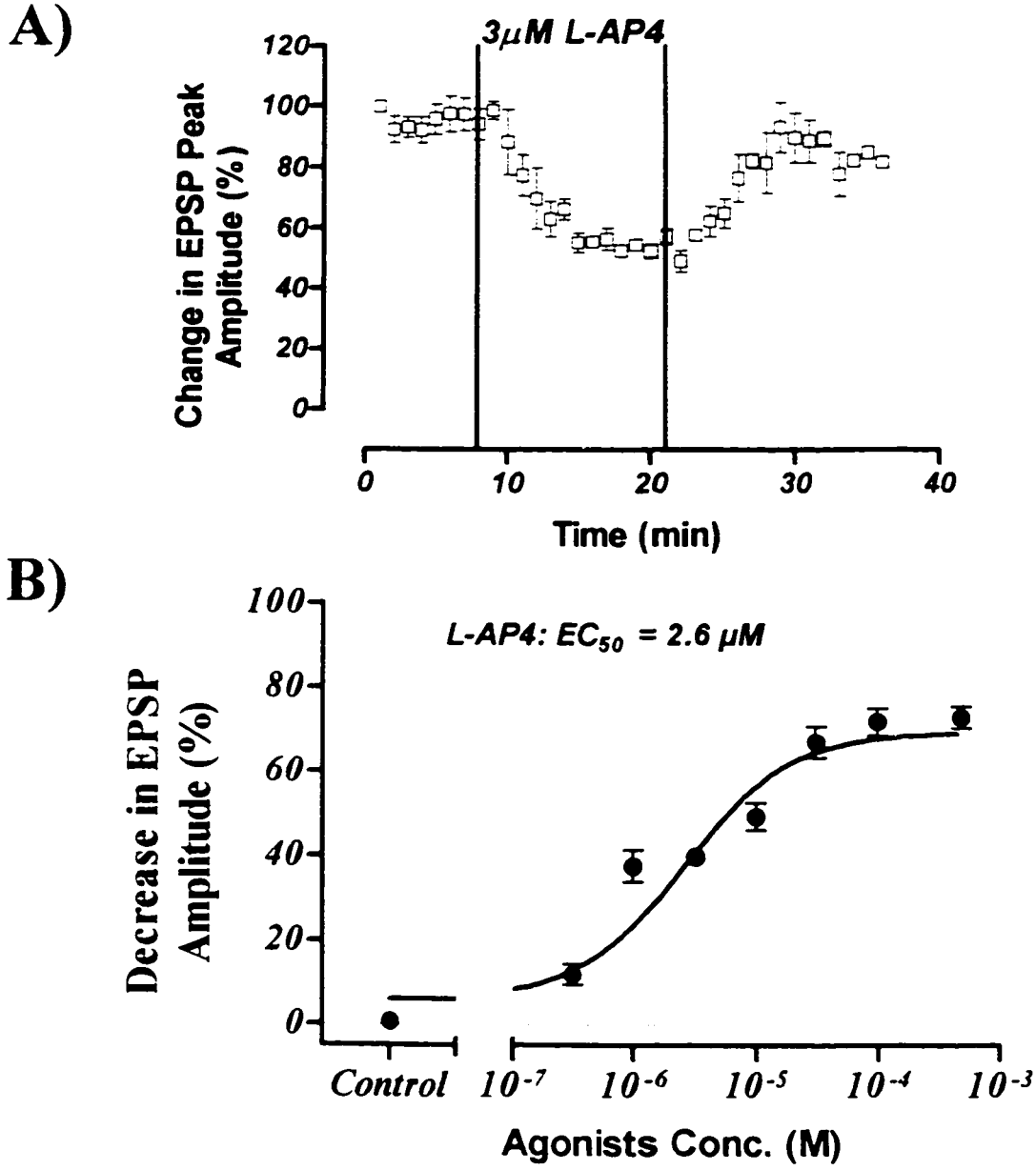


Figure 21: Time course and dose-response profile of the effects of L-AP4 on amplitude of EPSPs in rat LC neurons. A) Averaged (± SEM) time course for inhibition of EPSPs by L-AP4 (n=4 slices, only one cell per slice). The period of drug application is indicated on the graph by vertical lines. B) Dose-response relationship for the inhibition of EPSPs by L-AP4. The estimated EC50 value (as extrapolated by computer with a single binding site, i.e., Hill slope=1) for the inhibitory effect was 2.6 μM (R=0.89). The control point represents the average of seven experiments where no drugs were added for the first 45 min, a period that represents the time necessary to record a baseline, test, and washout periods. Each point represents an average of 3-25 independent experiments ± SEM.

Table 2: Lack of effect of mGluR agonists on membrane potential and input resistance in LC neurons

Agonist	Membrane potential (mV)	Input Resistance* (MΩ)
Control**	-63.0 ± 2.7	82.5 ± 7.9
500 μM *t*ACPD	-63.0 ±3.4	82.2 ± 8.2
Control**	-64.8 ± 3.0	71.2 ± 7.6
500 μM L-AP4	-66.7 ± 4.9	68.8 ± 5.9

* Mean steady-state chord resistance measured after a -600 pA hyperpolarizing pulse

** n=4 slices, no significant differences between control and agonist

3.3.2. Effects of co-application of *t*-ACPD and L-AP4

Recent reports have suggested that, in some systems, activation of different groups of mGluRs could produce similar effects on synaptic transmission to the same cell (Baskys and Malenka, 1991; Burke and Hablitz, 1994; Bushell et al., 1995; Gereau and Conn, 1995b; Salt and Eaton, 1995). Therefore, in an attempt to establish whether in my system both mGluR agonists are acting at the same receptor or signaling pathway, *t*-ACPD and L-AP4 were applied together to slices. To determine whether concomitant application of mGlur agonists produced additive effects, I used the value of "% of control" values for an agonist in the following relationship: $\text{Inhibition}_1 \times \text{Inhibition}_2 = \text{additive}$.

3.3.2.1. Additivity at submaximal concentrations

In a first set of experiments, *t*-ACPD (30 μM) and L-AP4 (10 μM) were applied together to slices. These concentrations correspond approximately to the EC_{70} and EC_{80} (with respect to the dose-response curve) for *t*ACPD and L-AP4 respectively. When compared to the effects of either agonist alone, the combination of both *t*-ACPD and L-AP4 produced a significantly greater depression in EPSP amplitudes (fig. 22). Figure 22A shows the time course of the effect of the agonists alone, or in combination, on four LC neurons. Results from pooled data from each test period (fig. 22B) indicate that the combination of both agonists reduced EPSP amplitude to an average of 38.5% ± 1.3% of the pretest control (mean ± SEM, n=4). This inhibition was significantly greater than that observed with *t*-ACPD alone ($p<0.01$), or L-AP4 ($p<0.01$). This suggests that the effects of both agonists are fully additive at these doses (i.e., $Inhbition_{L\text{-}AP4} x Inhibition_{t\text{-}ACPD}$ ➜ 59.5% 63.6% = 37.8%).

3.3.2.2. Partial additivity at high doses

In this set of experiments, concentrations of agonists that produced 90% (EC_{90}) inhibitions were used, to avoid some nonspecific effects observed with maximal doses. When tested, the combination of both *t*-ACPD (104 μM) and L-AP4 (21 μM) produced a greater depression of the EPSPs when compared to the effect of either agonist alone (fig. 20). Results from pooled data from each test period indicate that the combination of both agonists reduced evoked EPSP amplitudes to an average of 24.2% ± 4.2% of the pretest control (n=4). This value was significantly lower than that observed with *t*-ACPD alone in the same

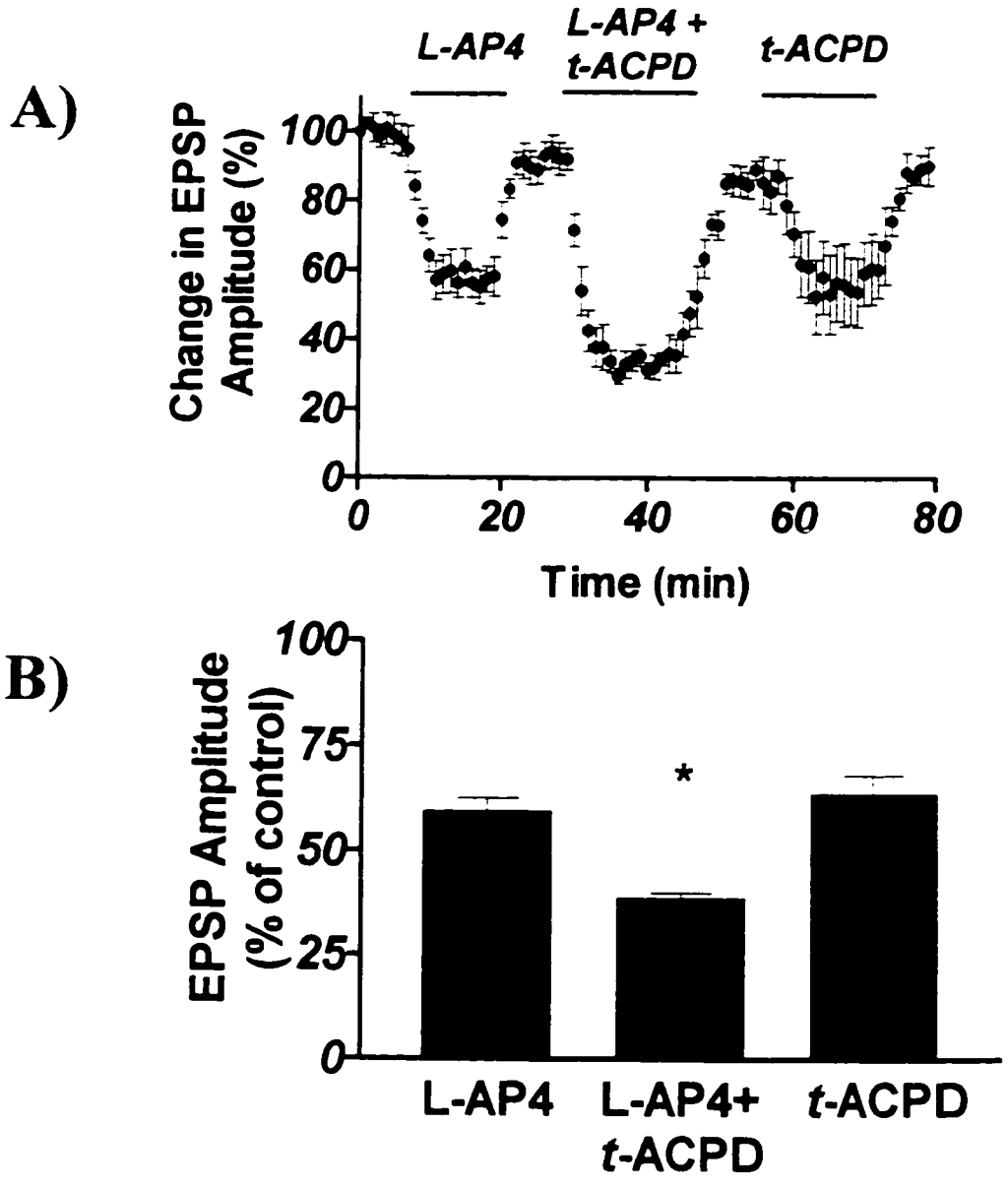


Figure 22: Additive inhibitory effects of submaximal concentrations of L-AP4 and *t*-ACPD on EPSP amplitude in LC neurons. In four separate experiments, the effects of 10 μM L-AP4 and 30μM *t*-ACPD were tested individually and in combination on EPSP amplitude. (A) shows the time-course of agonist effects, measured every minute. (B) represents the same four experiments showing pooled data from periods when the responses reached steady levels. Values are the percentage of the mean pooled value of EPSP amplitude measured during the test period divided by the average of the mean pooled EPSP amplitude measured during the control and washout period. In both graphs, values represent mean ± SEM (n=4). The asterisk indicates statistical difference as assessed using paired t-test analysis: L-AP4 vs L-AP4+*t*-ACPD: p=0.006, *t*-ACPD vs L-AP4+*t*-ACPD: p=0.006, L-AP4 vs *t*-ACPD: p=0.8.

experiments (41.8% ± 6.7%, n=4, $p<0.01$) or L-AP4 alone (35.0% ± 4.7%, n=4, $p<0.01$), but not significantly different from the inhibition observed with 500 μM L-AP4 alone (the averaged maximal inhibition measured with one only agonist; tested by unpaired t-test). Furthermore, the total inhibition observed with both agonists is less than the inhibition expected if both agonists had produced a fully additive response (i.e., $Inhbition_{L\text{-}AP4} x Inhibition_{t\text{-}ACPD}$ → 35.0% * 41.8% = 14.6% = full additivity. This is smaller than the experimental value).

3.3.3. Pharmacological characterization of the mGluR subtype(s)

Since *t*-ACPD has mixed agonist effects (Group I and II), additional tests were required to determine which group(s) of mGluR mediated the effects of this agonist. Therefore, additional, more specific agonists and several selective antagonists were tested. Conversely, L-AP4 is one of the most specific group III mGluR agonists, with very little actions on group I and II mGluRs at doses used in this work. However, to confirm this, antagonism of the effect of L-AP4 with specific antagonists was also investigated. For details on the agonists and antagonists used below please refer to section 1.2.4.2. of the Introduction.

3.3.3.1 Specific agonists

To assess whether specific activation of group I mGluRs could mimic some or all of the effects observed with *t*-ACPD described above, (RS)-DHPG, a highly selective agonist for this group, was tested for its effects on EPSPs. However, DHPG failed to depress evoked

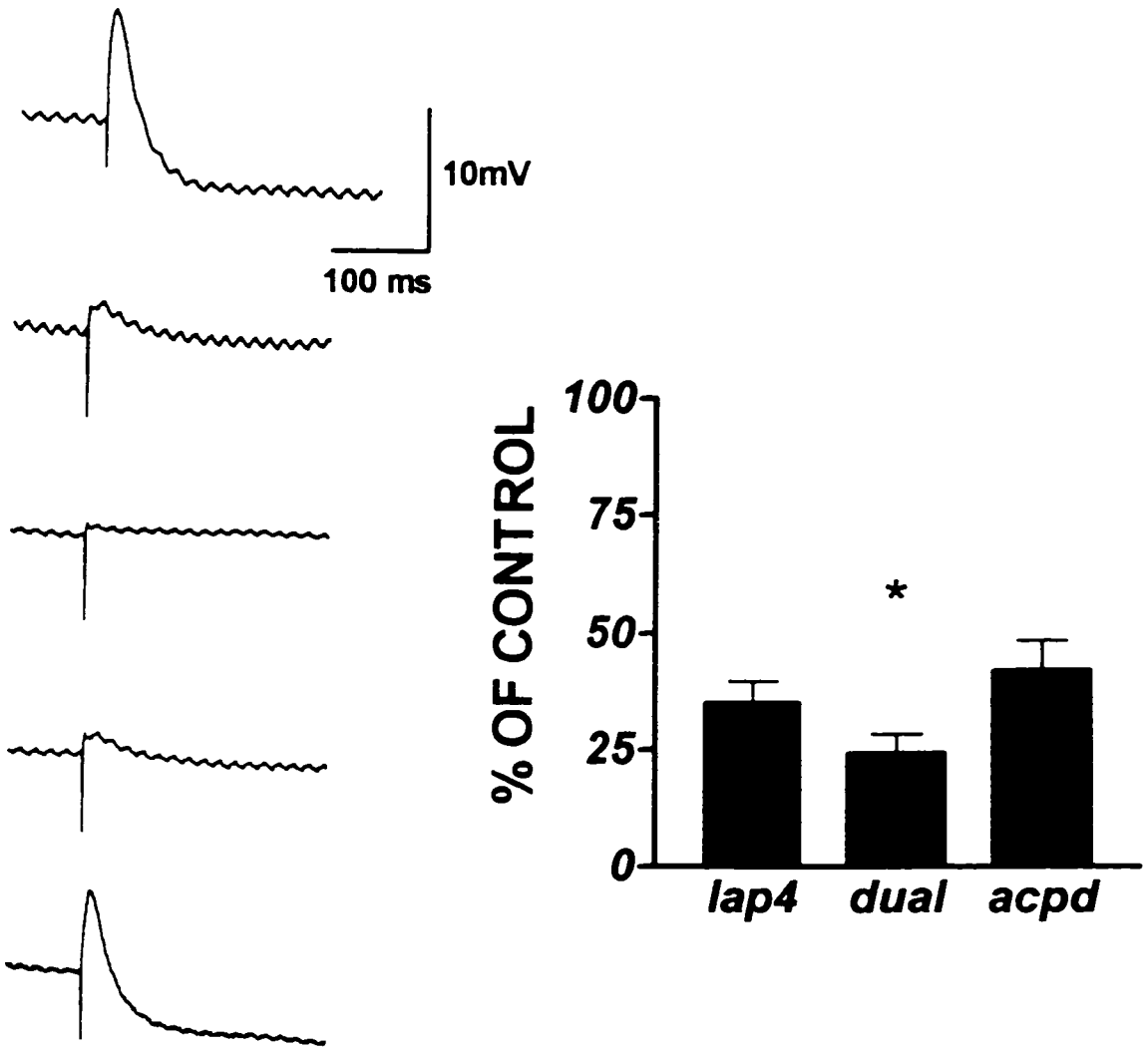


Figure 23: Partially additive inhibitory effects of large concentrations of L-AP4 and *t*-ACPD on EPSP amplitude in LC neurons. A) Representative example of additivity recorded in the same cell and depicting the effect of 104 μM *t*-ACPD plus 21 μM L-AP4 applied individually or concomitantly. Individual concentrations were chosen to produce 90% of the maximal inhibition (EC90) with respect to the respective dose-response profiles (fig. 2). Each waveform is an average of three trials performed one minute apart, each trial consisting of eight consecutive sweeps. In this example, the mean depression (% of control) for *t*-ACPD, *t*-ACPD plus L-AP4, and L-AP4 was 23.2%, 12.37%, and 23.6% respectively. Each test period was preceded by a control period. B) Average pooled data from periods when the responses reached steady levels (n=4). Values are the percentage of the mean pooled value of EPSP amplitude measured during the test period divided by the average of the mean pooled EPSP amplitude measured during the control and washout period. In both graphs, values represent mean ± SEM (n=4). The asterisk indicates statistical difference as assessed using paired t-test analysis: L-AP4 vs L-AP4+*t*-ACPD: p=0.006, *t*-ACPD vs L-AP4+*t*-ACPD: p=0.02.

EPSPs in my preparation when tested at concentrations of up to 100 μM,(fig. 24). The second compound tested, 4C, 3H-PG, was chosen based on its agonist activity for group II mGluR and is also a group I antagonist. Bath application of 4C, 3H-PG (100 μM and 500 μM) resulted in a significant decrease in EPSP amplitude (fig. 24). Finally, additional support for group II mGluR in EPSP inhibition was obtained with the used of the new and selective group II agonist, 2R-4R-APDC, the actions of which mimicked the actions of *t*-ACPD (fig. 24). Results of all agonists tested in this study are summarized in table 3.

3.3.3.2. Specific antagonists

The pharmacological identities of the receptors activated by *t*-ACPD and L-AP4 were further investigated using different mGluR antagonists. A summary of these results is contained in tables 4 and 5. The first antagonist, (+) MCPG (500 μM), was tested on the effect of *t*-ACPD (10 μM) or L-AP4 (3 μM). This antagonist was chosen based on its action on group I and II mGluRs and its relative lack of antagonist activity for mGluR4 and 7 which belong to the group III (L-AP4-sensitive) mGluRs. MCPG blocked the effect of *t*-ACPD on evoked EPSPs, but was ineffective in blocking the effects of L-AP4. Several more specific antagonists were also tested. Of these, MCCG and MAP4 have been commonly used to distinguish between the actions of *t*-ACPD and L-AP4 respectively. However, MCCG (500μM) did not block the effects of *t*-ACPD-(30 μM) on EPSPs, in my preparation, nor did MAP4 (500μM). Another selective mGluR antagonists, MSOPPE (500 μM), a potent group II antagonist, and AIDA (500 μM), a potent and selective group I mGluR antagonist, also failed to antagonize significantly the actions t-ACPD. Finally, a recently developed group II-

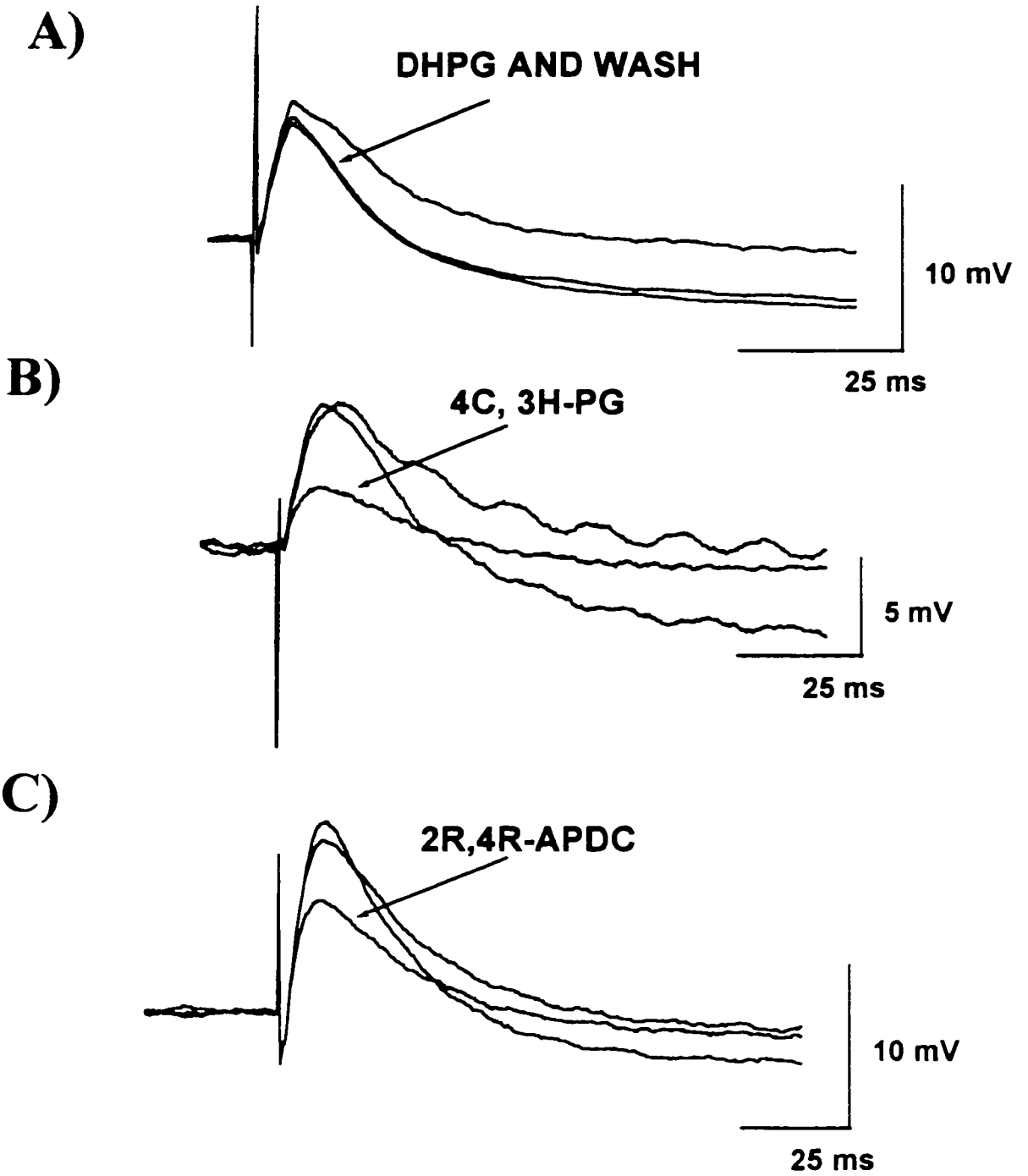


Figure 24: Effect of other selective mGluR agonists on EPSP in rat LC neurons. Representative traces showing the effect of A) the selective group I mGluR agonists DHPG (100 μM), B) the group I antagonists/group II agonist 4C, 3H-PG (500 μM), and C) the groupII selective agonist 2R, 4R-APDC (50μM) on EPSP amplitudes. Recordings were made on three different cells.

specific antagonist, EGLU, (500 μM), significantly inhibited the effects of t-ACPD (30 μM). These data are summarized in tables 4 and 5

Table 3: Pharmacological profile of inhibition of excitatory postsynaptic potentials by metabotropic glutamate receptor agonists

Agonists		Mean depression (% of control)	n	
Vehicle		95.25 ± 5.4	7	
Group I/II	30 μM *t*-ACPD	52.5 ± 3.7	19	p<0.001*
Group I	100 μM DHPG	92.6 ± 4.1	7	n.s.**
Group II	500 μM 4C, 3H-PG	49.8 ± 8.6	3	P<0.02*
	50 μM APDC	56.4 ± 4.9	5	p<0.002*
Group III	3 μM L-AP4	60.1 ± 1.7	27	p<0.001*

*** significantly different from vehicle**

Table 4: Antagonist profile of inhibition of EPSPs by *t*-ACPD

Agonist/Antagonist combination		Mean depression (% of control)	n	
Group I/II	30 μM *t*-ACPD	63.9 ± 4.35		
	+500 μM (RS)-MCPG	97.5 ± 3.54	3	p<0.005*
Group I	30 μM *t*-ACPD	57.7 ± 6.2		
	+500 μM AIDA	65.0 ± 3.2	3	n.s.
Group II	30 μM *t*-ACPD	62.0 ± 7.0		
	+500 μM MCCG	64.5 ± 6.0	4	n.s.
	30 μM *t*-ACPD	46.4 ± 6.6		
	+500 μM MSOPPE	44.3 ± 7.0	6	n.s.
	30 μM *t*-ACPD	39.0 ± 6.7		
	+500 μM EGLU	65.3 ± 2.5	4	P<0.02*
Group III	30 μM *t*-ACPD	45.8 ± 5.2		
	+500 μM MAP4	59.2 ± 8.9	3	n.s.

The L-AP4 antagonist profile was also investigated. Figure 25 displays the effect of 500 μM MAP4 on the L-AP4 (3 μM)-induced depression of evoked EPSPs. This L-AP4-mediated inhibition was blocked when MAP4 was included in the perfusate. Results from pooled data for each period showed a highly significant difference between the agonist alone and with the antagonist (table 5). MCCG (500 μM) caused no significant change of L-AP4-induced EPSP depression (table 5). In Summary, only MCPG and EGLU were found to antagonize *t*-ACPD inhibition of excitatory synaptic transmission to LC, and MAP4 antagonized L-AP4.

Table 5: Antagonist profile of inhibition of EPSPs by L-AP4

Agonist/Antagonist combination		Mean depression (% of control)	n	
Group I/II	3 μM L-AP4	70.2 ±1.8		
	+500 μM (RS)-MCPG	71.0 ± 5.1	6	n.s.
Group II	3 μM L-AP4	67.8 ± 4.5		
	+500 μM MCCG	72.0 ± 3.1	4	n.s.
Group III	3 μM L-AP4	67.8 ± 4.5		
	+500 μM MAP4	91.2 ± 3.9	6	$p<0.005$*

*** significantly different from conditions without antagonist**

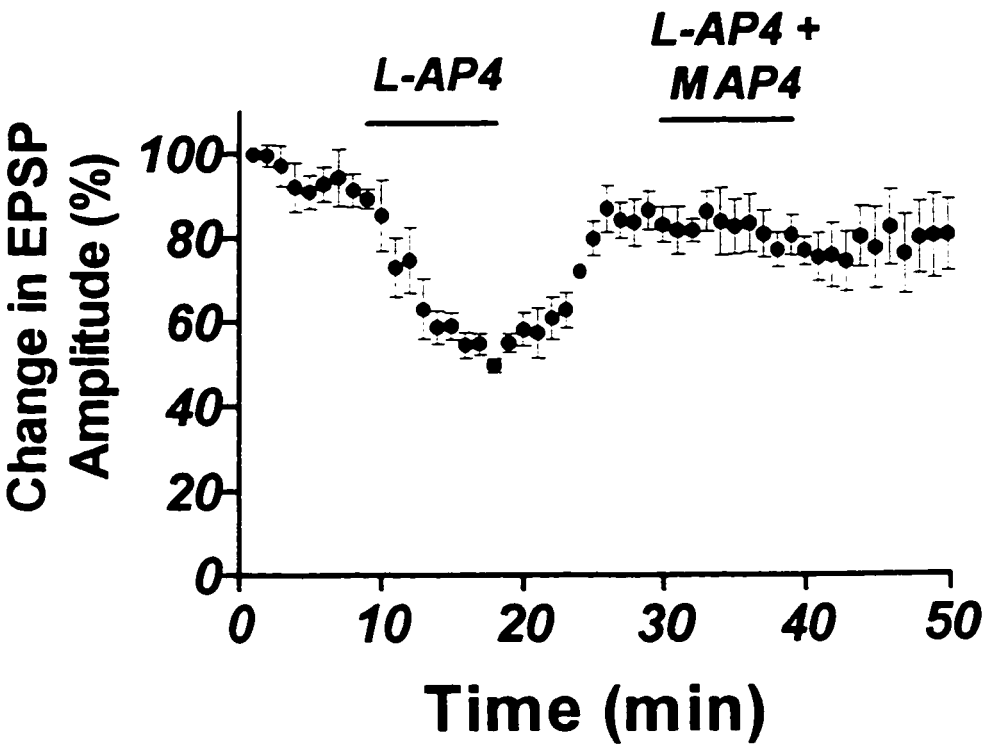


Figure 25: Effect of the group III GluR antagonist MAP4 on L-AP4 inhibition of EPSPs in LC neurons. The antagonist MAP4 (500 μM) was introduced in the superfusate concomitantly with 3 μM L-AP4 in an identical fashion to experiments testing the agonist alone. The graph depicts the time-course of the effects of L-AP4 alone or in combination with MAP4 showing that MAP4 antagonises the effects of L-AP4 (mean ± SEM, n=4 slices). Drug applications are indicated by horizontal bars.

3.3.4. Loci of action of mGluR agonists

The results presented above clearly demonstrate that *t*-ACPD and L-AP4 inhibit EPSPs in LC nucleus. However, other than their lack of effect on postsynaptic markers such as Em or input resistance, the locus/loci of action of these agonists, i.e., pre- or postsynaptic is not known.

3.3.4.1. Effects on focally applied Glu

In a first set of experiments, the effects of both agonists were tested on the response of LC neurons to sub-maximal doses of exogenously applied Glu. Figure 26 presents a representative example of such an experiment. No significant changes in the LC response to exogenous Glu were observed, whether agonists were applied alone or in combination. Out of 15 cells tested for an effect on focally applied Glu, 14 cells showed little or no change, despite the magnitude of the depolarization produced by the Glu, while one cell showed a small and reversible decrease in responsiveness (20%) with 20 μM *t*-ACPD.

3.3.4.2. Effects on paired-pulse facilitation

Another test often used to differentiate between presynaptic and postsynaptic inhibitory effects of a specific drug or transmitter is to measure changes in paired-pulse facilitation (Harris and Cotman, 1983; Stuart and Redman, 1991; Burke and Hablitz, 1994). It is generally accepted that the ratio of amplitude of two EPSPs, consecutively evoked with a small interval, is increased during presynaptic inhibition, as compared to control conditions (increase in paired-pulse facilitation). Therefore, I used this test in an attempt to delineate the

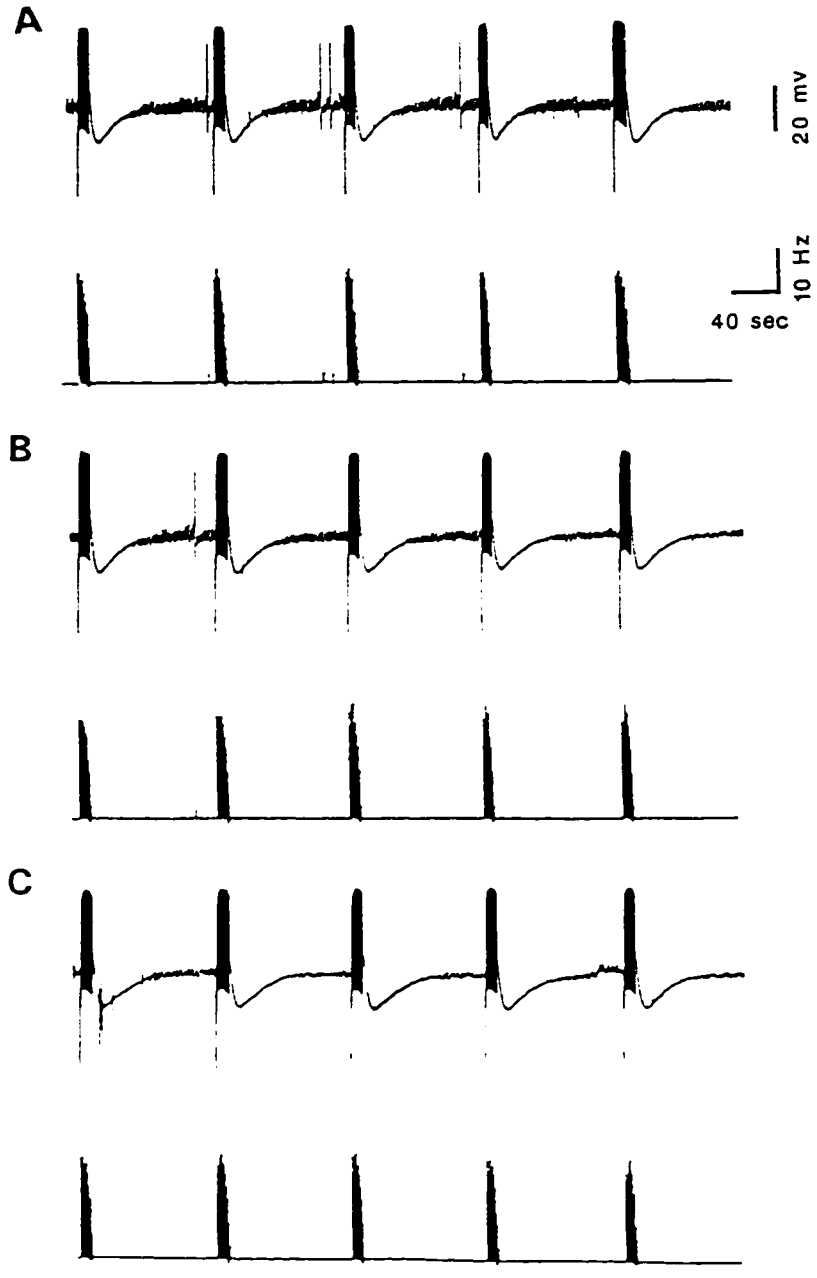


Figure 26: Lack of effect of both *t*-ACPD and L-AP4 on the response of LC neurons to focally applied exogenous Glu. Representative on-line recording of an intracellularly recorded LC neuron responding to periodic focal applications of glutamate before (A), during (B), and after (C), perfusion with 10 μM *t*-ACPD and 3 μM L-AP4 applied together. Upper trace in each section shows the intracellular potential (Em 55mV); lower trace is ratemeter output indicating action potential frequency. The extent of depression in EPSPs caused by the agonists combination was close to 70 % in this particular experiment.

site of action of *t*-ACPD and L-AP4. Figure 27 shows the effect of L-AP4 on facilitation observed during the paired-pulse protocol as compared to that observed without any drug. Both L-AP4 (3 μM) and *t*-ACPD (30 μM) were found to produce significantly greater facilitations than control. The average inhibition produced by L-AP4 and *t*-ACPD was 59.6% ±2.0% and 58.3 ± 5.0, respectively. The percent increase of facilitation over control is 123% and 160% respectively for L-AP4 and *t*-ACPD. In contrast, CNQX at a dose (2.5 μM) which causes similar inhibitions to those observed with the mGluR agonists, and has a well-established postsynaptic locus of action, did not produce any significant change in paired-pulse facilitation.

3.3.4.3. Interactions between mGluR agonists and adenosine

It has been recently shown in certain systems that *t*-ACPD produced presynaptic inhibition through the stimulated release of adenosine from glial cells (Schoepp et al., 1995b). Under these conditions, the released adenosine then acts on excitatory presynaptic terminals to decrease neurotransmitter release through its action on adenosine A_1 receptors. Since such an effect could represent an indirect mechanism of mGluR inhibition of EPSPs, the role of adenosine in *t*-ACPD-dependent presynaptic inhibition was investigated in LC. Bath application of 30 μM *t*-ACPD, alone or in the presence of 0.1 μM DPCPX, a potent and selective adenosine A_1 receptor antagonist, was tested on evoked EPSPs. This concentration of DPCPX has been used successfully in many studies (e.g., Breakwell et al., 1997) and specifically on LC (Nieber et al., 1995) where it was found to block the actions of adenosine on A_1 receptors without direct effects on LC neurons by itself. Similarly in this study,

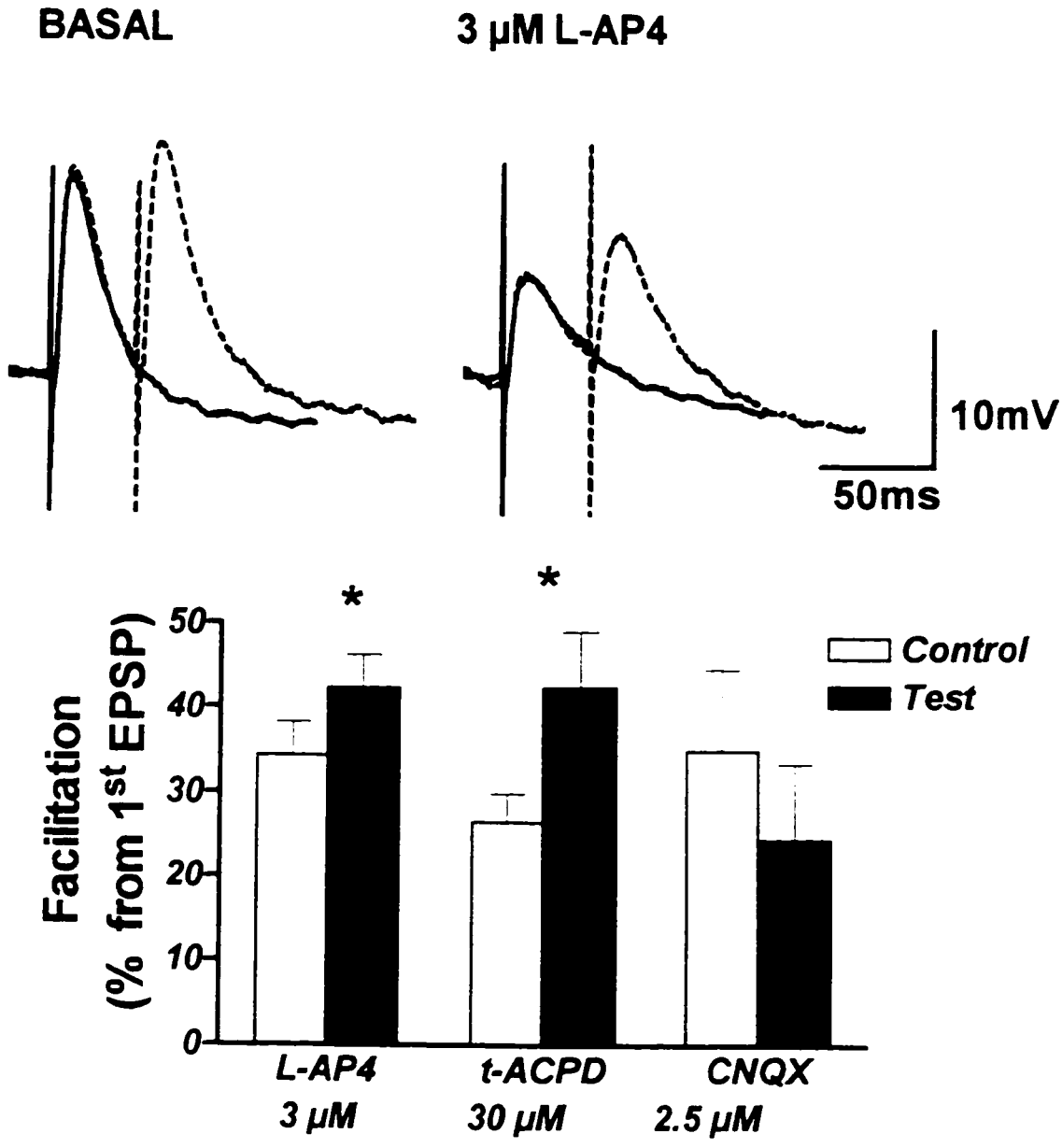


Figure 27: Paired-pulse facilitation is increased in the presence of mGluR agonists. A) A representative example of a single evoked EPSP (dark line) superimposed with a double EPSP (dotted line) resulting from a paired-pulse protocol under control condition and in the presence of 3 µM L-AP4. Each trace is an average of eight consecutive sweeps. In this example, mean potentiation [(second EPSP - first EPSP)/first EPSP x 100] under control conditions was 22% versus 53% in the presence of L-AP4 (Em=-94 mV after a -700pA pulse was applied to hyperpolarize the cell). B) Bar graph comparing facilitation between control and different treatments. Values are as follows: control vs L-AP4: 34.3% ± 3.9% vs 42.3% ± 3.9%, n=14, p=0.040, paired t test; control vs *t*-ACPD: 26.5% ± 3.3% vs 42.4% ± 6.7%, n=7, p=0.040, paired t test; control vs CNQX: 34.9% ± 9.7% vs 24.4% ± 9.0%, n=6, n.s.

DPCPX alone did not affect evoked EPSPs [compared to control: 97.9% ± 7.8%, n=3]. Furthermore, when tested on the same neurons, no significant interaction between the effect of *t*-ACPD and DPCPX was found [change from control (%): 30 μM *t*-ACPD: 72.8% ± 9.4%, 30 μM *t*-ACPD + 0.1 μM DPCPX: 66.4% ± 11.8% (n=3)]. Hence *t*-ACPD actions in LC are not mediated through the active release of adenosine as suggested in other systems.

3.3.5. Actions of mGluR agonists on wild-type mouse and mGluR4-deficient LC neurons

The pharmacological profile of mGluRs can help to delineate to which of the three specific groups a receptor belongs. However, discrimination of the different subtypes within a specific group is less obvious. Consequently, I took advantage of a recently developed mGluR4 KO mouse (Generous gift from Dr. David R. Hampson, University of Toronto) to verify whether the L-AP4-sensitive response in LC was dependent on the activation of mGluR4.

A total of 17 LC neurons from wild type mice (six mice) and 23 LC neurons from mGluR K.O. cells (11 mice) were recorded from. Table 6 compares the electrophysiological characteristics between wild type and mutant mice. There was no significant difference between the two groups. The effects of *t*-ACPD (100 μM) and L-AP4 (10 μM) were then investigated. Figure 28 shows representative examples of the effect of the two mGluR agonists on evoked EPSPs in wild-type and mGluR4-deficient mice LC neurons. No significant differences were found in the response of either agonist in either mouse model.

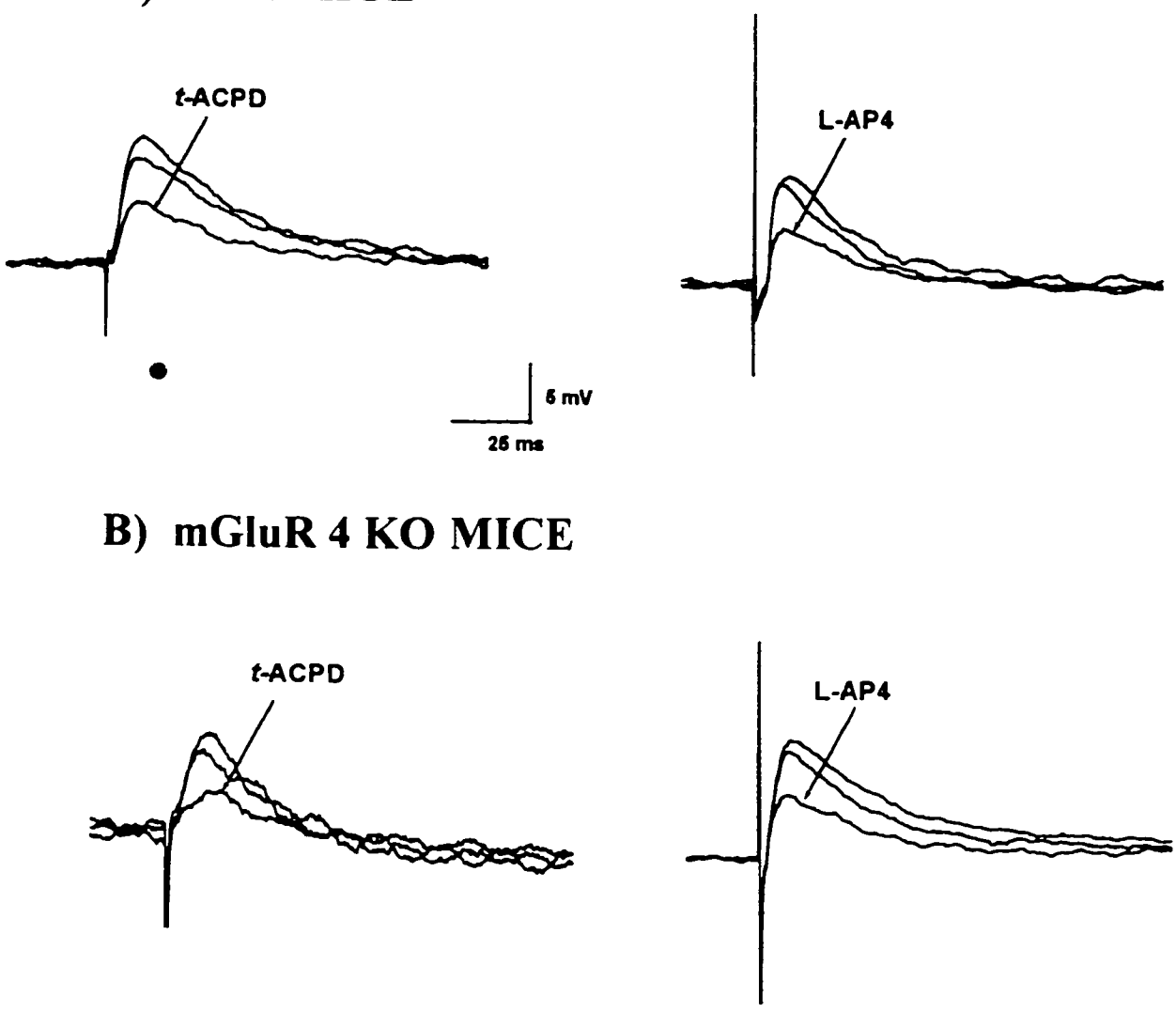


Figure 28: Comparison between the effect of *t*-ACPD and L-AP4 on EPSPs evoked in wild-type mice and mGluR4 KO mice. Representative tracings of EPSPs evoked in A) wild type and B) mGluR4 KO. The three superimposed traces represent EPSPs evoked under basal and wash conditions, or in the presence of *t*-ACPD (100 μM) or L-AP4 (10 μM) (indicated with an arrow). Each set of tests was recorded from different animal.

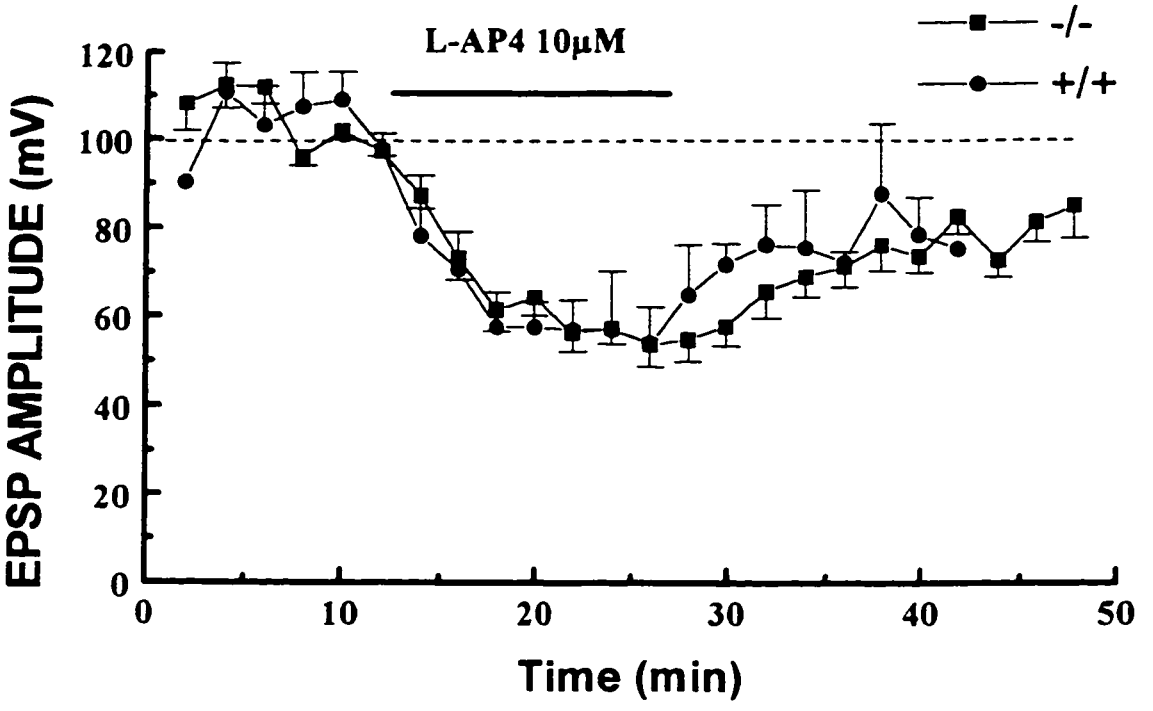


Figure 29: Comparison of the time-course of action of L-AP4 on EPSPs evoked in WT or KO mouse LC. The agonist L-AP4 (10 μM) was introduced in the superfusate concomitantly. The graph depicts the time-course of the effects of L-AP4 on EPSPs recorded in LC from WT (+/+) mice and mGluR4 KO (-/-) mice. There were no significant differences in the response to L-AP4 between the two genotypes.

This is further exemplified by the time course of the effects of L-AP4 for each mouse genotype, plotted in figure 29. Hence, average inhibitions produced by *t*-ACPD and L-AP4 for wild type and mutant mice were comparable. A summary of these results is found in table 6.

Table 6: Summary of data collected from wild-type and K.O. mouse LC neurons

	Wild-type mice	**mGluR4 K.O. mice**
Input resistance (MΩ)	192.5 ± 20.0 (17)	217.2 ± 15.2 (23)
AP amplitude (mV)	63.3 ± 0.8 (15)	65.5 ± 1.5 (20)
Mean EPSP amplitude (mV)	9.41 ± .82 (14)	9.15 ± 0.82 (20)
Range of EPSP amplitudes (mV)	4.46 - 16.17	4.08 - 17.28
Inhibition by 100 μM *t*-ACPD (%)	50.9 ± 7.2 (3)	50.4 ± 5.6 (5)
Inhibition by 10 μM L-AP4 (%)	56.7 ± 5.3 (5)	62.2 ± 3.5 (13)

(#) number of cells used

3.3.6. Signal transduction mechanisms mediating the effects of *t*-ACPD and L-AP4 in rat LC neurons

The mGluRs have been grouped based on their pharmacology, gene similarity and signal transduction system mediating their effects. When investigated in expression systems,

group I mGluRs are coupled to an IP_3/intracellular calcium pathway while group II and III are preferentially coupled to a $G_{i/o}$-dependent pathway, often mediating a decrease in the AC activity. Since two mGluRs from two distinct groups are causing presynaptic inhibition of excitatory transmission to LC, investigation of their respective cellular coupling mechanisms became an obvious next step to delineate their identities and functions.

The first group of experiments was designed to test the G-protein coupling of the *t*-ACPD- and L-AP4-sensitive receptors. As the pharmacological profile of the mGluRs in LC suggested that a group II and III are involved, the possibility of inhibiting the actions of either or both receptors by blocking $G_{i/o}$-actions was tested. Two compounds have been used for this purpose (Dolphin and Scott, 1991). First, the bacterial enzyme pertussis toxin produces a specific inhibition of $G_{i/o}$-actions by ADP-ribosylation of specific residues on the G-protein. However, this compound requires a long incubation period (12-24h) and its effects are irreversible (Dolphin and Scott, 1991). Therefore, I opted for a second compound, N-ethyl-maleimide (NEM), an agent that akylates residues of certain proteins thus disrupting their functions. Although the effects of NEM are less specific than that of pertussis toxin, it has been shown to alkylate and inactivate $G_{i/o}$ preferentially within minutes (Dolphin and Scott, 1991; Shapiro et al., 1994). Therefore, NEM (50 μM) was used in an attempt to disrupt the action of *t*-ACPD and L-AP4 on excitatory synaptic transmission. NEM alone produced either a decrease (four neurons, range=2.5% to 20.6%), or an increase (6.9% and 31%) so that no significant changes in the mean amplitude of EPSPs were found [mean change: 0.5% ± 8.9%, (n=6)]. However, a ten minute application of NEM completely abolished the effect

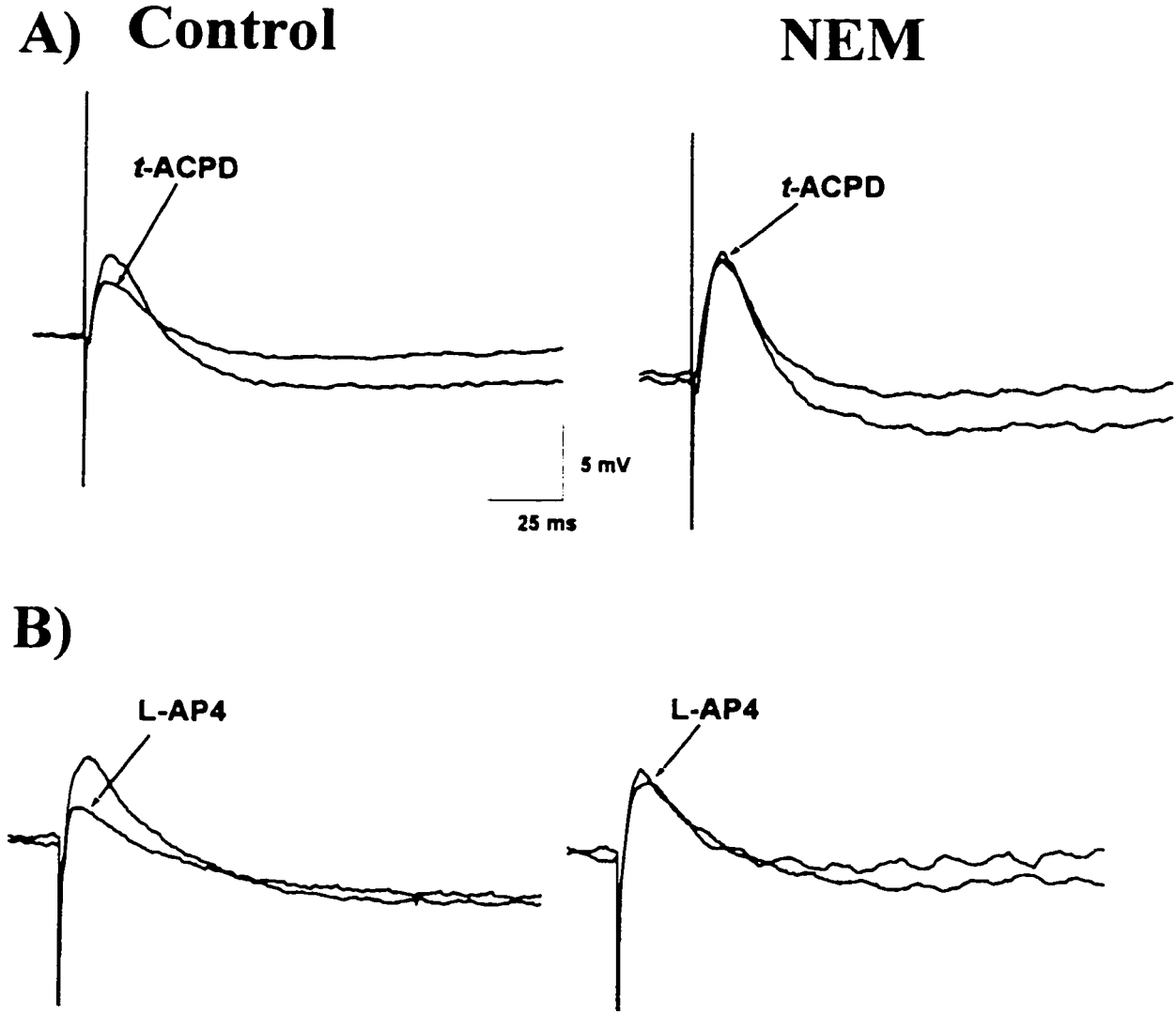


Figure 30: Blockade of the effect of *t*-ACPD and L-AP4 on EPSP evoked in rat LC neurons by the alkylating agent NEM. Representative tracings of EPSPs evoked in rat LC neurons showing the effect of NEM (50 μM) on A) *t*-ACPD and B) L-AP4-mediated inhibition of EPSP. Both *t*-ACPD (100 μM) and L-AP4 (10 μM) effects were fully blocked by a 10 min pretreatment of the slice with NEM.

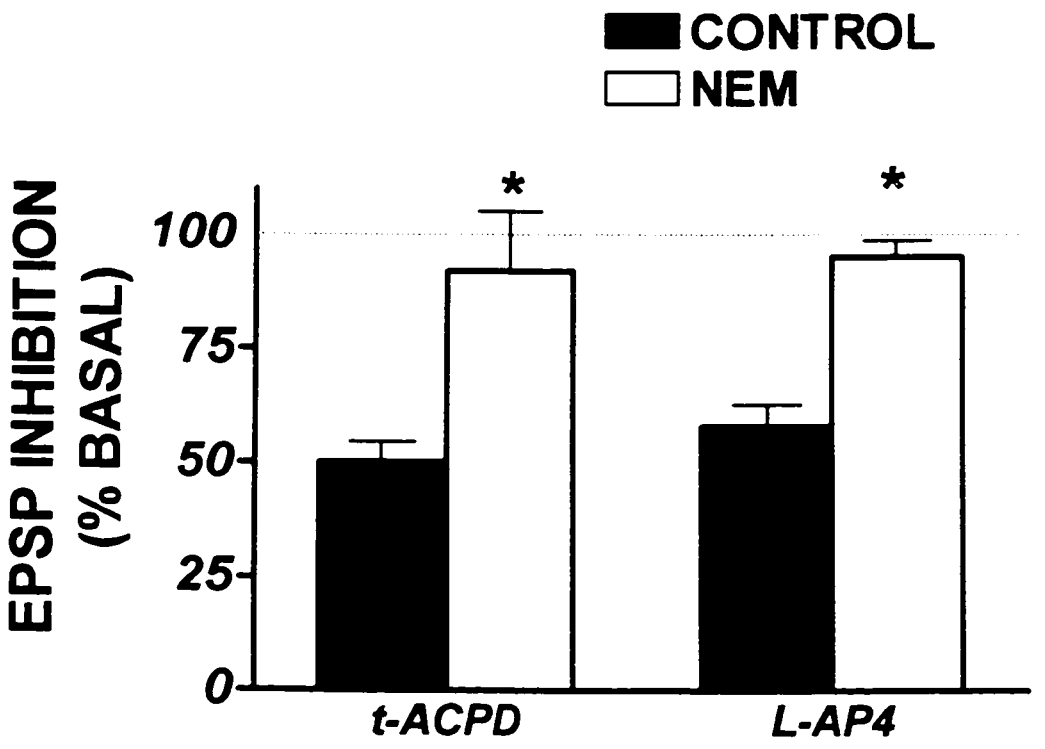


Figure 31: Lack of effects of group II and III mGluRs on excitatory synaptic transmission in rat LC in the presence of NEM. Bar graph summarizing results from the NEM tests. The graph shows the average depression caused by *t*-ACPD (100 μM; n=3) and L-AP4 (10 μM; n=4) under control conditions (black bars) and after a 10 min pretreatment of the same slice with 50 μM NEM (open bars). The treatment with NEM completely blocked the effects of either mGluR agonist (* indicates a significant difference between control and NEM-treated conditions with $p<0.05$).

of either mGluR agonist. Figure 30 shows a representative examples of the effects of *t*-ACPD and L-AP4 on the amplitude of EPSPs before of after treatment of the slice with 50 μM NEM for 10 min (NEM was added to the perfusate). A summary of these results is presented in figure 31.

As both *t*-ACPD- and L-AP4-sensitive receptors are NEM-sensitive (suggesting coupling to $G_{i/o}$), and display pharmacologies identical to mGluRs reported to be negatively coupled to AC, a series of tests was conducted to determine whether their actions were mediated through a decrease in cAMP production. The effects of either agonists, applied sequentially, was tested alone or in the presence of a cocktail aimed at "clamping" the levels of cAMP in the slice. The cocktail contained the non-hydrolysable and cell permeant cAMP analogue 8-bromo-cAMP (200 μM), the phosphodiesterase inhibitor IBMX (1 mM) and the AC agonist forskolin (20 μM). This cocktail has been successfully used to maintain high cAMP levels in cells, in studies looking at the function of cAMP-modulated CFTR Cl^- channels (Kartner et al., 1991). By itself, application of the cAMP cocktail alone produced a significant potentiation of the amplitude of the EPSPs (fig. 32 and 33). In seven experiments, the increase in amplitude of the EPSPs had a range from 34.0% to 94.0% over control amplitudes (mean increase 67.3% ± 8.2%, n=7). This was accompanied by a significant decrease in paired pulse facilitation (fig. 32). Under these conditions, no notable changes in firing rate, R_{in}, or Em were observed. Furthermore, in four experiments, depolarizations evoked by focal applications of Glu were not notably changed. The effects of cAMP persisted for up to 30 min after removal of the cocktail, a period longer than that

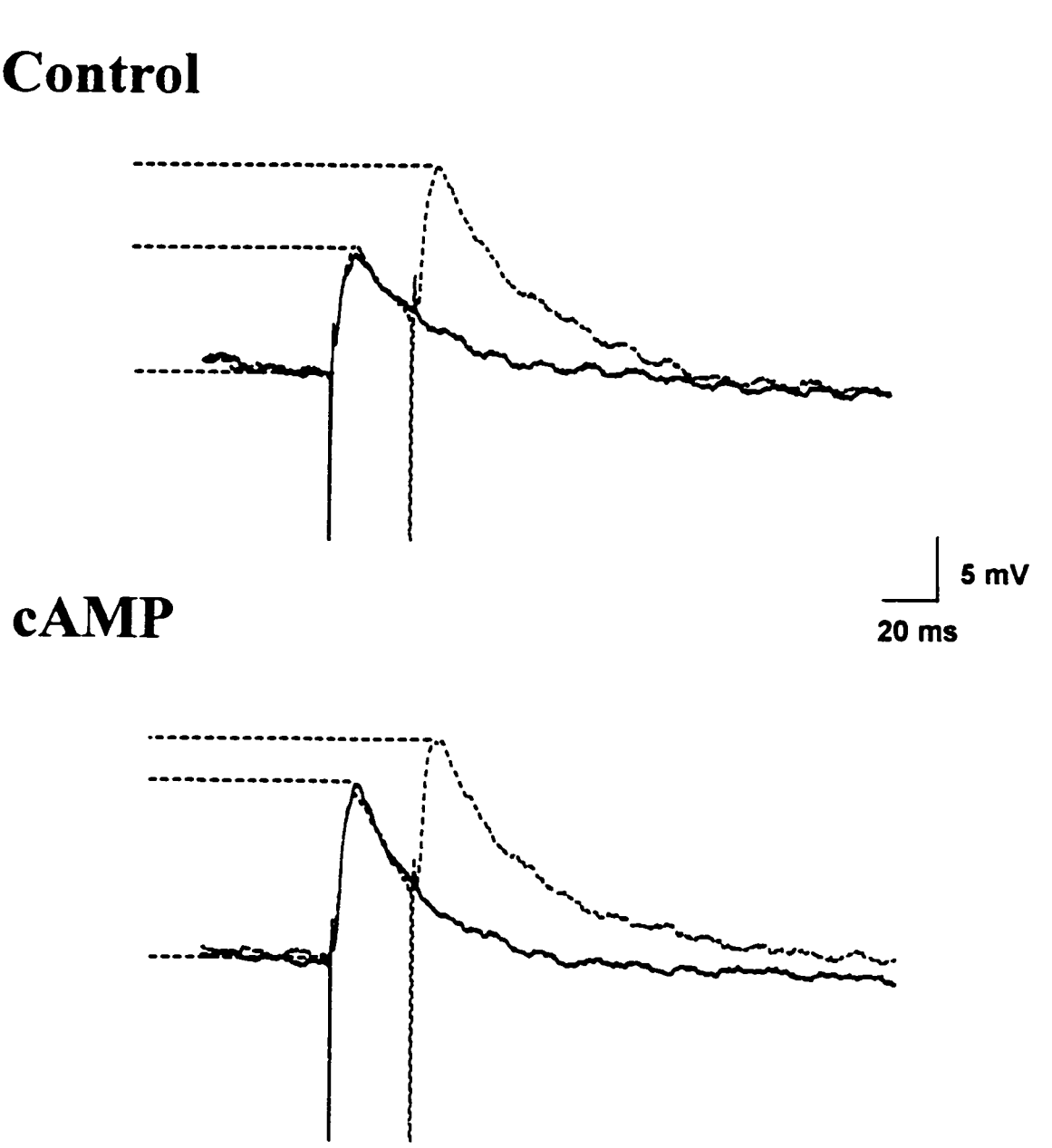


Figure 32: Paired-pulse facilitation is decreased by increasing excitatory synaptic transmission with cAMP. A representative example of a single evoked EPSP (dark line) superimposed with a double EPSP (dotted line) resulting from a paired-pulse protocol. Tests were carried-out under control conditions and after 15 min in the presence of a cAMP cocktail containing 200 μM 8-Br-cAMP, 10 μM forskolin and 1 mM IBMX. Each trace is an average of four consecutive sweeps. In this example, the amplitude of EPSPs was increased by 57.7% and the mean facilitation [(second EPSP - first EPSP)/first EPSP x 100] under control conditions was 59% and 8% in the presence of cAMP (Em=-72 mV after a -200pA pulse was applied to hyperpolarize the cell). Values are as follows: (control+wash/2) vs cAMP: 56.0% ± 6.6% vs 17.4 ± 0.8%, n=5, $p<0.04$, paired t test. Dashed lines indicates to the increase in amplitude of EPSPs in the presence of cAMP. Horizontal dashed lines were included in the figure to provide a visual impression of the relative changes in amplitudes of EPSPs and paired-pulse facilitation.

required for a full washout of other drugs (e.g., mGluR agonists). The effects of *t*-ACPD (30 μM) and L-AP4 (10 μM) were tested under normal conditions, and during the "cAMP clamp" (the cAMP cocktail was applied to the slice by perfusion 15 min before mGluR agonists' tests and remained for the entire test and wash periods). Figure 33 shows representative examples of the actions of either mGluR agonist before and during the "cAMP clamp". When amplitudes of the EPSPs were normalized to the pre-agonist control amplitude, EPSPs under either condition displayed similar shapes, and durations. Furthermore, the time-course of action of either mGluR agonist was not significantly changed by the "cAMP clamp" (fig. 34). Pooled data are as follows [%control ± SEM for agonist vs. agonist + cAMP]: 10 μM L-AP4 (n=3) -- 43.7 ± 3.7 vs. 54.3 ± 8.0, and 30 μM *t*-ACPD (n=4) -- 52.4 ± 6.9 vs. 67.6 ± 6.4. Together, the last two sets of experiments demonstrate that the *t*-ACPD- and L-AP4-sensitive mGluRs are coupled to $G_{i/o}$, consistent with the pharmacological classification of these receptors. However, the results also suggest that their inhibitory effects on excitatory synaptic transmission are independent of the levels of cAMP in the cells, ruling-out that the effects of these receptors are associated with AC activity.

3.4. SYNAPTIC ACTIVITY-DEPENDENT DEPRESSION OF EPSPs IN RAT LC NEURONS

The following sections present the characterization of a paradigm aimed at demonstrating endogenous activation of presynaptic mGluRs. These experiments were developed to test the hypothesis that either or both mGluRs, characterized in rats and mice

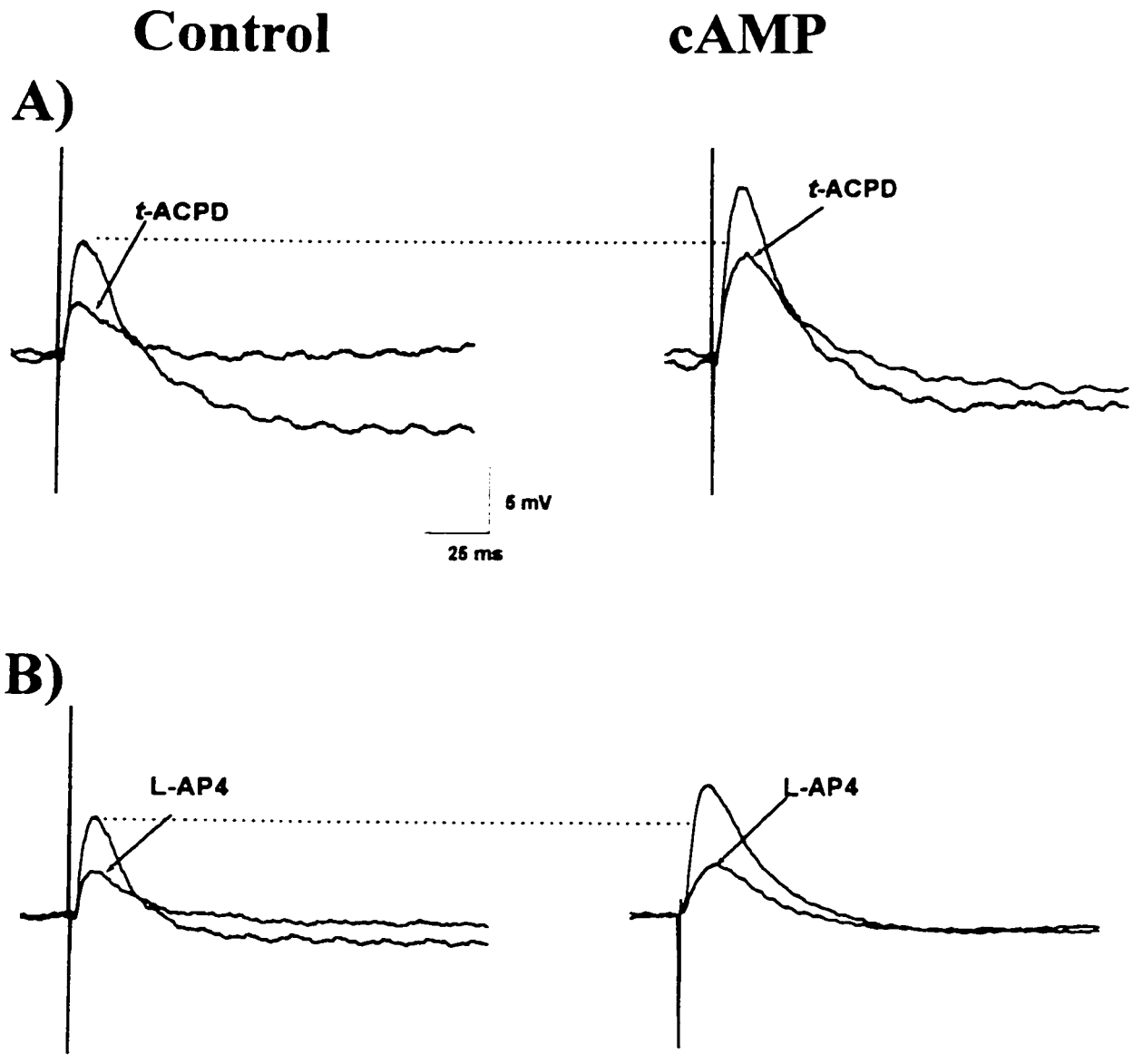
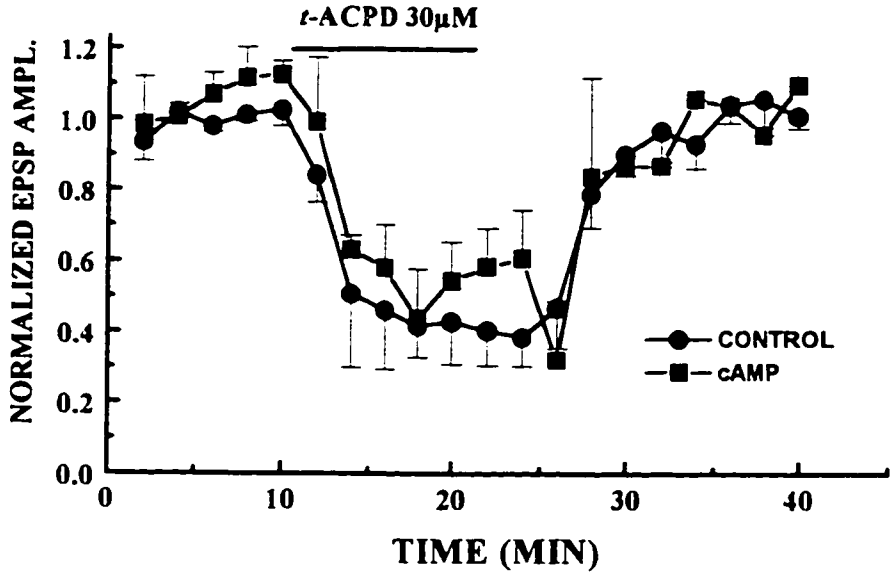


Figure 33: Effect of cAMP on the effects of *t*-ACPD and L-AP4 in rat LC neurons. Representative tracings of EPSPs evoked in rat LC neurons showing the effect of the cAMP cocktail (200 μM 8-Br-cAMP, 20 μM Forskolin and 1 mM IBMX) on A) 30 μM *t*-ACPD- and B) 10 μM L-AP4-mediated inhibition of EPSP. Dashed line indicate the increased amplitude of EPSPs in the presence of cAMP

A)



B)

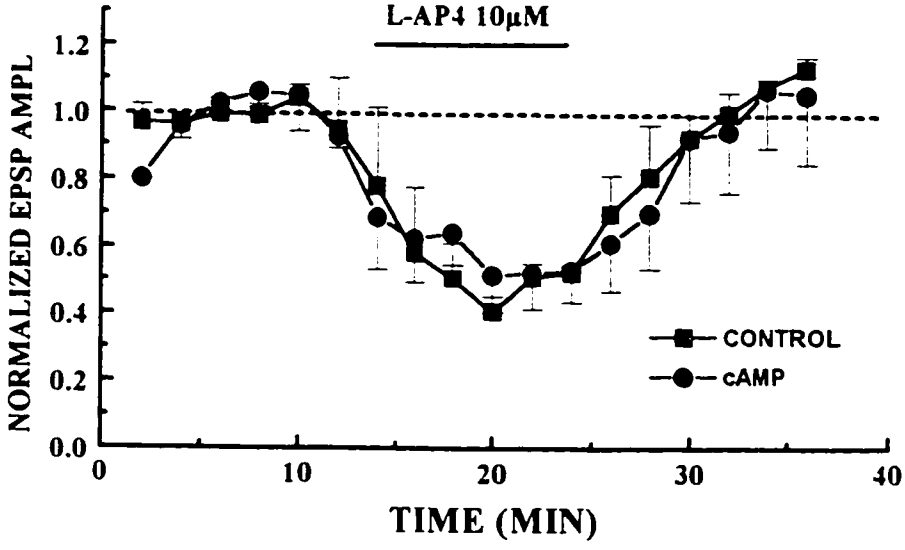


Figure 34: Comparison of the time-course of action of *t*-ACPD and L-AP4 on EPSPs evoked under control conditions and in the presence of cAMP in rat LC neurons. The graph depicts the time-course of the effects of A) 30 μM *t*-ACPD (n=4) and B) 10 μM L-AP4 (n=3) on EPSPs recorded in LC during control conditions and in the presence of the cAMP cocktail containing 200 μM 8-Br-cAMP, 20 μM forskolin and 1 mM IBMX. There were no significant differences in the response to *t*-ACPD or L-AP4 between the two conditions.

LC, are presynaptic autoreceptors which can be activated in response to the accumulation of EAA in the synaptic cleft.

3.4.1. Basic characterization of activity-dependent depression of EPSPs

The principle behind the following test was to compare the amplitude of a control EPSP (C) to a test EPSP (T) separated by a period of time during which a stimulation volley of the afferent caused a train of EPSPs. I hypothesized that if the stimulation volley caused enough release of Glu in the cleft, some of this Glu would activate presynaptic receptors. The first series of experiments were designed to determine the range of durations and frequencies of the stimulation volley, and the interval between the end of the volley and T, which resulted in optimal activity-dependent depression (ADD) of T with respect to C. In all experiments, Bic (10 μM) or picrotoxin (50 μM) was used to block $GABA_A$-mediated postsynaptic potentials resulting from the possible release of GABA. No notable differences were observed between experiments using either $GABA_A$ antagonist. An interval of 5 sec between consecutive measurements was used to allow for the effects of the last stimulation to dissipate.

3.4.1.1. Determination of durations and frequencies of stimulation volleys for optimal ADD

In this set of experiments, C and T were arbitrarily separated by an 800 ms interval (corresponding to 0 Hz stimulation). It was shown earlier in the Results section that in the

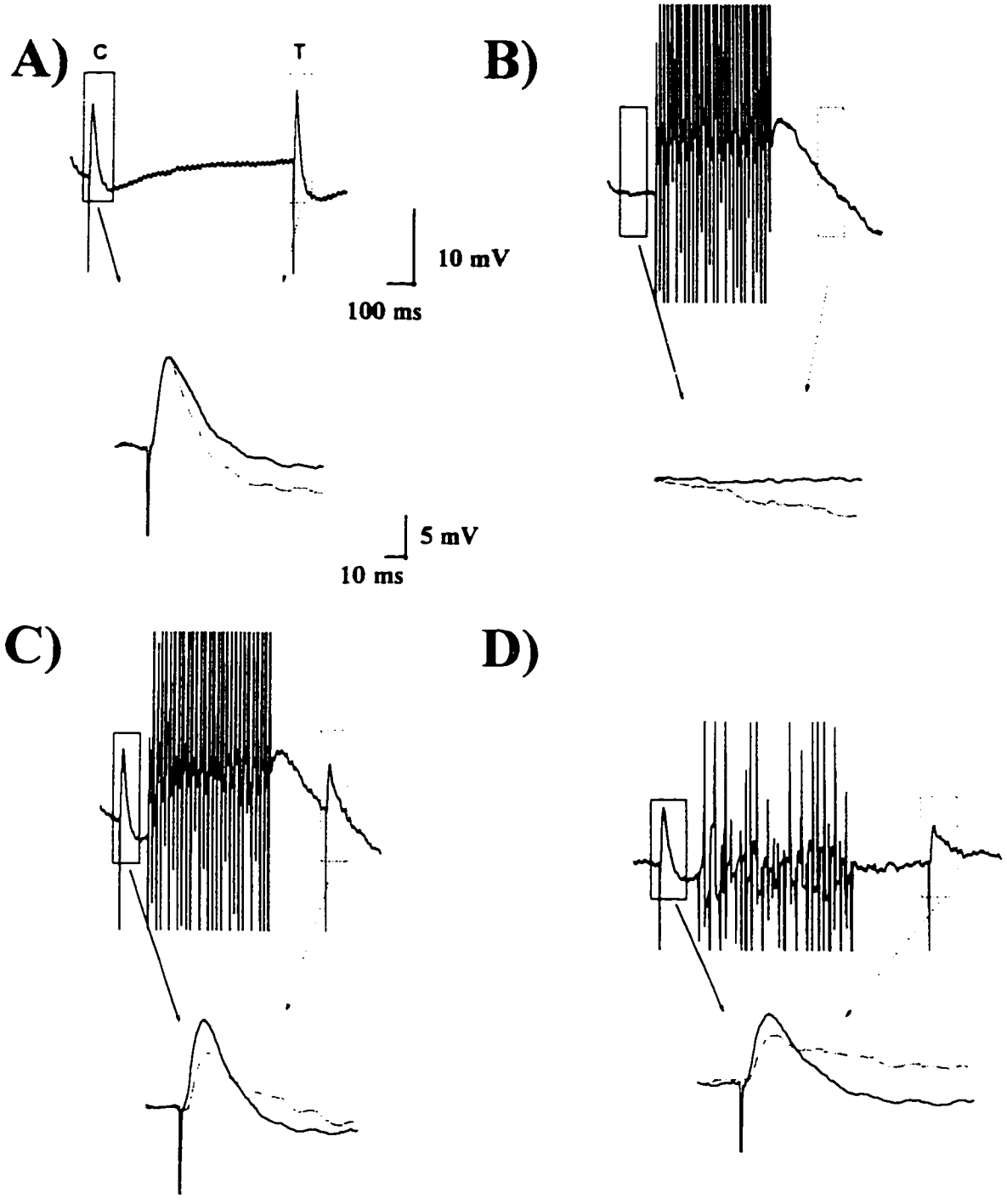
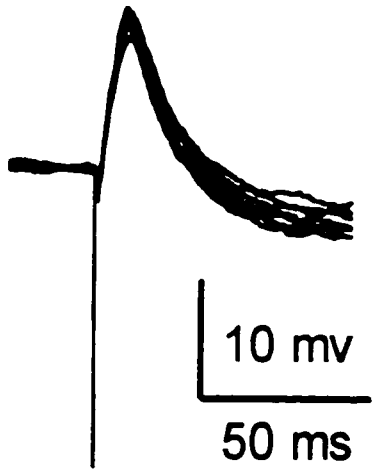


Figure 35: Representative examples of activity-dependent inhibition of tests (T) EPSP amplitude in LC neurons following trains of stimulation of afferents. Average of two to six consecutive sets of measurements representing the following: A) Two consecutive EPSPs (**C** and **T**) evoked 800 ms apart, B) A 70Hz 300 ms train without **C** or **T**, and C) Combinations of **C** and **T** EPSPs together with a 70Hz - 300 ms train. D) represents the result of the subtraction of trace (B) from (C). This subtracted waveform was used to measure and compare the amplitude of individual **C** and **T** EPSPs between different conditions. Note that without the train of stimulation the amplitude of C and T EPSPs is comparable. However, when a train of stimulation was applied between the two EPSPs the **T** EPSP was consistently smaller than **C**.

absence of stimulation volleys, this interval did not lead to paired-pulse facilitation (fig. 14). Keeping the interval between C and T constant, 100 ms-, 200 ms- or 300 ms-long stimulation volleys were delivered to the LC afferents. Frequencies of stimulations between 10 and 90 Hz (increment of 10 Hz) were tested for each time duration and the amplitudes of T were compared to those of C. An example of such a test is depicted in figure 35. To measure the amplitudes of C and T following stimulation volleys, a control waveform containing only the response of the neuron to the volley (fig 35B) was subtracted, off line, from the test waveform containing both C and T and the volley (fig. 35C). The ratio of T to C amplitudes measured from the resulting waveform (Fig 35D) was used as a description of the activity-dependent effects on LC excitatory synaptic transmission. Ratios > 1 indicated a potentiation and ratios <1 indicated inhibitions. Results from these experiments showed significant ADD when 300 ms stimulation volleys were delivered to the LC afferents with frequencies ranging from 50 to 70 Hz (fig. 37). Figure 36 shows a representative example displaying how C and T EPSPs varied when 300 ms stimulation volleys of different frequencies (0-90 Hz) were applied. Results for all durations and frequencies tested are summarized in figure 37. Note the shape of the falling phase of the T EPSP as compared to C (fig. 36). This delay in repolarization of the EPSP was often observed and was seen when stimulation volleys were applied or not. The change in EPSP kinetic was insensitive to the NMDA antagonist, CPP (50μM) (3 out of 3 cells, not shown).

A) 300 ms TRAIN - CONTROL EPSPs (C)



B) 300 ms TRAIN - TEST EPSPs (T)

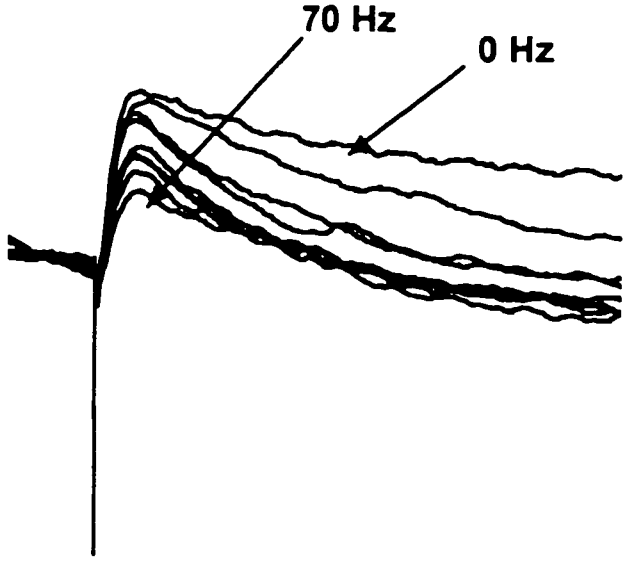


Figure 36: Inhibition of test (T) EPSP amplitude in LC neurons by stimulation volleys of LC afferents is frequency-dependent. A representative example displaying how the frequency of a 300 ms stimulation volley affects the amplitude of **C** (A) and **T** (B) EPSPs. Each trace in A and B represents an EPSP prior to or following a stimulation volley of increasing frequencies (0-90 Hz). As demonstrated in A, the amplitude of **C** is not affected by the stimulation volleys subsequently applied but the **T** amplitude (B) varies in relation to the frequency (compare 0Hz to 70Hz).

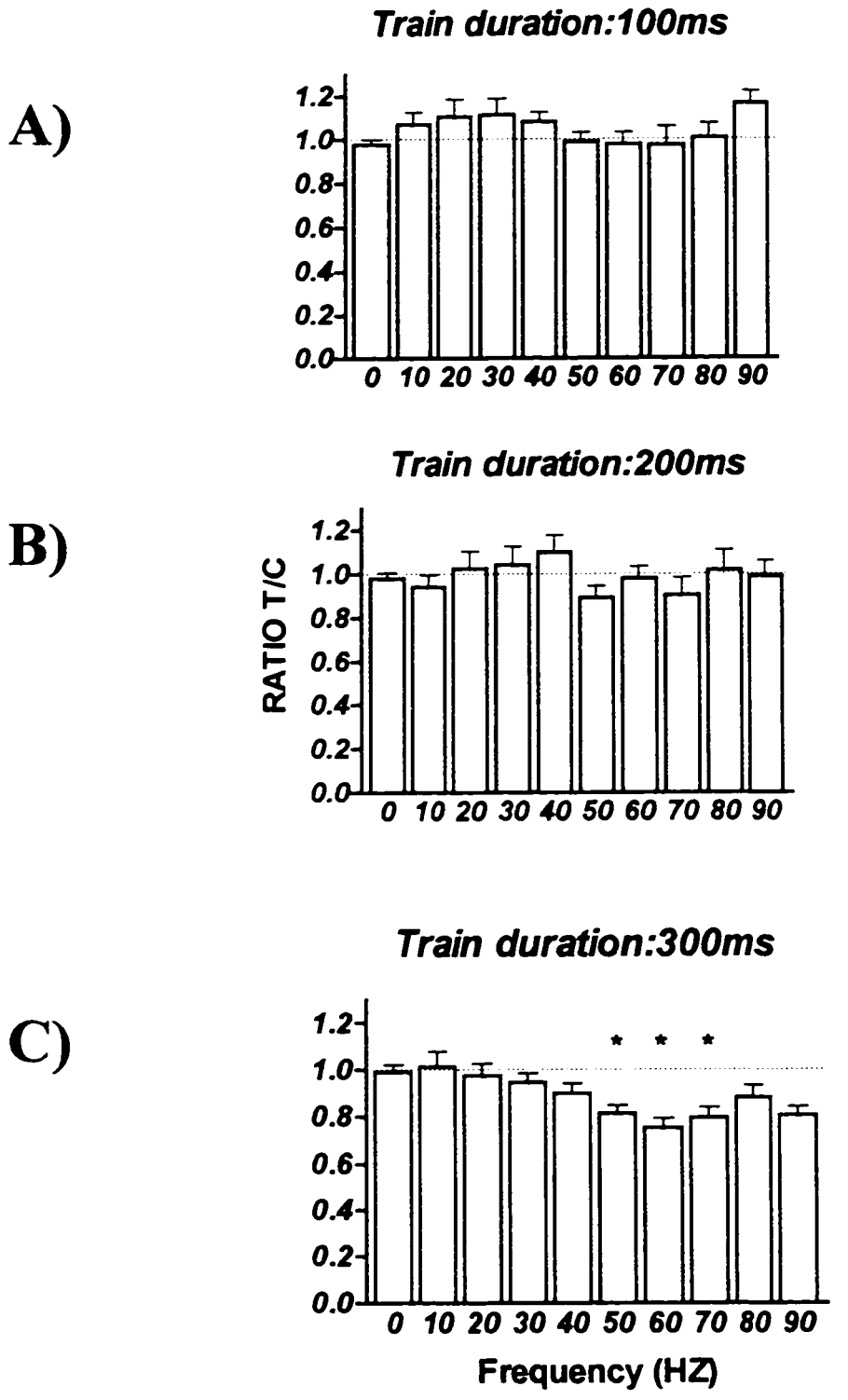


Figure 37: Inhibition of T EPSP amplitude in LC neurons by trains of electrical stimulation of afferents. Histograms displaying the effect of train duration (100, 200 and 300 ms) and frequency (0-90Hz) on ADD of the T EPSP with respect to the **C** (average of 8-12 slices). Trains of stimulations of 50, 60 or 70Hz for 300 ms all produced a statistically significant (* $p<0.05$, assessed by ANOVA followed, when overall means were found to be significantly different, by a tukey analysis) depression in the ratio of **T/C** with respect to control (0Hz). 70Hz, 300 ms was chosen for later tests.

3.4.1.2. Determination of the optimal interval between the end of the stimulation volley and T for ADD

With regards to the initial hypothesis, the next parameter to determine was the optimal interval separating T from the end of the stimulation volleys. This parameter can be viewed as the balance between the time needed for activation of presynaptic receptors and the time required for clearance of the EAA from the synaptic cleft, and termination of the presynaptic activation. To test this, optimal duration and frequency of stimulation volleys, determined above, were kept constant throughout the range of intervals tested between the end of the volley and T. T was measured at different times after the volley (from 200 ms to 2 sec.). Results from these experiments are outlined in figure 37A. Significant ADD was observed at intervals ranging from 200 ms to 300 ms. Inhibitions could not be measured at intervals shorter than 200 ms, for the cell response to the stimulation volley was very noisy during that period. A 300 ms interval was chosen as a default, unless no inhibition was observed.

Based on the nature of LC neurons as NA releasing cells, the involvement of α_2-adrenoceptor-mediated IPSPs in ADD of EPSPs was assessed. Figure 37B displays a representative example of the stimulation volley test under normal ACSF and in the presence of Yoh (1 μM). While no significant changes in T to C ratios were measured (fig. 37A), the long-lasting hyperpolarization observed following the stimulation volley was notably decreased in every cell examined (n=7). Nevertheless, as a precaution, Yoh was added to the perfusate in all following experiments.

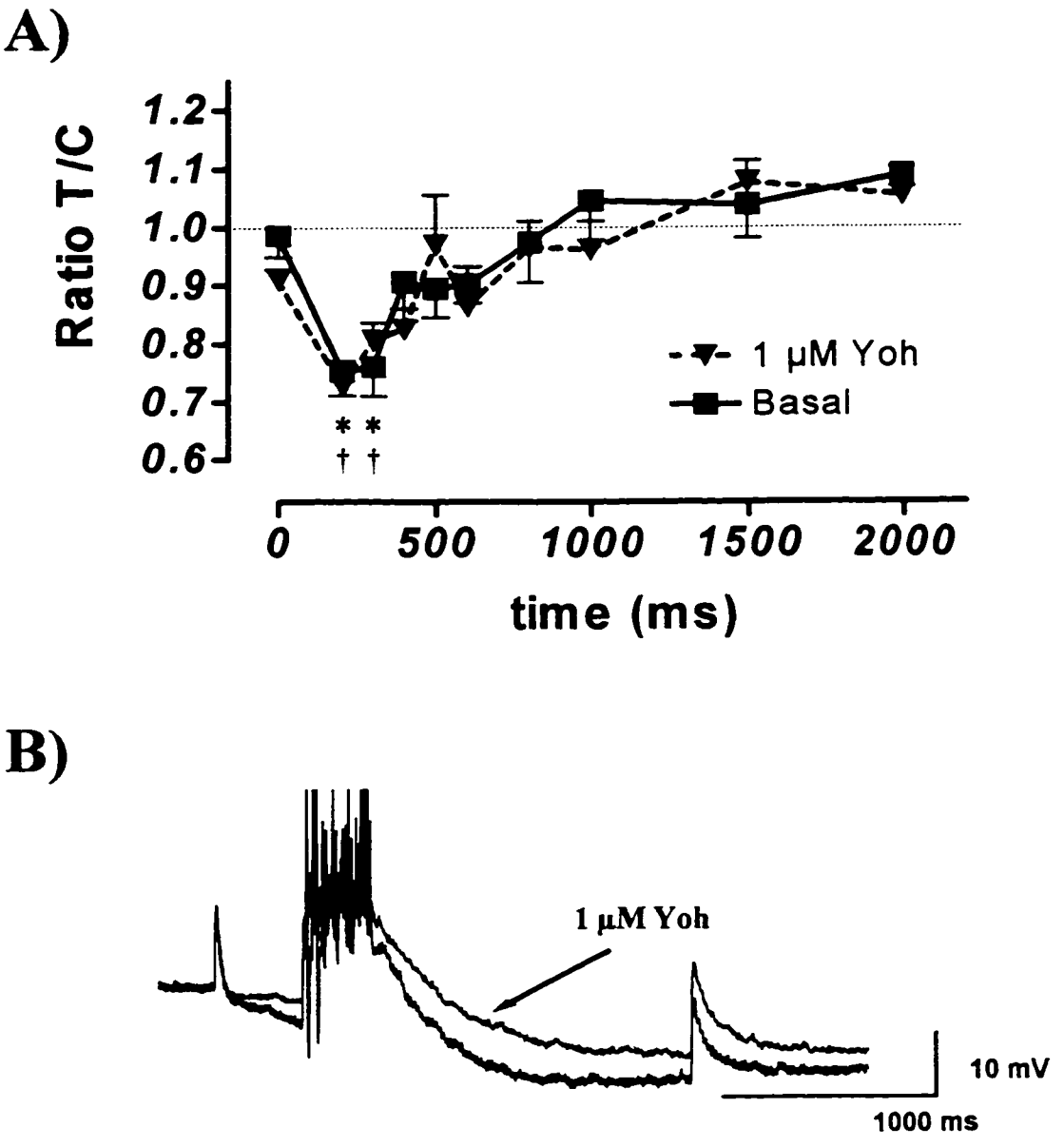


Figure 38: Time-course of ADD of EPSP in rat LC. A) Graph depicting the effect of varying the interval between T and the end of a 70Hz/300 ms train on ADD. Under these conditions the ratio of T/C was significantly reduced when T was delivered 200-300 ms after the train (* indicates significance from 0 ms $p<0.01$, n=7, assessed by ANOVA). Bath-applied Yoh (1 µM) did not affect the amplitude of the individual EPSP (dotted line, † indicates significance from 0 ms $p<0.05$, n=6, assessed by ANOVA), but decreased the hyperpolarization following the EPSP, as shown in the representative example in B).

3.4.2. Roles of mGluRs in ADD

To test the hypothesis that presynaptic mGluRs can be activated following stimulation volleys, through the release of high amounts of EAA in the synaptic cleft, specific mGluR antagonists were tested for effects on ADD. Three specific sets of experiments were performed: first, the effects of mGluR antagonists alone were assessed, second, the effect of an EAA uptake inhibitor was tested in an attempt to potentiate the ADD and third, the mGluR antagonists were tested in the presence of the EAA uptake inhibitor.

3.4.2.1. Effects of specific mGluR antagonists on ADD

In this set of experiments, three mGluR antagonists were tested, based on previous results (section 3.3.3.2.). Of these, both (R)-MCPG and EGLU were shown to inhibit the effects of exogenously applied *t*-ACPD on excitatory synaptic transmission, while MAP4 was found as a selective antagonist of L-AP4-dependent effects. Each of these agonists was tested individually on ADD. However, results from these experiments showed that, after 15 min. perfusion with the antagonist at 500 μM, none of these antagonists caused a significant change in the ratio of T to C. These results are summarized in figure 39 where both the individual values (circles and lines) and the mean values are displayed. Significance was tested on the difference between the control and antagonists and none were significantly different from zero.

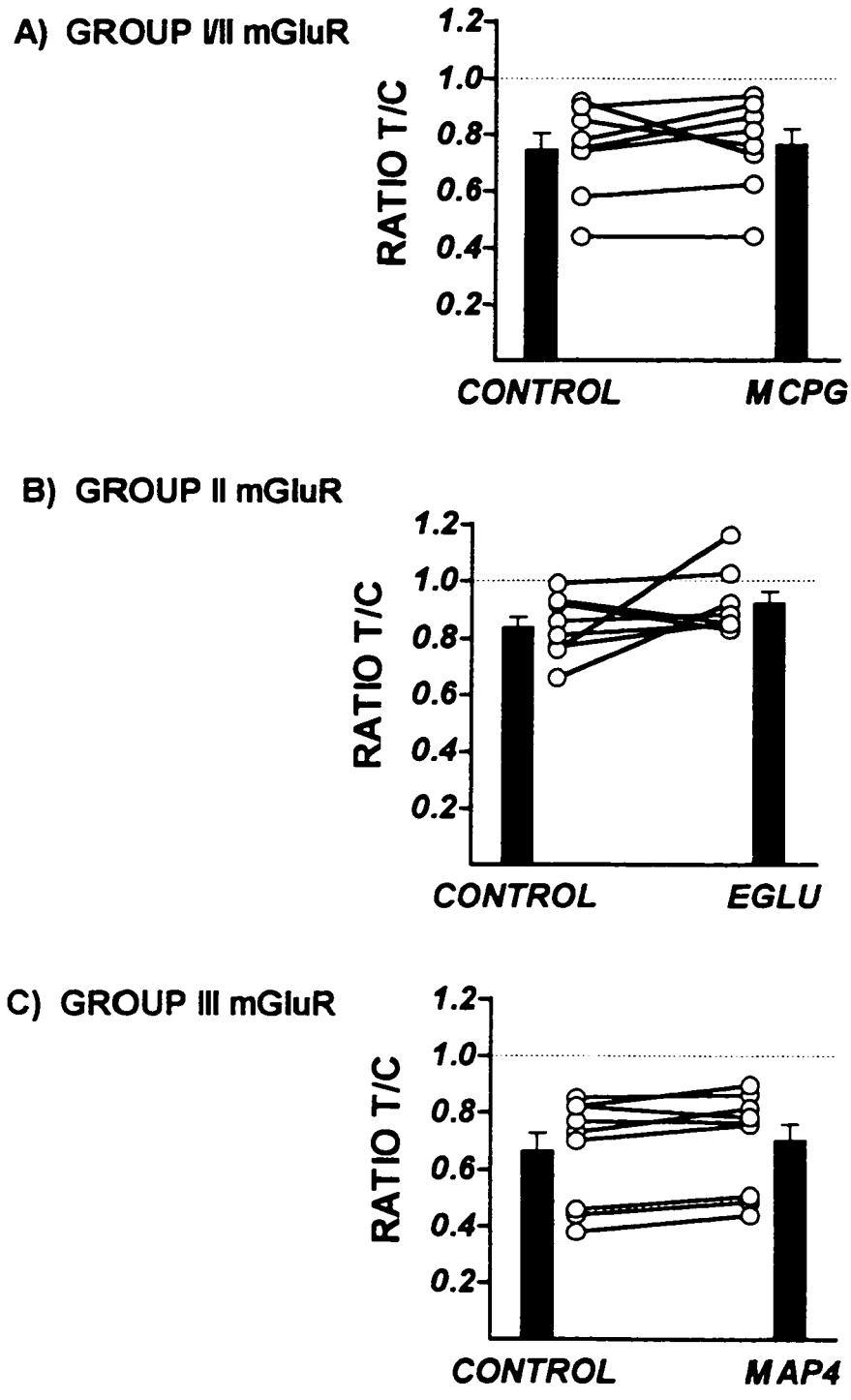


Figure 39: Effects of different mGluR antagonists on ADD of EPSP amplitude in rat LC. A) The group I/II [(±)- -MCPG, 500 μM], B) the group II (EGLU, 500 μM) and C) the group III (MAP4, 500 μM) mGluR antagonists were applied by bath 10-15 min prior to testing. Graphs display the average (bars) and individual experiments (open circles) for control (average of basal and washout values) and during antagonist application. The differences between the mean test value and mean control value for antagonists are as follows: MCPG 0.04 ± 0.04, EGLU 0.11 ± 0.06 and MAP4 0.05 ± 0.02. None of these values were different from zero.

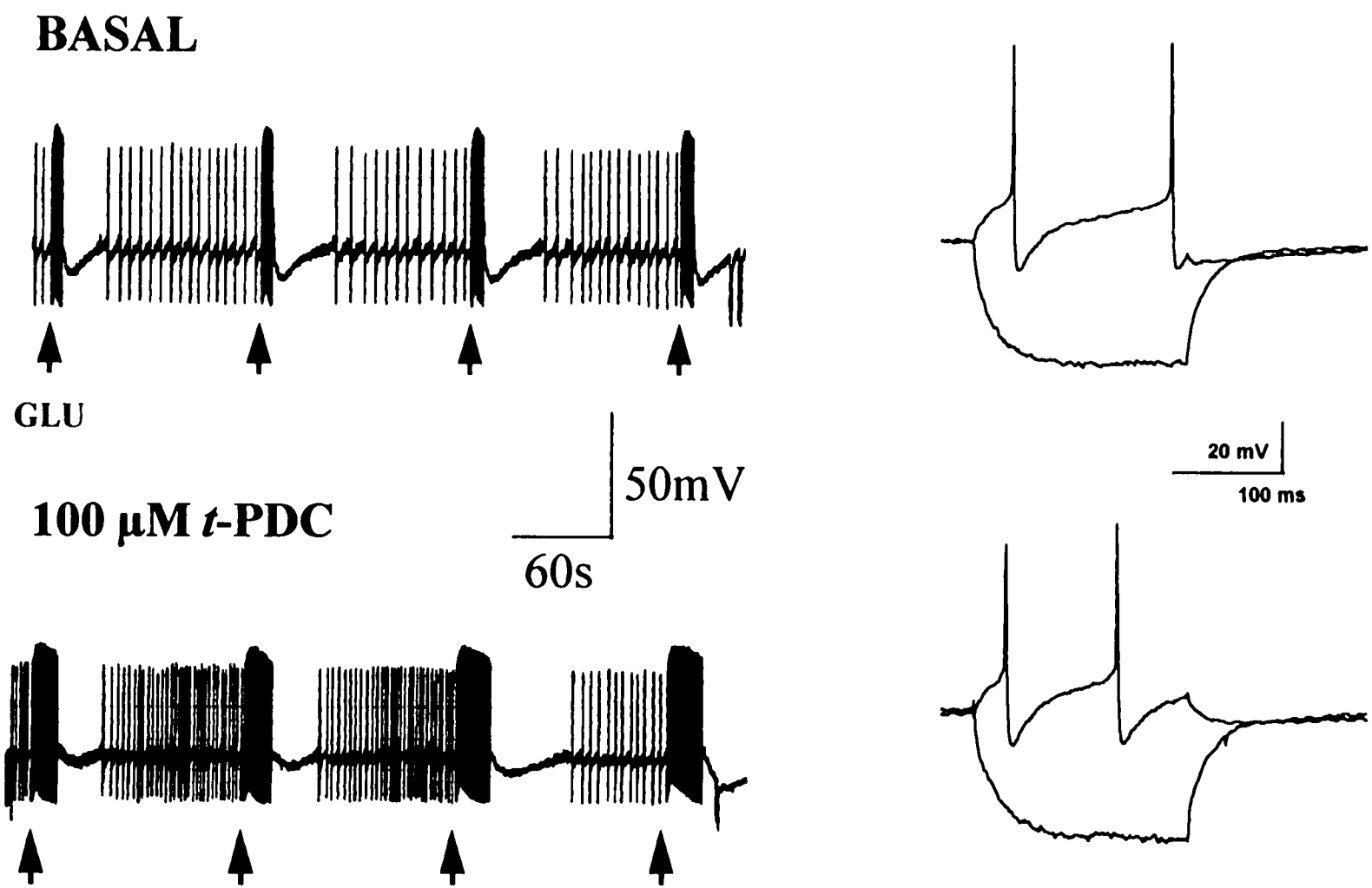


Figure 40: Potentiating effect of Glu uptake inhibition on exogenous Glu-evoked depolarizations in LC neurons. A representative example of the response of an LC neuron to focally applied Glu (pressure application) under control conditions (top trace) and during bath-application of *t*-PDC (100 µM). *t*-PDC consistently potentiated the effects of exogenous glutamate applications on LC neurons in all tests (n=6). There were no significant changes in cell input resistance (right) under these conditions.

3.4.2.2. Effects of the specific EAA uptake inhibitor *t*-PDC on ADD

The following set of experiments was to test the hypothesis of whether ADD could be potentiated by blocking the uptake of EAA by specific transporters. In a first step, the ability of *t*-PDC as an EAA uptake blocker was assessed on focally applied Glu-evoked depolarizations of LC neurons. As exemplified in figure 40, Glu-evoked depolarizations were markedly potentiated, and the duration of the responses increased, following the bath application of 100 μM *t*-PDC in all of the five cells tested, suggesting that the clearance of the focally applied Glu depends, in part, on the activity of the Glu transporters. In two of the cells tested, the firing frequency of the impaled neuron also increased in the presence of *t*-PDC (fig. 40), further supporting a role for *t*-PDC in blocking the clearance of the Glu. In a second step the effect of 100 μM *t*-PDC was tested for its effect on ADD. Figure 41 displays a representative example of such an experiment. As depicted in this example the amplitude of the C was not affected by the uptake inhibitor. Furthermore, when no stimulation volleys were delivered between C and T, the amplitude of T and the ratio of T to C remained unchanged as compared to control. Conversely, under the same conditions, when stimulation volleys were applied to the afferents, the amplitude of T was further reduced in the presence of *t*-PDC. This is depicted in figure 41 (compare B and D) and summarized in figure 42, where results are presented for all these tests. ADD in the presence of *t*PDC was significantly larger than ADD under control conditions (mean T/C control vs. *t*-PDC: 0.84 ± 0.05 vs. 0.69 ± 0.04. $p<0.0001$, n=9).

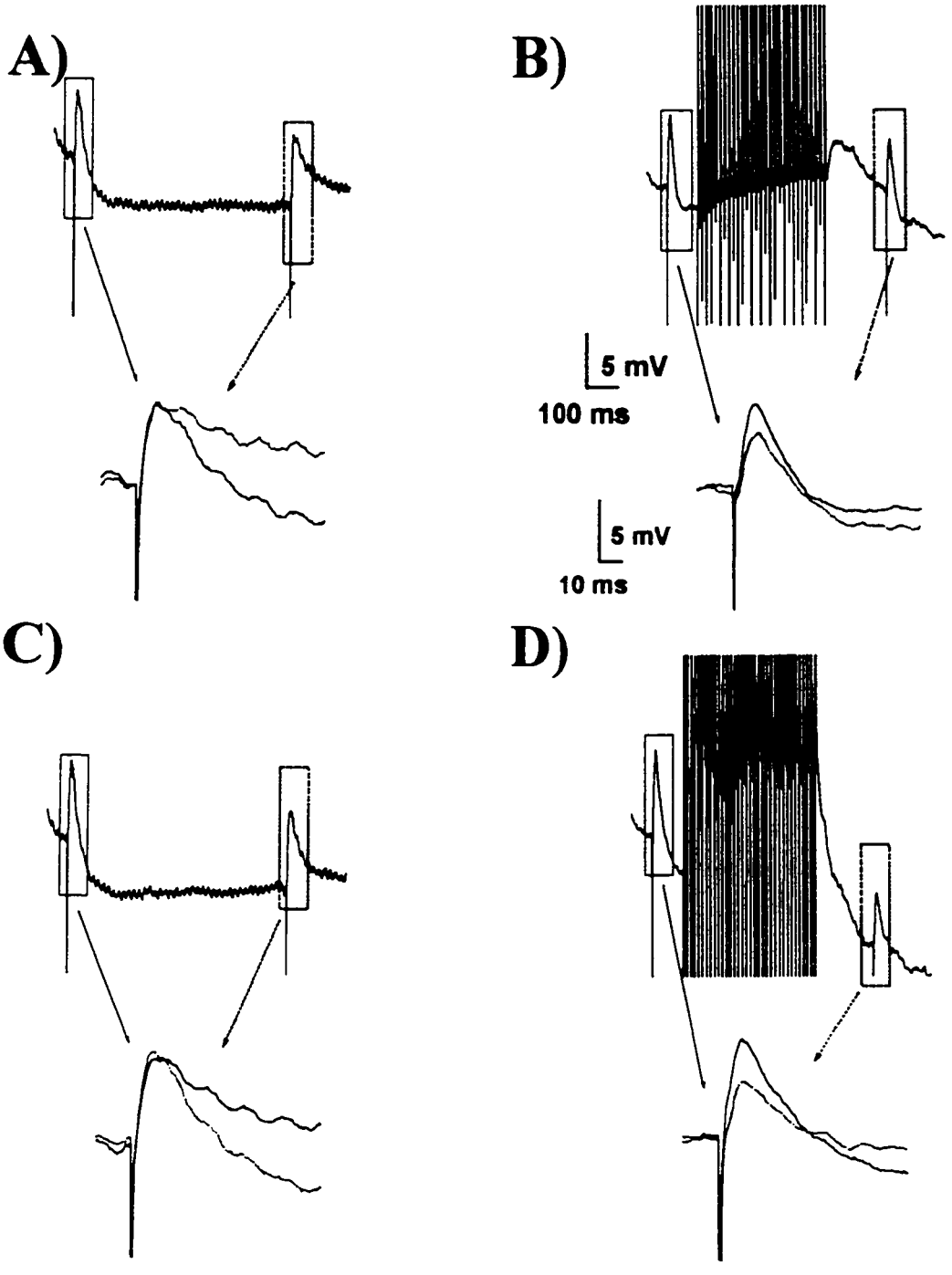


Figure 41: Potentiating effect of the glutamate uptake inhibition on ADD of evoked EPSPs in LC neurons. A) A representative example of ADD under control conditions and B) after 15 min of perfusion with 100 μM *t*-PDC (average of 2 to 4 consecutive sets of measurements). Note that *t*-PDC caused an increase in ADD but failed to change the response of the cell when stimulation volleys were not applied.

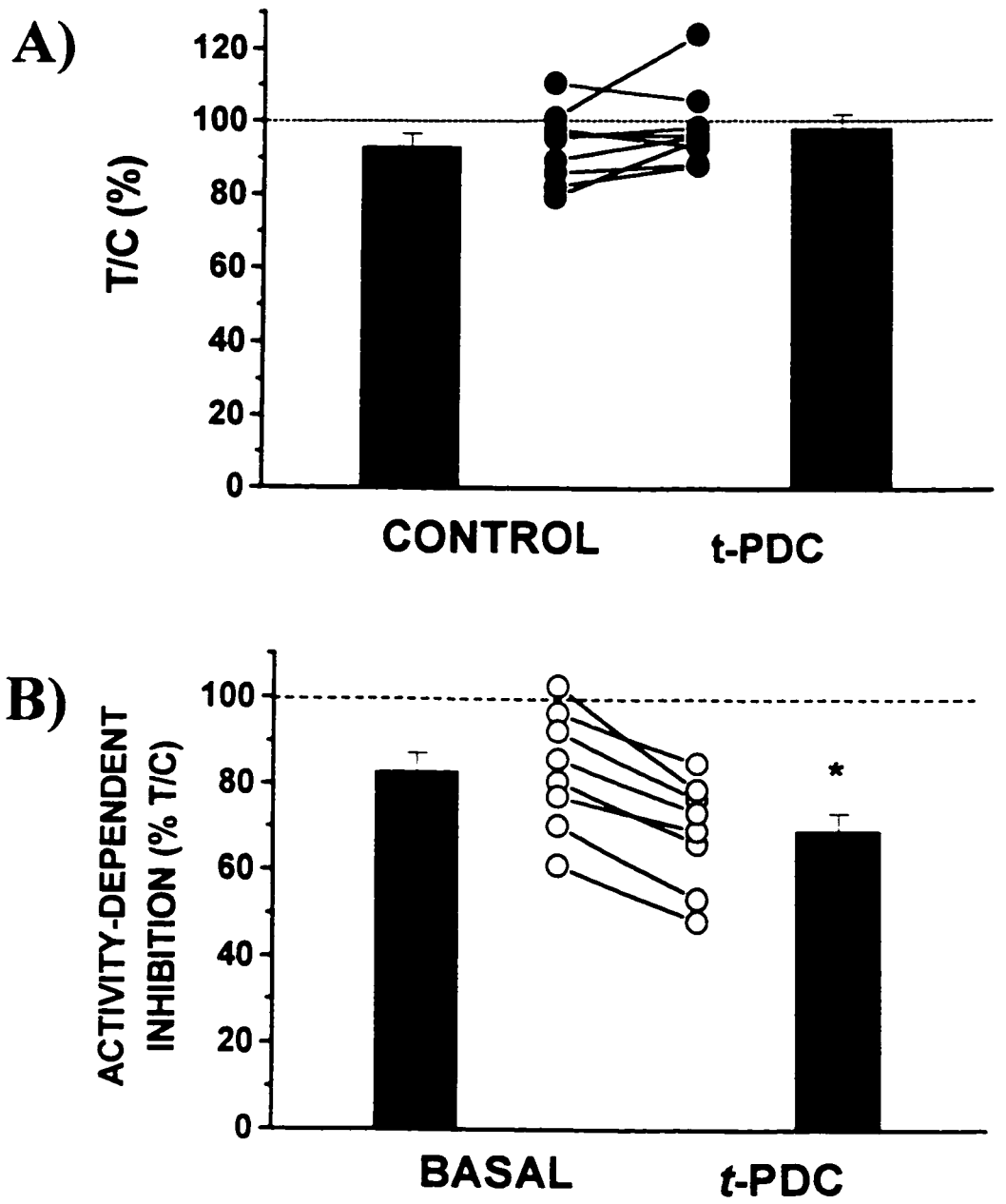


Figure 42: Summary of the potentiating effect of the glutamate uptake inhibition on ADD of evoked EPSPs in LC neurons. Graphs displaying the average (bars) and individual values for each experiment (open circles) testing the effects of *t*-PDC on ADD. A) Conditions where no stimulation volleys were applied between the C and T (no ADD) and B) Conditions where stimulation volleys (300 ms, 70Hz) were delivered to the afferents. The effect of *t*-PDC on ADD was significantly different from control ($p<0.0001$; paired ttest).

3.4.2.3. Effects of mGluR antagonists on the *t*-PDC-potentiated ADD

In the previous section, it was shown that ADD could be potentiated by EAA uptake inhibition. It can hypothesized that, under these conditions, part of the inhibition observed with *t*-PDC likely results from the action of EAA accumulating in the synaptic cleft. As such, the aim of the following experiments was to test the hypothesis that the potentiation of the depression observed in the presence of *t*-PDC is mediated through the activation of either or both groups of mGluRs characterized in this work. For these tests, two of the same antagonists tested before were used, i.e., EGLU (500 μM) and MAP4 (500 μM). Figures 43 and 44 display representative examples of the effects of the EGLU and MAP4 on the relative amplitude of T, in the presence of *t*-PDC. Application of EGLU (fig 43) failed to alter *t*-PDC-potentiated decrease in the T to C ratio. A summary of all EGLU tests is presented in figure 45A. It shows that, as expected, *t*-PDC significantly potentiated the T to C ratio, and shows the failure of the group II mGluR antagonist EGLU to affect the ratio suggesting that this receptor was not responsible for ADD.

When the group III mGluR antagonist MAP4 was tested, the relative amplitude of T was increased in the presence of this antagonist. As outlined in the representative example (fig. 44) and in the summary graph (fig. 45B), the ratio of T to C was significantly increased not only as compared to the condition where *t*-PDC was present but also with respect to the control without stimulation volleys. Thus, application of MAP4 under these conditions significantly reversed the *t*-PDC-dependent and independent inhibition of T by stimulation

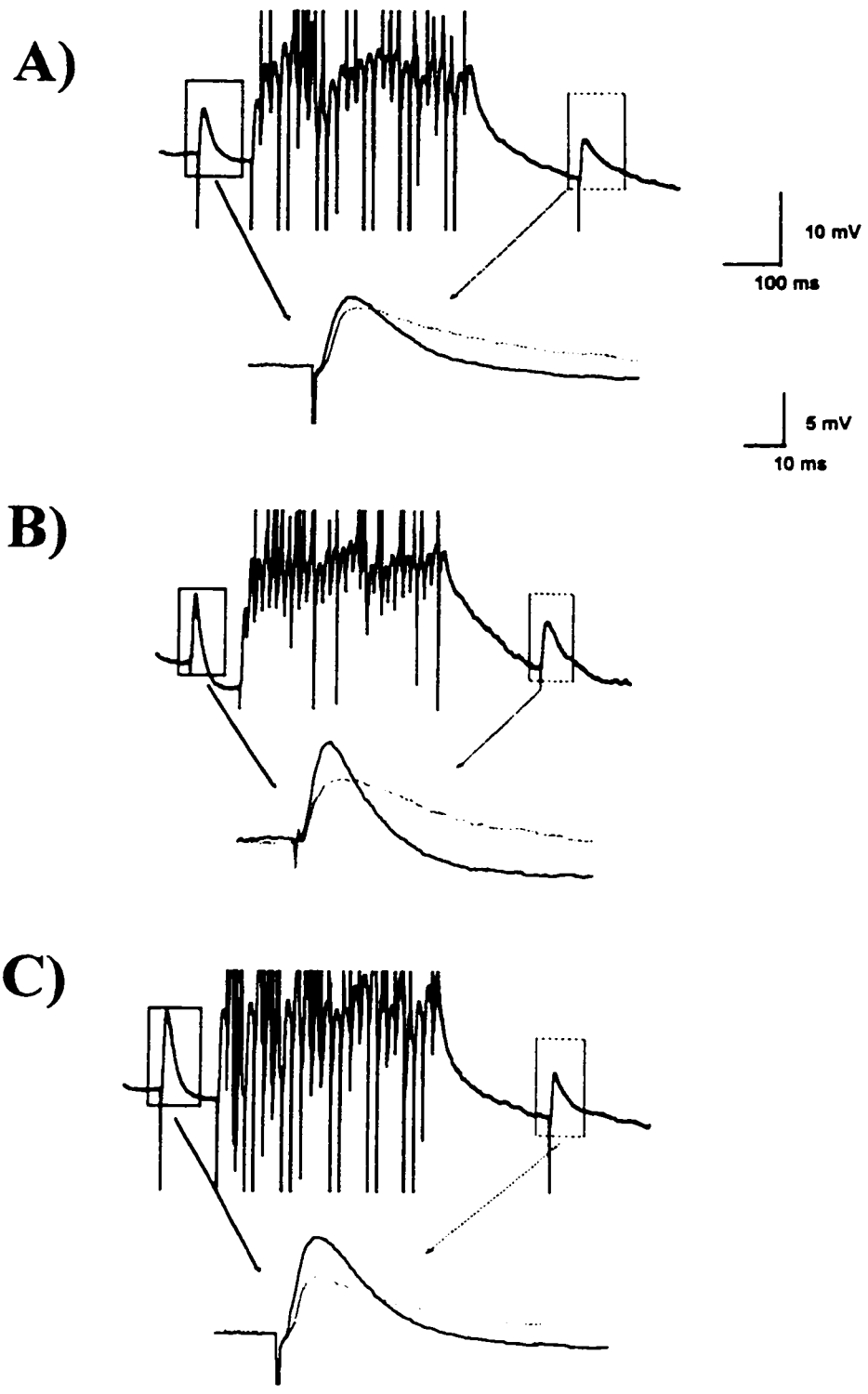


Figure 43: Lack of effect of group II mGluR antagonist on ADD of EPSP amplitudes in the presence of *t*-PDC in rat LC. A) A representative example (average of 3 consecutive sets of measurements in the same cell) displaying ADD of EPSPs under control conditions, B) in the presence of t-PDC (100 μM) and C) in the presence of *t*-PDC and a group II mGluR antagonist (EGLU, 500μM). *t*-PDC and EGLU were applied by bath 10-15 min prior to testing.

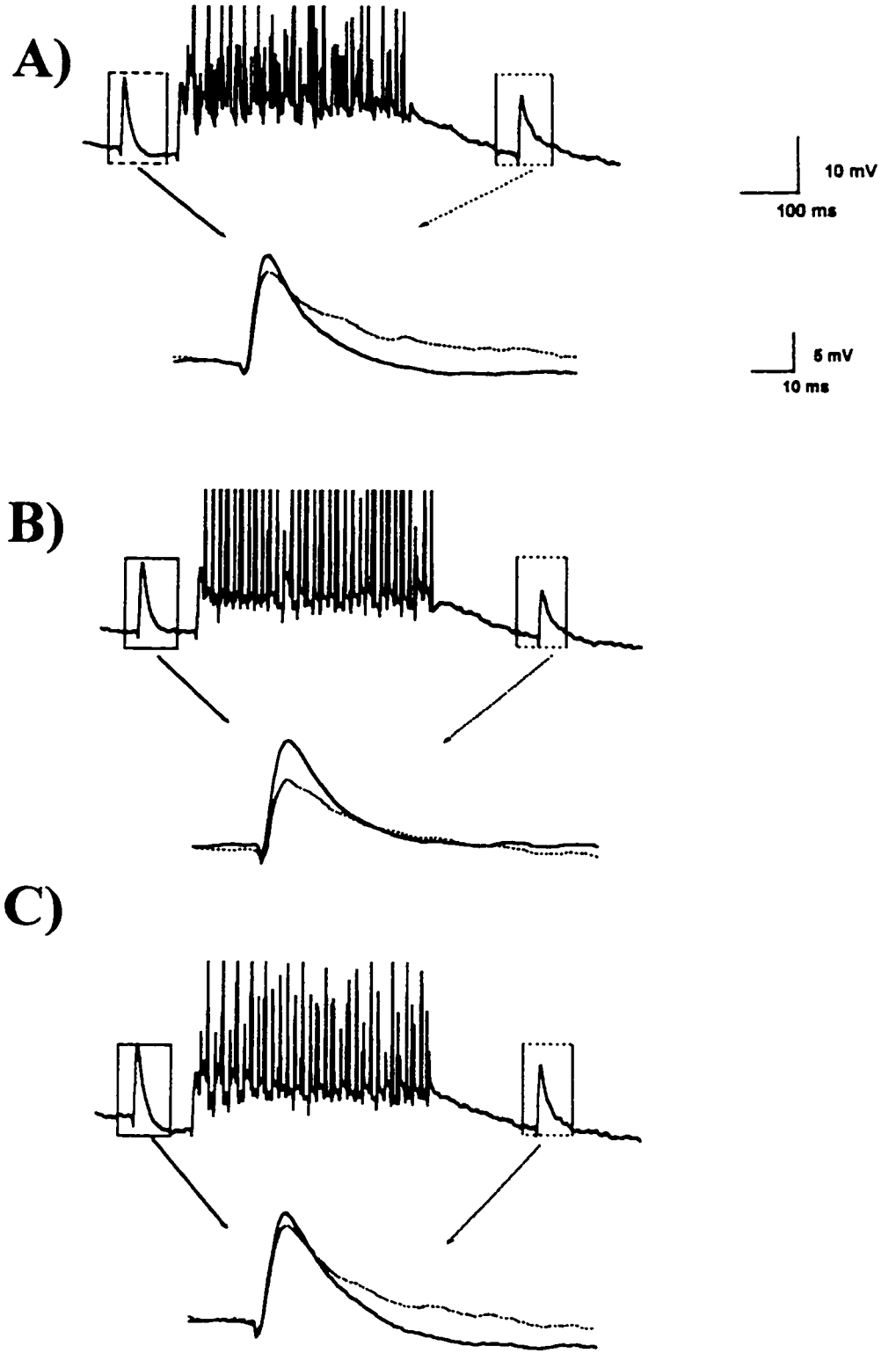


Figure 44: Restoring effect of group III mGluR antagonist on ADD of EPSP amplitude in the presence of *t*-PDC in LC neurons. A) A representative example (average of 2 to 4 consecutive sets of measurements) displaying the ADD of EPSPs under control conditions, B) in the presence of *t*-PDC (100 μM) and C) in the presence of *t*-PDC and a group III mGluR antagonist (MAP4, 500μM). *t*-PDC and MAP4 were applied by bath 10-15 min prior to testing.

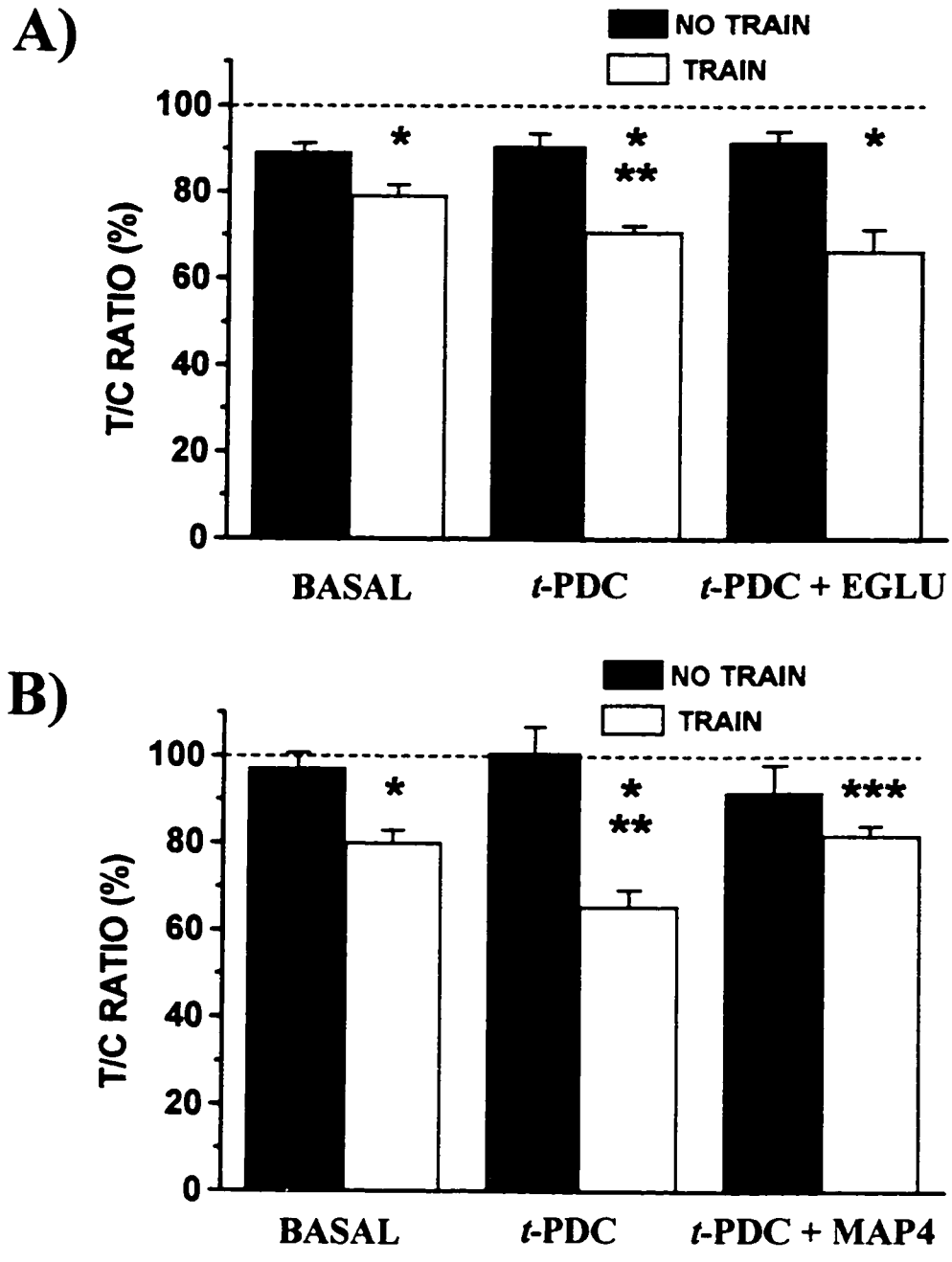


Figure 45: Summary of the effects of group II and group III mGluRs on ADD of EPSP amplitude in the presence of *t*-PDC in LC neurons. A) Summary of the effects of EGLU with *t*-PDC: bar graph comparing the average T/C ratios (index for ADD) in the absence and presence of trains under the conditions indicated [n=8, * significantly different from no train ($p<0.02$); ** significantly different from basal with trains ($p<0.01$)]. B) Summary of the effects of MAP4 with *t*-PDC: bar graph comparing the average ratios in the absence and presence of trains under the conditions indicated [n=5 individual experiments. * significantly different from no train ($p<0.005$); ** significantly different from basal with trains ($p<0.02$); *** significantly different from *t*-PDC with trains ($p<0.006$)].

3.4.2.4. Involvement of other neurotransmitters in ADD

The protocol described above presents certain caveats. One of these is that the focal stimulation applied to the slice in order to evoke synaptic potentials results in the potential release of several neurotransmitters in addition to EAA. Roles for two of these, i.e., GABA and NA, have previously been addressed. While the fast and slow IPSPs mediated by these are blocked by inclusion of specific antagonists in the perfusate, other actions of these and neurotransmitters could potentially interfere with the measurements. As such, the possible involvement of three likely candidates, i.e., GABA (through its action on the $GABA_B$ receptor), adenosine (a known neurotransmitter released during high synaptic activity), and serotonin (shown to depress excitatory synaptic transmission in LC) was investigated. Specific antagonists for each of the three neurotransmitters were tested in a fashion identical to those for mGluRs. Table 7 summarizes the results obtained will all antagonists tested.

Three specific antagonists of $GABA_B$ receptor were tested. 1) Phaclofen is of the first generation of $GABA_B$ receptors antagonist developed and its actions on this receptor are quite weak (Misgeld et al., 1995). 2) Saclofen is part of the second generation of antagonists for $GABA_B$ receptors and while its potency is greater than the former, I (not shown) and others (Misgeld et al., 1995; Kaupmann et al., 1997) have found this compound to be a partial agonist for $GABA_B$ receptors. 3) CGP35348 displayed both high selectivity and potency [IC_{50} ranging from 4-20 μM (Kaupmann et al., 1997)] for $GABA_B$ receptors and is specifically antagonistic. In my hands, CGP35348 (100 μM) reversed the $GABA_B$-(baclofen 3 μM)

ranging from 4-20 μM (Kaupmann et al., 1997)] for $GABA_B$ receptors and is specifically antagonistic. In my hands, CGP35348 (100 μM) reversed the $GABA_B$-(baclofen 3 μM) induced presynaptic inhibition of excitatory synaptic transmission in LC (unpublished observations). At 100-500 μM concentation, neither of these antagonists significantly affected ADD (Table 7). The second set of compounds were antagonists of adenosine receptors. Aminophylline and 8-phenyl-theophylline are antagonists of both the A_1 and A_2 receptors subtype while DPCPX, as described above (section 3.3.4.3.), is a potent and selective A_1 antagonist. Again, neither of the antagonists affected ADD when tested in a manner identical to that described for mGluRs (table 7). Finally, the broad range serotonin antagonist mianserin (500 μM) was also tested, and had no effect on ADD (table 7).

Table 7: Effects of specific $GABA_B$-, adenosine-, and serotonin-receptor antagonists on ADD

Receptors	antagonist	Control T/C	Test T/C	n
$GABA_B$	Phaclofen (500 μM)	0.67 ± 0.08	0.70 ± 0.09	4
	Saclofen (500 μM)	0.68 ± 0.03	0.73 ± 0.06	3
	CGP35348 (100 μM)	0.79 ± 0.04	0.75 ± 0.02	3
Adenosine	Aminophylline (1 mM)	0.70 ± 0.02	0.67 ± 0.04	4
	8-ρ-theophylline (500 μM)	0.74 ± 0.03	0.74 ± 0.02	3
	DPCPX (0.1 μM)	0.79 ± 0.05	0.83 ± 0.09	4
Serotonin	Mianserin (500 μM)	0.78 ± 0.03	0.72 ±0.08	5

4. DISCUSSION

As thoroughly reviewed in the introduction section of this document, the LC is the most prominent noradrenergic nucleus in mammalian brain, sending noradrenergic innervations throughout the neuraxis, including the exclusive supply of NA to the neocortex and the hippocampus. The widespread efferent distribution of LC neurons is in sharp contrast to the restricted number of structures impinging on it. Yet, a large number of compounds has been identified as potential neurotransmitters/modulators acting on LC. As such, the factors, mechanisms and stimuli that control and modulate the activity of the LC nucleus and the outflow of NA throughout the CNS are poorly understood.

The aim of the current study was to further our knowledge of the mechanisms regulating excitatory synaptic transmission to LC. In this work, two mGluRs have been identified and characterized. Activation of these receptors produced a significant reduction of the excitatory transmission to LC neurons. Both receptors were functionally localized to the presynaptic axons impinging on LC. Interestingly, attempts to determine their functions through endogenous activation revealed that only one of these receptors could functionally be activated under conditions that promote excess accumulation of neurotransmitters in the cleft. Therefore, results from this work demonstrate the existence two pharmacologically and functionally distinct mGluRs. In the discussion section, I will compare and contrast my results with current knowledge in the field, discuss the relevance of these finding, and

speculate as to how these receptors may contribute to the overall control of the activity of LC and the brain NA outflow.

4.1. ELECTROPHYSIOLOGICAL PROPERTIES OF LC NEURONS

In the first part of this investigation, LC neurons in brainstem slice preparations were characterized electrophysiologically. Several features of this preparation must first be highlighted, i.e., the orientation of the slice, the age of the animals and the species used. Some of these features have been reported to influence significantly the electrophysiological characteristics and behavior of the impaled neurons. The following is a discussion of some variables that have had an influence on this study.

4.1.1. LC neurons from rats

There are some basic features of LC neurons that have been described in various preparations. In some preparations, LC neurons have been described as spontaneously active displaying bursting activity (up to 25 Hz), as observed either in explant cultures of mouse (Finlayson and Marshall, 1988; Marshall and Finlayson, 1988), or *in vivo*, in unanesthetized rats or monkeys (between 1 to 5 Hz) (Aston-Jones and Bloom, 1981b; Foote et al., 1980). Interestingly, in brainstem slice preparations, bursting behavior is replaced by a basal tonic firing pattern with frequencies ranging between 0.5-5 Hz. This tonic firing has been observed in most neurons recorded in this study, and has been identified as a key characteristic of LC neurons in brain slice preparations (Nakamura and Sakaguchi, 1990; Williams et al., 1984; Williams and Marshall, 1987). It has been shown that the firing activity of LC is

developmentally regulated and LC neurons in fetal and neonatal rats were largely quiescent (Nakamura and Sakaguchi, 1990). With increasing age, the firing pattern changes from quiescent to sporadic burst of discharges to periodic tonic discharges (Nakamura et al., 1987; Sakaguchi and Nakamura, 1987). In my studies, young animals of 30-50 postnatal days were used. This age group is ideal for this kind of work, as young animals appear to be more resistant to ischemic damage, which may occur during the slice preparation. However, they are old enough to display electrophysiological features, such as a tonic firing pattern, which resemble those of adult animals. The difference in firing behavior between *in vivo* conditions and brain slice conditions has been attributed to an excitatory input on LC that is interrupted in brain slices but present in explants (Finlayson and Marshall, 1988; Marshall and Finlayson, 1988).

Another characteristic of LC neurons is the shape and time course of the action potentials. As shown in this study, the action potential could be divided in four distinct components. The fast rising phase was TTX-sensitive, suggesting the action of voltage-dependent Na^+-channels. This was followed by an initial fast repolarisation and the appearance of a second depolarizing phase forming a hump on the falling phase of the action potential. The latter was TTX-insensitive; it was shown elsewhere to be dependent on Ca^{2+} (Masuko et al., 1986). The last phase was characterized by a prolonged after-hyperpolarization merging into a slow depolarization or membrane potential oscillations. Both the slow depolarization and the potential oscillations brought the cell back to the threshold, and contributed to the spontaneous activity of the cells. While spontaneous activity

was blocked by TTX, action potentials could still be evoked but with a threshold several mV higher than under control conditions. This threshold corresponded to the activation potential for the calcium conductance and the resulting action potential displayed significantly slower kinetics. In this study, and others (Williams and Marshall, 1987), neither the after-hyperpolarization phase nor the potential oscillations were affected by TTX. The effect of TTX on spontaneous activity likely represents the inability of the slow depolarizing current to bring the neuron to the higher threshold.

Intrinsic membrane oscillations described in this study have been a source of interest for many years (Williams and Marshall, 1987; Christie et al., 1989). Slow inward movements of extracellular Ca^{2+} in the dendritic region was proposed as an underlying mechanism (Williams and Marshall, 1987). In horizontal brainstem slice preparations, most of the recorded neurons displayed membrane oscillations with frequencies ranging from 1 to 10 Hz and amplitudes ranging from 1 to 16 mV. When characterized in coronal slices, both the amplitudes and the frequencies were developmentally regulated (Christie et al., 1989; Marshall et al., 1991; Williams and Marshall, 1987). Indeed, LC from young animals, in coronal slices, displayed slow (< 1 Hz) and relatively large oscillations while older animals had faster (about 3 Hz) and smaller oscillations. By postnatal day 27, oscillations were seldom observed. This is in sharp contrast with results presented in this study. All animals used in this study were older than 30 days. Yet, oscillations of varying amplitude and frequencies were observed at all ages tested. When I tested for a correlation between age (weight of animals) and oscillation amplitude, a significant but small negative correlation was

found only when a large number of animals (n > 150) was used in the test. This difference in LC properties between different slice orientations is intriguing.

Oscillations in LC neurons are observed even under voltage-clamp, suggesting that the initial source for these is at location remote from the recording electrode (Williams and Marshall, 1987). It has been suggested that remote dendritic regions of LC neurons may contribute substantially to these. Electrotonic coupling between LC neurons has been reported and synchronized oscillations between pairs of neurons have been observed (Christie et al., 1989; Christie and Jelinek, 1993; Marshall et al., 1991). In my work, using horizontal slices, as in Christie et al. (1989) using coronal slices from young animals, the demonstration that LC neurons could be divided in two electrical compartments can be inferred. The results from the time constant analysis in this study indicated that most of the cells analyzed (>90% of the cells) were best fitted with a second order exponential with mean τ_1 and τ_2 values of 17.1 ms and 2.3 ms, respectively. Futhermore, dye-coupling between LC neurons was observed in my preparations.. Christie et al. (1989) reported that LC neurons, in coronal slices from young rats, could also be best fitted with a second order exponential; the mean time constant values in that case were 31.4 ± 2.0 ms and 7.9 ± 1.5 ms respectively for τ_1 and τ_2. [No valid comparisons can be inferred from these values as, in the latter study, cell conductances were significantly altered to inhibit oscillations (i.e., High Mg^{2+}/low Ca^{2+} external solution)]. Cells recorded from adult rat coronal slices were fitted with a single exponential (Christie et al., 1989). These cells did not display oscillations.

From the discussion above, the following question emerges: what are the differences between coronal and horizontal slices which could confer LC neurons with constant oscillatory properties in the latter but not the former? The answer could lie with organization of the neurons within the LC nucleus. Morphological analysis of the adult rat LC nucleus demonstrated that both the somata and the dendritic field of the majority of LC neurons are organized as disk-like shape with the major axis in the rostral caudal orientation (Grove and Wilson, 1980a). Clearly then, coronal sections of LC nucleus would sever the neurons' dendritic tree, the likely source of the oscillations and/or electrotonic coupling, while dendritic structures would be better preserved in horizontal slices. This dendritic tree could contribute to one of the two electrical compartments inferred from the exponential fit. Whether the organization of neonatal LC neurons is substantially different from adults to explain the developmental difference reported in coronal slices is not known. Smaller cells in young animals, which could be entirely contained with the slice, could be a factor which would affect oscillations. Furthermore, LC neurons display morphological plasticity (e.g., cells' nuclear area, or synaptogenesis in LC) throughout early postnatal development and sometimes for up to 60 days after birth (Lauder and Bloom, 1975). Christie et al. (1993) suggested that, while the occurrence of oscillation in LC neurons appears to correlate with that of electrotonic- (or dye-) coupling between neonatal rat LC neurons, other mechanisms, such as dendro-dendritic contacts (Groves and Wilsons 1980a), could become more important in adult rats. Many of these contacts would likely be cut in coronal slices. The function of the oscillations is not understood but it has been speculated that they serve a synchronizing

role bringing groups of LC neurons simultaneously to perithreshold or threshold, leading to synchronized discharge and NA release (Marshall et al., 1991).

4.1.2. LC neurons from mice

Increasingly, genetic approaches are becoming the tool of choice in solving many questions pertaining to current issues in neuroscience. These often make use of mice either in transgenic or functional deletion (KO) models. Some aspects of the research presented in this thesis made use of such an approach. Therefore, basic electrophysiological characterization of mouse LC was carried out, in a fashion similar to that described for rats. To my knowledge, this is the first account of electrophysiological characterization of mouse LC neurons in a brain slice preparations. Although no obvious differences between WT and mutant mice were found in this study, results were kept separate, and the following account of the basic electrophysiological properties of mouse LC neurons only made used of WT mice data.

As a whole, electrophysiological properties of mouse LC in horizontal brain slice preparations were very similar to those of rats except for a few differences. Action potentials displayed a similar shape, and cells were often spontaneously active, with firing frequencies comparable to those in rats. As for rats, the V-I relationship was linear but the input resistance was significantly larger (more than double) in mice. This could be due to smaller size neurons in mice, although no data confirming this is available. Smaller size neurons would help to explain the relatively low success rate for impalement, poor recovery from

impalement injury, and shorter impalement duration, all factors that made this study much more difficult as compared to the situation in rats. As for rats, the time constants of the cells were best fitted with a second order exponential. However, unlike rat LC, no neurons from mice displayed subthreshold oscillations, and spontaneous activity of these cells was driven by a slow depolarization of the membrane similar to that described in LC neurons in coronal slices from old rats. I did not investigate this further. However, this significant difference in behaviour between rat and mouse LC would be worth additional analysis. Results from such a study could reveal key elements for understanding the role, mechanism, and development of these oscillations.

4.2. SYNAPTIC TRANSMISSION TO LC

In the second part of the investigation, evoked synaptic potentials were recorded in LC neurons in horizontal brain slice preparations. Qualitatively similar synaptic potentials have been observed in both coronal and horizontal slices but those recorded from the latter were more effective (Williams et al., 1991). Synaptic potentials in LC have been previously characterized (Cherubini et al., 1988; Williams et al., 1991), and the following is a discussion of my results on the basic properties of synaptic transmission compared to those observed in other studies.

In the current study, EPSPs were isolated from other potentials in two ways. First, Bic (or sometimes picrotoxin) was used to block fast GABAergic IPSPs. Furthermore, I chose potassium acetate over potassium chloride as the electrode solution. This prevented

a shift in chloride reversal potential (E_{Cl}) from around -70 mV to higher potentials. Such a shift would lead to a shift from inhibitory potential (or no potential if the membrane potential is close to E_{Cl}) of chloride-dependent IPSPs (e.g., GABA, glycine) to depolarizing potentials, thus interfering with true excitatory potentials. Under these conditions, stimulations, rostrolateral to the recording site, resulted in a transient depolarization in neurons, i.e., EPSP. While many other transmitters and modulators have been shown to cause a response in LC, only EAA, GABA, glycine and NA-mediated synaptic potentials have been observed in LC (Williams et al., 1991).

The general principles underlying excitatory synaptic transmission are now well characterized, and up to a few years ago, synaptic activation of glutamatergic synapses seemed relatively simple (Mayer and Westbrook, 1987a). Release of EAA at a central synapse resulted in the activation of two distinct classes of ligand-gated channel, the non-NMDA and NMDA, both of which can be localized to the same synapses (Bekkers and Stevens, 1989). Fast synaptic potentials were mediated by the non-NMDA-gated channels and the slower responses were mediated through the opening of the Ca^{2+}-permeable and voltage dependent NMDA receptor channels.

Results from the work presented here demonstrated that the EPSPs evoked in LC (in rats and mice) are principally mediated by CNQX-sensitive receptors, i.e., AMPA- or Ka-gated channels. Conversely, the specific NMDA receptor antagonist, CPP, caused only a small (about 5%) decrease in the amplitude of the EPSPs, in the presence of physiological

Mg^{2+} concentrations and assessed at hyperpolarized membrane potential. Additional evidence supporting a prominent role of non-NMDA receptors in excitatory synaptic transmission in LC is the linear voltage dependency of EPSP amplitudes. This is consistent with previous reports in LC (Cherubini et al., 1988; Ennis et al., 1992; Xiong and Marshall, 1994). It has been shown in hippocampus that NMDA channels, at resting membrane potentials, contribute to a small and variable portion of the EPSPs (Nicoll et al., 1990).

This simple view of excitatory synaptic transmission in LC is not entirely accurate, and a comprehensive discussion of any glutamatergic synapse should now consider several aspects. One of these is the repertoire of different gene families coding for a large number of different receptor subunits. Beside the eight mGluRs, which are the primary focus of this thesis, these subunits comprise the AMPA (GluR1-4), Ka (GluR5-7, Ka1, 2) and the NMDA (NR1, NR2A-D) subunits (Hollmann and Heinemann, 1994). Experiments in expression systems have clearly shown that different combinations of subunits confer distinct channel properties.

In LC, the Ka subunits GluR6 and Ka1 display low expression but Ka2 expression is high (there is no detectable expression of GluR5 or 7 in LC) (Herb et al., 1992; Wisden and Seeburg, 1993). As outlined in the Introduction, GluR5 or 6 is essential for a functional receptor. The co-assembly of either of these with other subunits results in ligand-gated channels with distinct properties (Herb et al., 1992). For example, homomeric GluR6 are absolutely insensitive to AMPA but the presence of Ka2, a very likely composition in LC,

makes the Ka receptor significantly sensitive to AMPA. Besides the Ka subunits, high levels of NR1 subunit immunoreactivity, essential for functional NMDA receptors, and low to moderate levels of NR2A and B (not C or D) subunits have been found in LC (Watanabe et al., 1994; Petralia et al., 1994; Petralia et al., 1994). Receptor channels comprising such subunits will likely display a high magnesium block and a marked voltage sensitivity (Hollmann and Heinemann, 1994). Furthermore, NMDA receptors containing NR2A will display high Ca^{2+}-dependent inactivation (Medina et al., 1995; Krupp et al., 1996). AMPA receptor subunits have not been reported in LC (Petralia and Wenthold, 1992). It is not likely that Wenthold's laboratory overlooked the LC nucleus, during the screening for specific structures expressing GluR1-4. Indeed smaller structures adjacent to LC, such as MeV, have been characterized. LC neurons respond vigorously to focally applied AMPA, and cyclothiazide, a compound that specifically blocks GluR1-4 desensitization (Partin et al., 1993), potentiates the response of LC to both AMPA and Glu (Dubé and Marshall, unpublished observations). However, responses to Ka in hippocampal neurons were reported to be significantly potentiated by cyclothiazide (Patneau et al., 1993). Thus, it is not clear at this point, which non-NMDA receptors may be mediating the EPSPs in LC.

4.3. ROLE OF mGluRs IN EXCITATORY SYNAPTIC TRANSMISSION TO LC

As explained above, excitatory synaptic transmission, or chemical transmission as a whole, is not a process as simple as originally thought. Besides subunit heterogeneity in ligand-gated channels, many different mechanisms such as splice-variants, RNA editing, the

actions of phosphatases and kinases, provide individual neurons with tools to modify the behaviour of a channel. Furthermore, investigators in the field are faced with emerging new facts regarding receptor localization and functional expression. As such, besides the receptors located at the postsynaptic density, recent evidence now places "classical postsynaptic" receptors, such as NMDA (Cheramy et al., 1994; Liu et al., 1994; Alford and Dubuc, 1993; Van Bockstaele and Colago, 1996), AMPA (Cheramy et al., 1994; Alford and Dubuc, 1993; Petralia and Wenthold, 1992) and Ka (Cheramy et al., 1994; Chittajallu et al., 1996; Petralia et al., 1996b) away from the synapse or at presynaptic locations. Finally, a newer subset of receptors is now emerging as significant contributors to synaptic transmission. These receptors are not directly coupled to ion channels, so that their action was not obvious at first. However, activation of these receptors, by the same neurotransmitters, often leads to significant modification of synaptic transmission. As such, their actions are sometimes called "slow" synaptic potentials (Baskys, 1992). The following is a discussion of the characterization of two such receptors, members of the mGluR family, which I have shown to cause a significant decrease in excitatory synaptic transmission to LC.

4.3.1. t-ACPD receptors

In this work, I reported that two mGluR agonists, with mutually exclusive pharmacological profiles could produce similar effects. The first one, *t*-ACPD, is a racemic mixture containing equal amounts of 1R, 3S-ACPD and 1S, 3R-ACPD, of which 1S, 3R-ACPD is the active isoform. It was shown that *t*-ACPD produced a reversible dose-dependent inhibition of evoked EPSPs in LC, with an estimated half-maximal effect achieved

with tens of micromolar. Similar effects of *t*-ACPD in other parts of the CNS have been previously reported (Baskys and Malenka, 1991; Crépel et al., 1991; Calabresi et al., 1993; Glaum and Miller, 1993b; Burke and Hablitz, 1994; Lovinger and McCool, 1995; Poncer et al., 1995). Two groups of mGluRs have been reported to be activated by *t*-ACPD, i.e., those referred to as group I and II (Nakanishi, 1992; Nakanishi, 1994; Roberts, 1995), with group II being relatively more sensitive when tested in expression systems. The pharmacological profile of *t*-ACPD suggests that a group II mGluR (2, 3) is involved. First, two group II-specific agonists mimicked the effect of *t*-ACPD, while the group I-specific agonists produced no change. Second, EGLU, a group II-specific antagonist, antagonized the effects of t-ACPD, while AIDA, the group I-specific antagonist was ineffective. It is not clear why other reported antagonists specific for group II were not able to block the effects of *t*-ACPD. However, it has recently been reported that the antagonistic effects of MCCG on group II receptors can be quite weak (Bushell et al., 1996).

4.3.2. L-AP4 receptors

The second compound tested, L-AP4, has long been identified as an agonist for an elusive Glu autoreceptor (Harris and Cotman, 1983; Cotman et al., 1986a). With the cloning of many different mGluRs, it is now known that the "L-AP4 receptor" is represented by a subset of cloned mGluR (called group III) which comprises four different receptor subtypes (mGluR 4, 6, 7, 8) (Nakanishi, 1992; Thomsen et al., 1992; Nakajima et al., 1993; Tanabe et al., 1993; Saugstad et al., 1994). In this work, L-AP4 also produced a reversible and dose-dependent depression of evoked EPSPs, but with an estimated half-maximal effect achieved

at concentrations several times lower than for *t*-ACPD. The antagonist profile of the L-AP4 response suggests that mGluR 4 or 7 is involved. First, MAP4, a group III mGluR-specific antagonist, blocked the L-AP4 response, but MCCG did not. Furthermore, MCPG, a mGluR antagonist with a broader spectrum of action, was unable to reverse the effects of L-AP4. To date, of the group III mGluR, only mGluR4 and 7 are known to be insensitive to MCPG (Hayashi et al., 1994; Saugstad et al., 1994). Since the affinity of mGluR7 for L-AP4 is several fold lower than that of mGluR4 (Pin and Duvoisin, 1995), these results suggest that the L-AP4 effect is mediated through mGluR4.

To test the hypothesis that mGluR4 was the receptor mediating the effects of L-AP4, I took advantage of a newly developed functionally-deleted mGluR4 (KO) mutant mice (Pekhletski et al., 1996). The mouse was reported to have normal gross motor abilities and no major learning deficiencies [(Pekhletski et al., 1996), and oral communication at the EAA97 meeting, Waterville Valley, NH, July 16-20, 1997]. However, electrophysiological analysis of cerebellar slices indicated that, as compared to WT mice, L-AP4 in mutant mice was unable to inhibit synaptic transmission at the parallel fibre-Purkinje cell synapse, confirming the functional deletion of the receptor (Pekhletski et al., 1996). Thus, it was interesting to find that EPSPs in LC of mutant mice were inhibited to the same degree by L-AP4 as those observed in LC from wild-type mice. This inhibition was comparable in size to that observed in rat LC at the dose of L-AP4 used. The conclusion emerging from these results is that mGluR4 is not the receptor mediating the effect of L-AP4 in mice. Furthermore, it does seem a reasonable extrapolation to conclude that the situation in the rat

is the same, i.e., mGluR4 is not the receptor mediating the effects of L-AP4 on excitatory synaptic transmission in rat LC. Thus, asuming that there are no problems with the KO mouse, the only alternative explanation which would reconciliate all these results is that L-AP4 on excitatory synaptic transmission in LC is mediated through a novel, uncharacterized receptor.

4.3.3. Sites of action of the metabotropic glutamate receptor agonists

I have provided evidence that both agonists, at the doses used in this study, displayed a presynaptic locus of action, and did not alter any of the postsynaptic parameters examined, i.e., membrane potential, input resistance, frequency of firing, intrinsic oscillation, or the response of LC neurons to focally applied Glu. Furthermore, both agonists produced significant increases in paired-pulse facilitation. Paired-pulse facilitation in CNS neurons is observed when two stimuli are applied to the afferents in short succession causing an increase in the amplitude of the second EPSP compared to the first (Cummings et al., 1996; Debanne et al., 1996; Leung and Fu, 1994). This in contrast with the neuromuscular junction, where this procedure usually results in paired-pulse depression (Betz, 1970; Thies, 1965). However, in either model, when the two stimuli are applied under conditions where the release probability of neurotransmitter is reduced, the ratio of the second EPSP to the first is usually lager than that observed in control [e.g., Creager et al. (1980), Debanne et al., 1996, Katz and Miledi, (1968), Manabe et al. (1993) and Thies (1965)]. This change in facilitation is traditionally accounted for by the residual calcium hypothesis (Katz and Miledi, 1968). According to these authors, a small fraction of Ca^{2+} that enters in response to the action

potential mediating the first EPSP, remains in the terminal for several hundred milliseconds, a time course that roughly corresponds to the one described for paired pulse facilitation in this study (~100 ms). This Ca^{2+} is too small to trigger neurotransmitter release by itself but could add significantly to the calcium entering during the second action potential. As a result, the increase in the probability of vesicle docking and fusion produced by the second action potential is greater than that achieved by the first. Futhermore, it has been suggested that during presynaptic inhibition, the "immediately releasable pool of neurotransmitter" would not be entirely used-up and would be available for release following subsequent action potentials which, in combination with the residual calcium, could result in increased probability of release of neurotransmitter (Katz and Miledi, 1968; Stuart and Redman, 1991). Thus, in the present work, results from the paired-pulse facilitation in the presence of *t*-ACPD and L-AP4 are consistent with presynaptic inhibition. Similar results with mGluR agonists have been reported in other parts of the CNS (Baskys and Malenka, 1991; East and Garthwaite, 1992; Rainnie and Shinnick-Gallagher, 1992; Kemp et al., 1994; Burke and Hablitz, 1994; Gereau and Conn, 1995b). Together, these results are consistent with a presynaptic locus of action for mGluRs.

A note on the time-course of PPF should be made. In this study, PPF was observed 20 ms following the first EPSP and remained for more than 100 ms. The decay in the PPF with time could be fitted with a single exponential. This is slightly different from time-course results in hippocampal cultures presented by Debanne et al. (1996) which showed an increase in PPF between 30 and 70 ms and a subsequent decrease with time similar to that observed

here. Alternatively, Stuart and Redman (1991) used interstimulus intervals of only 4 ms in group Ia spinal cord afferents. Thus, the stimulus interval may be a function of the system studied, and sensory neurons may have faster kinetics than other neurons. No additional characterization was carried out in LC.

4.3.4. mGluRs, LC and LC afferents

The LC nucleus has been found to express a few mGluR subtypes. Out of the eight mGluRs cloned, the LC nucleus displays very low levels of mGluR3 mRNA (Ohishi et al., 1993), but very high mGluR7 mRNA expression (Kinzie et al., 1995; Ohishi et al., 1995). Furthermore, low levels of mGluR5 receptor protein immunoreactivity have been detected (Romano et al., 1995). Finally, no significant mRNA expression of mGluR8 was found associated with LC (Saugstad et al., 1997, and G. Westbrook, personal communication). The presence of mGluR5 or others could explain the postsynaptic depolarization observed with high concentrations of *t*-ACPD (50-500μM). Gereau and Conn (1995b) demonstrated L-AP4 presynaptic activity on synaptic transmission to CA1 hippocampal neurons with agonist concentrations in the hundreds of micromolar range. They have suggested that mGluR7 is involved as this receptor displays low sensitivity for L-AP4 (the sensitivity of mGluR7 to L-AP4 is roughly a 100-200 fold lower than that of mGluR4 and mGluR8) and is not affected by ACPD (Okamoto et al., 1994; Saugstad et al., 1994). High concentrations of L-AP4 (500 μM) did not produce any notable postsynaptic changes. This was somewhat surprising since mGluR7 is apparently very prominent in LC. However, I only surveyed a few

electrophysiological parameters at high doses of L-AP4 and more systematic investigations should be used to determine the function of this receptor in LC.

As the majority of the mGluR effects described in this thesis apear to be presynaptic, however, the distribution of mGluRs to the LC afferents maybe more pertinent to the discussion of my results. As such, low to moderate levels of immunoreactivity for mGluR2/3 were associated with PGi and PrH nuclei (Petralia et al., 1996a) and low levels of mRNA of mGluR3 in PrH (Ohishi et al., 1995). Moderate levels of mGluR7 mRNA were localized to PrH (Ohishi et al., 1995). Finally, mGluR4 mRNA was undetectable in PrH (Ohishi et al., 1995) while mGluR8 was not reported for these structures. Therefore, the two main structures which innervate LC express members of mGluR group II and III. However, the fact that some cells in these structures stained for specific mGluRs does not necessarily imply that these neurons are the ones synapsing on LC.

A major question is: where are the receptors? The ramifications from this question are many folds. For example, (1) are the afferent fibres stimulated part of a single population of axons, or a mixed population coming from different sources (i.e., PGi and PrH)? (2), Are both group II and III mGluRs present on the same excitatory presynaptic terminals innervating LC neurons? (3), If on distinct axons, are the latter coming from the same nucleus, or are receptors segregated based on the source of the afferents (i.e., one type on PGi axons and one on PrH)? (4) Are the effects of the receptors mutually exclusive, additive, or saturable? (5) Are the receptors coupled to similar signaling pathways? The following

sections will discuss these pertinent questions based on the results obtained in this study and others.

The afferent innervation of LC appears to be very limited (Aston-Jones et al., 1986); the one main structure found to send excitatory axons to LC neurons is the PGi (Ennis and Aston-Jones, 1988; Ennis et al., 1992). However, excitatory synaptic input from PrH cannot be excluded, as this nucleus was also shown to use EAA as neurotransmitters (Aston-Jones et al., 1991), although in a minor proportion. It is reasonable to assume, based on these anatomical observations, that, even a full and specific inhibition of the PrH pathway would only result in a small decrease in EPSP amplitude. This situation is reflected by data presented in this work, as inhibitions observed with either mGluR agonist seem too large to be the exclusive result of PrH inhibition. Alternatively, either or both receptors could be on both PGi and PrH terminals. If this were the case, the dose-response curve would be better fitted with a double sigmoidal curve with different maxima. A close inspection of the data fails to suggest this with *t*-ACPD. However, the dose response curve for L-AP4 (fig. 21) did display a small biphasic shape between the concentrations of 10^{-6} and 10^{-5} M. However, upon reexamination of the dose-response for L-AP4, this time comparing a one binding site vs. a two binding site model, it is clear that a one binding site model fitted the data significantly better ($p<0.05$). Thus, although the possibility that a heterogeneous population of afferents participate in the L-AP4 actions cannot be completely excluded, the data clearly suggest that most of the effect of L-AP4 is a result of a homogeneous population of receptors/afferents, i.e., PGi axons.

The next logical question is whether both receptors are present on the same terminal or is there heterogeneity between PGi axons. From the results, I must conclude that both receptors are present on some if not all axons. The first line of evidence supporting this conclusion comes from the maxima from the dose-response curve of each agonist. Indeed, if each receptor was segregated on mutually exclusive axons, the sum of the individual maximal effects would not exceed 100%. This is clearly not so as each agonist exhibited maximal inhibitions > 60%. A similar scenario, where two mGluRs are expressed on the same axon terminals, has been proposed to explain results in other systems (Baskys and Malenka, 1991; Burke and Hablitz, 1994; Bushell et al., 1996; Gereau and Conn, 1995b; Salt and Eaton, 1995).

Both group II and group III receptors are thought to be coupled to the same signaling pathway (Pin and Duvoisin, 1995). This raises interesting questions as both receptors must be co-expressed at least on a fraction of the terminals, each producing similar decreases in excitatory synaptic transmission. In addition, results show that concomitant application of both agonists produces an inhibition superior to the maximal inhibition observed with either agonist alone at a given dose. However, at high doses, the inhibition was only partially additive. This suggests that upon activation with high concentrations of agonists, a saturation of the signal occurs at a site downstream from the receptors. This is a plausible hypothesis, especially if both receptors share a common signaling pathway. To address this, two tests were performed on the premise of the reported coupling of group II and III. Thus, while the complete mechanism of action of mGluRs at the presynaptic terminal could not be

determined, I have shown that the two agonists acted on receptors that depended on the functional coupling of the receptor to a $G_{i/o}$ member of the G-protein family, in accordance to a group II and III receptor.

The best described consequence of the activation of group II and III mGluR is the reduction of the forskolin-stimulated cAMP levels through the coupling of the receptor to G_i. However, in experiments where levels of cAMP were "clamped" to high concentrations (using non-hydrolysable cAMP analogues, the AC stimulating compound forskolin, and the cAMP degrading enzyme inhibitor IBMX), clearly showed that both L-AP4 and *t*-ACPD actions were unaffected. Thus, the mechanism of inhibition of these two agonists in LC is independent of the activity of AC and intracellular levels of cAMP. These results are similar to those reported for the presynaptic actions of other neurotransmitters (e.g., muscarinic M_4, α_2-adrenergic, somatostatin, adenosine A_1, dopamine D_2, $GABA_B$) (Campbell et al., 1995; Shapiro et al., 1994; Toth et al., 1996; Wickman and Clapham, 1995; Yan et al., 1997). In these studies, the inhibition was attributed to the inhibition of N-type calcium channels [for review, see Wickman and Clapham (1995)]. It was recently reported that mEPSCs in mitral cells of the olfactory bulb were inhibited by activation of mGluR8 (Schoppa and Westbrook, 1997). Like evoked EPSPs in our study, L-AP4-mediated inhibition of mEPSCs in these cells was independent of cAMP levels (Schoppa and Westbrook, 1997). Although the specific mechanism mediating the inhibition could not be determined, it was suggested that this could result from the interaction of $G_{i/o}$ G-protein with a specific component of the secretory machinery.

Dunlap's laboratory, using chick embryo DRG neurons, has described two possible pathways that mediated N-type calcium current inhibitions as mediated by NA and GABA (Diversé-Pierluissi and Dunlap, 1993, 1995; Diversé-Pierluissi et al., 1995). The first was a G_i-mediated, PKC-dependent inhibition of the steady-state current activated by NA but not by GABA. This inhibition was mimicked by applications of ßγ subunits, and inhibited by anti-ßγ antibodies. These effects were attributable to the activation of PLC by these subunits. The second pathway was a steady-state inhibition of N-type currents, independent of G_i and PKC but was dependent, at least in part, on the activation of G_o. This has been the most commonly described pathway for many neurotransmitters, and is commonly referred to as the membrane-delimited pathway to contrast it from pathways dependent on soluble messengers (Shapiro et al., 1994; Wickman and Clapham, 1995). The G-proteins involved in membrane-delimited N-type (also L-, and possibly P/Q-type) calcium channel inhibitions are PTX-/NEM-sensitive (Shapiro et al., 1994; Wickman and Clapham, 1995). Furthermore, evidence suggests that $G\alpha_o$ is preferentially involved over $G\alpha_i$. Firts, whole cell perfusion of G protein heteromeres into PTX treated DRG neurons demonstrated tha $G\alpha_i$ was more efficient than $G\alpha_i$ in ihnibiting VDCC (Hescheler et al., 1987). Second, antisense nucleotides specific to $G\alpha_o$, but not $G\alpha_i$, reduced receptor-dependent inhibition of VDCC in sympathetic neurons (Campbell et al., 1993). Finally, injection of $G\alpha_o$ antibodies into sympathetic neurons reduced receptor-dependent N-type Ca^{2+} channel inhibition (Caulfield et al., 1991). Furthermore, while G_o-dependent inhibition is caused by the α subunit (which determines the class of the G-protein), additional evidence clearly demonstrated that the ßγ subunit can also cause membrane-delimited inhibition (Bourinet et al, 1996; Herlitze et al., 1996; Ikeda, 1996; Patil

et al., 1996). As demonstrated for α_2-adrenoceptors (Diversé-Pierluissi and Dunlap, 1995; Diversé-Pierluissi et al., 1995; Shapiro et al., 1994), PKC- and G protein-mediated inhibition are not mutually exclusive. Recently, protein segments of the N-type calcium channels which could be involved in the crosstalk modulation of N-type calcium channels between a PKC-sensitive and a ßγ-sensitive pathway have been described (Zamponi et al., 1997), lending support to the concept of dual modulation of presynaptic Ca2+ currents by G-proteins.

While the exact mechanisms involved in the *t*-ACPD and L-AP4-mediated inhibitions could not be determined in this study (brain slice preparations are not suitable for in-depth studies required for this), interaction between VDCC and mGluRs have been reported in other systems. All three groups of mGluR have now been implicated in the modulation of different VDCC in a variety of neuronal and glial cells. Inhibition has been found to occur at the N-type Ca^{2+} channel following activation of group II (Sayer et al., 1992; Sahara and Westbrook, 1993; Swartz et al., 1993; Hay and Kunze, 1994; Rothe et al., 1994; Stéfani et al., 1994; Chavis et al., 1995; Choi and Lovinger, 1996), and group III mGluRs (Sahara and Westbrook, 1993; Rothe et al., 1994). Inhibition of the L-type Ca^{2+} channels was also reported with group II (Sahara and Westbrook, 1993; Chavis et al., 1994; Chavis et al., 1995), and possibly group III (Sahara and Westbrook, 1993) [group III mGluRs have been shown to decrease Ca^{2+} through undetermined Ca^{2+} channels (Trombley and Westbrook, 1992; Herrero et al., 1996; Stéfani et al., 1996)]. Modulation of P-type Ca^{2+} channels by mGluRs has been shown (Glaum and Miller, 1995; Choi and Lovinger, 1996). In sympathetic neurons, functional coupling btween native N-type Ca^{2+} channel, and recombinantly expressed

mGluR2, has been demonstrated (Ikeda et al., 1995). In the majority of reports, inhibition of VDCC by mGluR has been shown to result from a membrane-delimited signal transduction system. However, as for other neurotransmitter receptors, activation of a PKC-dependent pathway was found to reverse the effects of mGluR on Ca^{2+} channels in some systems (Swartz et al., 1993; Herrero et al., 1996). Co-expression of specific group II mGluRs and N-type calcium channels in HEK cells provided evidence for a functional coupling that was PTX- and NEM-sensitive (McCool et al., 1996). This study is consistent with the findings presented here for synaptic transmission in LC.

Therefore, in answer to the questions outlined above, data presented here, with information from the literature, provide evidence for two mGluRs, a group II and a group III, located presynaptically on PGi axon terminals impinging onto LC neurons (PrH involvement could not be ruled-out but its contribution would be very small). These receptors mediate their effects through a NEM-sensitive receptor but whether it is G_i or G_o could not be determined in my preparations. The effects of both agonists were independent of cAMP-dependent pathways. Furthermore their effects were only partly additive suggesting that they share a common, saturable pathway. Whether they use the same pathway (e.g., G-protein-mediated inhibition of N-type calcium channels) or different pathways that converge at a specific site downstream from the receptor activation is not known. Obviously, the usefulness of LC in brainstem slice preparations to answer the remaining questions is limited, and additional investigations on the mechanisms underlying the effects of group II and III mGluRs on synaptic transmission will be better served in preparations where the presynaptic neurons

can be directly studied (e.g., isolated PGi neurons or in brain slices containing PGi). Such experiments may be challenging as, unlike LC, PGi neurons are very diffuse and poorly characterized.

To come back to cAMP, it is clear from my experiments that this compound did produce presynaptic modulatory effects on synaptic transmission. As demonstrated in many other preparations, activation of the cAMP/PKA pathway was found to facilitate synaptic transmission (Chavez-Noriega and Stevens, 1994; Capogna et al., 1995; Dixon and Atwood, 1989; Salin et al., 1996; Trudeau et al., 1996). Application of the "cAMP cocktail" to LC preparations resulted in a large and significant increase in EPSP amplitude, with no change in post-synaptic responses to exogenously applied Glu. This increase in EPSP amplitude was accompanied by a significant decrease in paired-pulse facilitation. This suggests that, under these experimental conditions, a positive presynaptic modulation of synaptic transmission was observed, in full agreement with previous observations. Furthermore, the time course for the washout of the potentiation outlasted that of the washout of the cAMP cocktail, reminiscent of a kinase action (Dixon and Atwood, 1989; Weisskopf et al., 1994).

The lack of postsynaptic effects of the cAMP cocktail is puzzling. Both NMDA and non-NMDA currents are potentiated by a cAMP/PKA-dependent pathway (e.g., Greengard et al., 1991; Wang et al., 1991; Cerne et al., 1993; Blackstone et al., 1994; Wyllie and Nicoll, 1994; Raman et al., 1996). Furthermore, cAMP, cAMP analogues or activation AC were found to increase significantly the rate of firing of LC neurons (Wang and Aghajanian, 1987;

Alreja and Aghajanian, 1991a, b, 1995) in transverse slice preparations. Compounds were applied by bath (during intracellular recording) or through the patch pipet. The reason for the lack of effect of the cAMP cocktail on the response to exogenously applied Glu or on the firing rate of LC in my preparation is unknown. However, the methodology used by Wang and Aghajanian varried somewhat from mine. For example, low resistance (about 15 MΩ) electrodes filled with 2M KCl were used. In this study, I used higher resistance electrodes (about 80 MΩ) and potassium acetate solution (2M). Hence with the former approach, low resistance KCl electrodes leading to a change in E_{Cl}^- could contribute to the difference in observation. Alternatively, the orientation of the slice (coronal v.s. horizontal) could also be implicated. However this is speculative and additional tests are required to clarify the reasons for these differences.

4.4. ACTIVITY-DEPENDENT MODULATION OF EPSP IN LC NEURONS: A POSSIBLE PHYSIOLOGICAL ROLE FOR mGluRs

The discussion up to now has focussed on the interpretation and implications of the pharmacological activation of two mGluRs. This is very important as it helped to delineate the identity, location and mechanisms associated with the receptors. However, as for the majority of the transmitters and modulators found to produce an action on LC neurons, a physiological role for mGluRs remained speculative, and endogenous activation of these receptors in LC remained to be demonstrated. The fact that mGluR antagonists alone had no obvious effects on the amplitude of EPSPs was indicative that these receptors were not activated following single stimulations. From this and other results presented here, I

hypothesized that, in LC, one or both of the mGluRs, present presynaptically on the afferents, played a role of autoreceptors with the ability to reduce synaptic transmission if the latter became highly active. To test this hypothesis, the conditions to produce excess neurotransmitter release had to be established. As an introduction, it would be useful to review the events that occur following a single EPSP. Therefore, in the following section, I will discuss various events underlying a single EPSP with an emphasis on those events which could determine the activation of mGluRs.

4.4.1. The dynamics of simple synaptic transmission

The time course of transmitter concentration in a synaptic cleft following a single stimulus can be influenced by a number of parameters. These were recently delineated by Clements (1996) as follow: EPSPs are dependent on 1) the number of vesicles simultaneously released into the cleft; 2) the location at which they are released; 3) the concentration of neurotransmitters in the vesicle; 4) the rate of transmitter release through the vesicle fusion pore into the cleft; 5) the neurotransmitter diffusion coefficient; 6) the geometry of the cleft and the extrasynaptic space, 7) the distribution and affinity of the receptors and transporters (binding sites for neurotransmitters) 8) the transporter uptake rate. Obviously, most of these parameters have not been accurately determined experimentally and will likely vary between synapses and between systems. However, keeping in mind the variability, certain parameters can be estimated. The concentration of Glu in vesicles has been estimated to be between 60-120 mM (Burger et al., 1989; Nicholls and Attwell, 1990). Once the content of one vesicle is released, the concentration of Glu in the cleft is estimated to peak

to 1.1 mM and remains >1 mM for the first 100-200 μs. It then decreases to >100 μM for 1 ms and to >50 μM for the following 5 ms (Clements et al., 1992). These numbers were estimated assuming no uptake mechanisms for Glu. With low uptake rates, the lifespan of Glu is shortened so that its concentration is reduced to <50 μM within 0.9 ms (Holmes, 1995) (these values are predicted at physiological temperature). After 5 ms, the concentration of extrasynaptic Glu is estimated to be >10 μM (in the absence of uptake) and full diffusional clearance would occur within 50-200ms. At rest (when no relase occurs) it was calculated that the transporter could buffer extracellular Glu concentrations down to as low as 0.6 μM (Bouvier et al., 1992) and experimental measurements indicate extracellular concentrations of Glu of about 1 μM (Benvenisite et al., 1984).

Upon contrasting these concentrations with the respective affinities for different Glu receptors, one can better appreciate the intricacies underlying synaptic transmission. Indeed, the average K_D for NMDA receptors is 1 μM, while that of AMPA receptors ranges from 250 to 1500 μM (Clements, 1996). The EC_{50}s of Glu for mGluRs range between 4-50μM, with the exception of mGluR7 which is about 1000 μM (Pin and Duvoisin, 1995). Thus, the concentration of Glu stays at AMPA-activating concentrations for only about 1 ms, but is high enough to bind to NMDA and mGluRs for a relatively long time after release. While activation of NMDA receptors is restricted by the magnesium block, it is likely that mGluRs, if localized to the synaptic cleft, would be activated. However, the effects of this activation would not be readily observed as the rate of activation of G-coupled receptors is markedly slower than that of ionotropic receptors with the fastest effects occurring only after 50 ms.

Overall, G-coupled receptors elicit their effects several hundreds of ms, even seconds, after activation (Wickman and Clapham, 1995).

However, the question is: are the mGluRs localized close enough to the release sites to be influenced by the above described schemes? Peter Somogyi's laboratory (Baude et al., 1993; Nusser et al., 1994; Luján et al., 1996; Shigemoto et al., 1996) and others (Martin et al., 1992; Romano et al., 1995; Petralia et al., 1996a) have addressed this question using combinations of electron microscopy and immunocytochemistry. First, these results demonstrated that the group I mGluRs (mGluR1α and mGluR5) appeared to be restricted (with some exceptions) to the postsynaptic side of the synapse but in the periphery of the PSD or on the dendrite shaft (segregated away from GluR immunoreactive signals), i.e., perisynaptic (Martin et al., 1992; Baude et al., 1993; Nusser et al., 1994; Romano et al., 1995) although staining of the PSD occasionally occurred. Second, group II mGluRs were found to have a dual distribution with postsynaptic staining localized to the lateral edges of the PSD (perisynaptically), but presynaptic signals were often in marked association with the entire presynaptic terminal, consistent with a role for these as autoreceptors (Petralia et al., 1996a). Little is known about group III mGluRs and only mGluR7 has been characterized. In hippocampus, this receptor densely labelled the presynaptic terminals in a cell specific manner (Shigemoto et al., 1996).

Therefore, to summarize data and estimates, it is suggested that there is enough Glu, for a long enough time to activate most mGluRs, if the are localized close enough to the

synaptic cleft. Once activated, the elicited effects would likely be seen after a very minimum of 50 ms delay. Under these conditions, and in accordance to the microscopical data, presynaptic mGluRs, which are located to the release site, could be activated, but those located to the perisynaptic regions would have low probabilities of activation. How do these observations fit with results presented here? First, it was shown in the last section of this work that the amplitude of the control EPSPs, during activity-dependent experiments, were not affected by antagonists to group II or III mGluRs, i.e., single evoked EPSPs were not affected. This is consistent with the time required for elicitation of the mGluRs effects (>50ms), but also indicates that these receptors are not tonically activated. Conversely, others have shown that a MAP4-sensitive mGluR was tonically active in SON magnocellular neurons (Schrader and Tasker, 1997).

If mGluRs were activated following a single elicited EPSP, would later EPSPs be affected? Although results from this work do not address this question directly, the finding that the PPF amplitudes decay with time following a single exponential process would suggest that no other mechanisms participate in the decrease. However, this is circumstantial and speculative, and additional work would be required to test thoroughly the hypothesis that mGluRs are activated following a single EPSP and participate in the decay of PPF. Fortunately, other experiments in this work demonstrated that activation of mGluRs requires more robust and lengthy stimulations than a single EPSP to cause a significant decrease in following EPSPs.

4.4.2. The dynamics associated with endogenous activation of mGluRs

As demonstrated, mGluRs do not likely influence excitatory synaptic transmission under the simple conditions described at the begining of this section. Therefore what are the conditions that allow for endogenous activation of either or both groups of mGluRs in LC? Intuitively, if dealing with autoreceptors or perisynaptic receptors, any attempts to increase Glu release, accumulation and/or spillover should increase the probability of activation of mGluRs. Furthermore, any tests for activation of mGluRs should consider the latency periods associated with G-coupled receptors. Going back to the eight parameters that influence synaptic transmission, one can appreciate that only two of these can be modified experimentally: A) the number of vesicles released (1) can be increased experimentally by increasing the strength, duration, number and frequency of stimuli applied to the afferent, and B) the transporter-mediated uptake of Glu (8) can be specifically reduced or blocked. These are the two approaches used in this study in an attempt to activate mGluRs.

4.4.2.1. ADD of EPSP and the role of mGluRs

From the PPF experiments, it was established that two EPSPs, delivered 800 ms apart, display identical amplitudes. This was the premise for the following tests. Trains of varying duration and frequencies (in effect increasing the release of transmitters) were applied 100 ms after the first EPSP was evoked and the amplitude of the second EPSP was compared to the first as a measure for any activity-dependent changes. It was interesting to find that large stimulations of the afferents were necessary to induce significant depressions of the second EPSP (ADD). Indeed 300 ms stimulation volleys at 50-70 Hz were needed to depress

significantly an EPSP evoked 200–400 ms later. This is in contrast with others, which showed ADD of EPSPs at 20 Hz following only 100-200 ms in cultured hippocampal neurons (Maki et al., 1995) and two seconds in NTS slices (Glaum and Miller, 1993b). To my knowledge, there is no published information regarding the frequency of firing of LC afferents (PGi) and only population frequencies were measured (Ennis and Aston Jones, 1988). Therefore I have no information to compare frequencies used in my study to conditions *in vivo*.

When tested on ADD in LC, I found that mGluR antagonists had little effect. The group I/II and group II mGluR antagonists failed to change significantly the depression while the group III antagonist MAP4 produced a significant but very small reversal. Together, these results provided poor evidence of marked involvement of mGluRs in ADD of EPSPs, suggesting one of two things: 1) the *t*-ACPD and L-AP4 receptors pharmacologically identified in this study do not function as autoreceptors, or 2) these receptors are not activated under the conditions tested.

In comparison, published work provides mixed results. In cultured hippocampal neurons, the frequency-dependent inhibition of EPSPs was not affected by (RS)-MCPG (500μM; RS is the racemic form of the antagonist) but other antagonists were not tested (Maki et al., 1995). In NTS brain slices, application of low doses (100 μM) (RS)-MCPG not only blocked the depression of excitatory synaptic transmission following 20Hz stimulations but the size of the EPSPs was increased over control amplitudes (Glaum and Miller, 1993b). Little explanation is provided, but the authors suggested that presynaptic mGluRs could be

tonically activated under basal conditions. In hippocampal slices, evidence was provided indicating that continuous stimulation at 1 Hz resulted in the activation of presynaptic mGluRs (Scanziani et al., 1997). Contrary to the results reported here, however, this kind of stimulation protocol produced an increase in the EPSP amplitudes as compared to control situations where low frequency stimulations (0.05Hz) were applied. Nonetheless, 1 Hz stimulation appeared sufficient to cause the activation of mGluRs, and blockade of these resulted in an ***additional*** increase in the amplitude of the EPSPs (Scanziani et al., 1997). It is noteworthy that, in the latter study, very high doses of antagonists were used (1.5 mM of (R)-MCPG, the active form of the antagonist), i.e., a concentration three times larger than that used in my work and 15 times larger than that reported by Glaum and Miller (1993). It is also three times larger than the maximal dose used in a previous study from the same lab (Manzoni et al., 1995). Similar findings in the same study were observed with 500 μM MCCG a group II-selective antagonist suggesting that the antagonism was specific to the inhibition of group II mGluRs (Scanziani et al., 1997). Finally, in the Calyx of Held synapse, 300 μM MCCP, a new antagonists to both group II and III mGluRs (Janes et al., 1996), and which was found to completely reverse the effects of both *t*-ACPD and L-AP4 at this synapse caused a modest but significant reversal (6%) of ADD induced by a 5-10 Hz stimulation of the presynaptic terminal (von Gersdorff et al., 1997). No tonic activation of MCCP-sensitive presynaptic mGluR were observed in this preparation. Together, these results suggest that mGluRs can be endogenously activated under specific conditions and that these may vary between different systems and protocols, likely due to the heterogeneity that exists between synapses in different parts of the CNS.

To test the hypothesis that the volley of stimulations applied to LC were insufficient to produce activation of presynaptic mGluRs, I attempted to experimentally increase the accumulation of Glu at synapses even more by blocking Glu uptake using *t*-PDC (100 μM), in combination with volley stimulations. As such, the two parameters that could be experimentally modified to increase the probability of activating mGluRs were altered. Note that successful blockade of Glu uptake was experimentally supported by the observations that the LC response to focally applied Glu was increased. As expected, under these conditions, ADD was increased. Conversely, the amplitudes of single EPSPs were not affected by this procedure. Accordingly, I retested the mGluR antagonists on ADD using this protocol. Unlike previous tests, results from these experiments provided convincing evidence for presynaptic mGluR activation. Furthermore, these tests allowed for a differentiation of the group II (*t*-ACPD/EGLU-sensitive) and group III (L-AP4/MAP4-sensitive) mGluRs, other than through agonist application. Under these conditions, only group III mGluR was activated and the blockade of this receptor by MAP4 reversed ADD of EPSPs. Thus for the first time, a MAP4-sensitive mGluR is shown as a potential autoreceptor with the ability to decrease excitatory synaptic transmission under conditions where release of EAA is increased and/or clearance decreased. This is the first functional differentiation between two groups of mGluRs present on the same terminals. The significance of the glutamate transporter physiology and pathophysiological conditions has recently been addressed. Significant down-regulation of the glial transporter GLAST (assessed by western blot analysis) was reported in the piriform cortex/amygdala as early as 24 h (stage 3 seizure) following kindling-induced epilepsy and persisted through multiple stage 5 seizure (Miller et al., 1997). In contrast,

levels of the neuronal transporter EAAC-1 were found to be up-regulated in the same areas but only when the animal had reached the stage 5 level. Hence the ability for glutamate buffering between stage 3 and 5 was likely markedly reduced. Thus while *t*-PDC application may not reflect physiological conditions, it clearly mimics conditions observed under specific pathologies such as epilepsy and likely other uncharacterized disorders.

Again, there are some differences between results from my study and others, although not many such studies have been reported. The lack of effects of *t*-PDC on single EPSPs is in contradiction with others that demonstrated depression of single EPSPs in hippocampal cultures with 250 μM *t*-PDC (Maki et al., 1994; Fitzsimonds and Dichter, 1996). In the latter reports, this depression of EPSPs was reversed by MCPG and other antagonists were not tested. Conversely, results from hippocampal slices showed that *t*-PDC did not affect basal synaptic transmission, i.e., when EPSPs were evoked at a 0.017 Hz frequency (Scanziani et al., 1997), in agreement with results in this work. However, in a fashion similar to that presented in this project, *t*-PDC significantly decreased the amplitude of EPSPs evoked at 1Hz (Scanziani et al., 1997). Again, in this study, MCPG (1.5 mM) reversed the effects of *t*-PDC at 1 Hz and had no effects on low frequency synaptic transmission (Scanziani et al., 1997). In the three studies mentioned above, either mixed CA3-CA1 cultures or Schaffer collateral-CA1 synapses in slices were studied. This synapse was found to display presynaptic group II mGluRs but low sensitivity to L-AP4, likely suggesting the presence of mGluR7 [e.g., Baskys and Malenka (1991), Watkins and Collingridge (1994), and Gereau and Conn (1995b)]. Microscopical findings clearly demonstrated presynaptic clustering of mGluR7 at

CA1 hippocampal synapse (Shigemoto et al., 1996). It will be interesting to see if endogenous activation of this receptor can be demonstrated, keeping in mind its low affinity for glutamate.

4.4.2.2. ADD of EPSPs and other neurotransmitters

The lack of action of antagonists to other neurotransmitters in ADD is interesting. I had hypothesized that, following the stimulation volley, a certain number of different neurotransmitters would be released, subsequently causing various pre and/or postsynaptic effects. Of those possible candidates, the actions of adenosine, GABA and serotonin were surveyed. The role of adenosine as a potent presynaptic inhibitor has been well characterized (e.g., Asztely et al., 1994; Bellingham and Berger, 1994; Hasuo et al., 1992; Li and Perl, 1994; Lupica et al., 1992; Scanziani et al., 1992; Wu and Saggau, 1994), and, as such, was an excellent candidate for ADD. Indeed, Mitchell et al. (1993), demonstrated activity-dependent release of adenosine in hippocampal slices, which mediated inhibition of excitatory synaptic transmission through an A1 receptor. In a manner similar to that presented here, the effects of adenosine were observed after 50 ms and peaked after 250 ms following the train of stimulation (up to 32 stimuli at 100 Hz). Furthermore, adenosine uptake inhibitors potentiated the inhibitions in their preparations, similar to that presented here. While ADD in LC was not affected by specific adenosine antagonists under the conditions tested, it is not

known whether uptake inhibitors to adenosine would change these observations as it did for EAA.

$GABA_B$ activation following trains of stimuli, and its involvement in heterosynaptic depression of EPSPs following repeated stimuli in hippocampus has also been reported (Isaacson et al., 1993). In this study, a similar protocol to that used here was tested, i.e., a control and a test EPSP separated by a stimulation volley. However, unlike the protocol in my work, two different pathways were stimulated, one which contained excitatory fibers exclusively and the other which contained mixed excitatory and inhibitory fibers. The latter pathway was used for stimulation volleys (50 Hz, 100 ms). The ADD observed in this study was fully blocked by the same $GABA_B$ receptor antagonist used in my study, i.e., CGP 35348. The extent (maximum ADD of about 60% of control) and the time-course (peaking at 200 ms post-train) of ADD was strikingly similar to that reported in the present work. This indicates that the process of activation of presynaptic receptors is similar for many neurotransmitters (either GABA, adenosine or Glu). Furthermore, specific inhibition of GABA uptake significantly potentiated the ADD (Isaacson et al., 1993), similarly to my observations and that of others (Mitchell et al., 1993, Scanziani et al., 1997). There could be several reasons for the lack of $GABA_B$ effects in my system. First, only a small number of GABAergic fibers are stimulated and not enough GABA is released to feedback onto excitatory fibers. Second, the uptake of GABA is very efficient and prevents accumulation and diffusion of GABA under the condition tested. Alternatively, it is possible that the anatomical space between excitatory and inhibitory synapses is too big to allow for

"crosstalk" between synapses (Clements, 1996, Barbour and Häusser, 1997) as proposed by Isaacson et al. (1993) for the hippocampus. However, the presence of $GABA_B$ receptors onto excitatory afferents of LC would suggest otherwise. The use of GABA uptake inhibitors in ADD protocols could answer some of these questions.

Finally, the last compound tested was the broad spectrum serotonin inhibitor mianserin. The rational for testing the participation serotonin in ADD comes from a series of reports demonstrating inhibitory action of this compound on excitatory synaptic transmission. However, the locus of action of serotonin in LC, i.e., presynaptic (Bobker and Williams, 1989) versus postsynaptic (Aston-Jones et al.1991; Charléty et al.1993) remains controversial. Results clearly show that mianserin did not affect ADD in LC. Serotonin has been localized to the LC nucleus [e.g., McRae-Degueurce and Milon (1983) and Iijima et al (1993)], however, modulation of excitatory synaptic transmission by endogenous serotonin remains to be demonstrated

4.4.2.3. Other effects of multiple stimuli on excitatory synaptic transmission

As noted in section 3.4.1.1., a change in the repolarization kinetics of test EPSPs was regularly observed whether stimulation volleys were applied or not. This was puzzling at first, as it suggested that a single EPSP could cause synaptic potentials which were revealed when a second EPSP was evoked 800 ms later. This was not affected by NMDA receptor antagonists or any of the mGluR antagonists tested. Recent reports describing late potentials now offer some explanations to this observation. First, two investigations made used of a

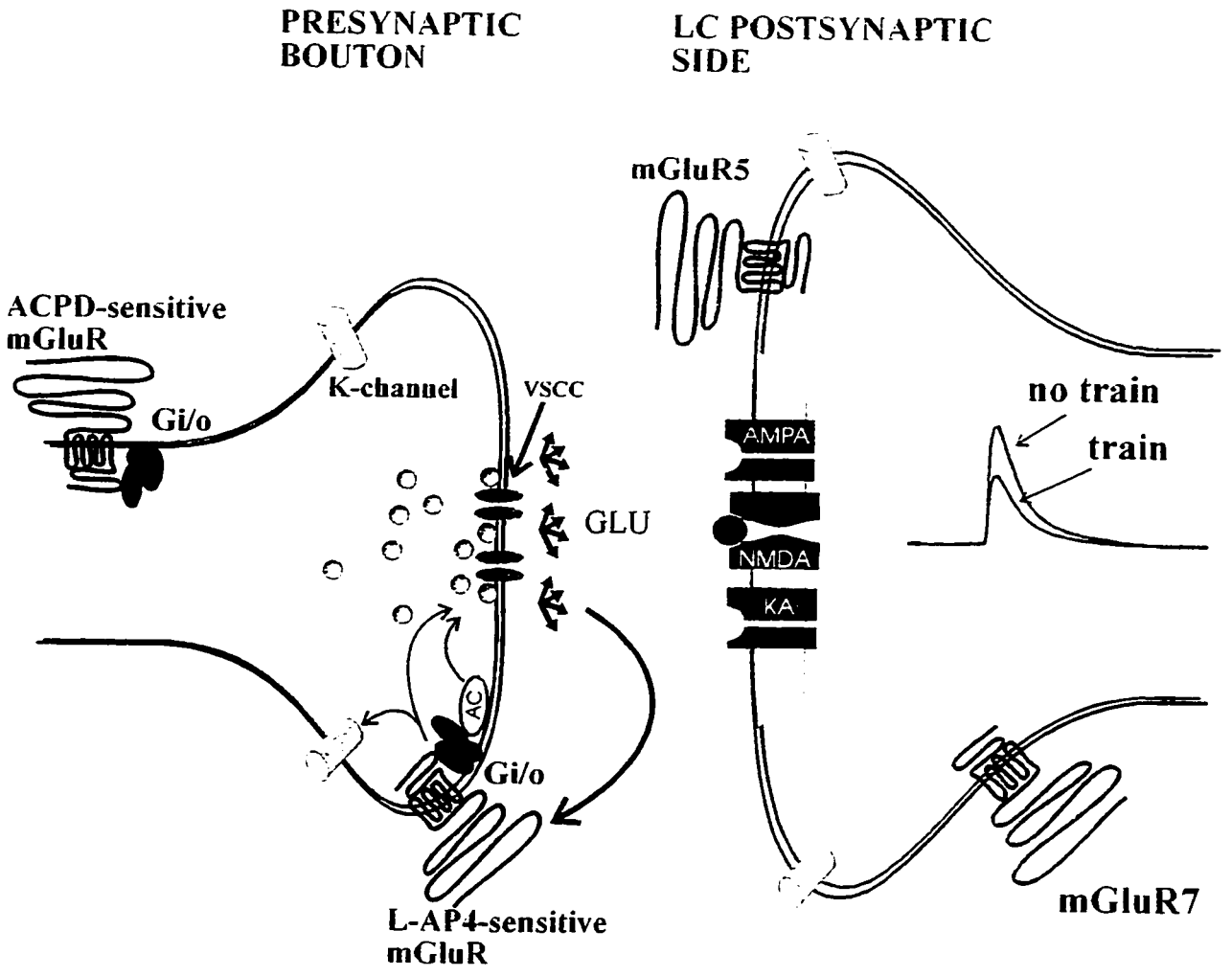


Figure 46: Schematic representation of a possible scenario explaining the mGluR group III- (but not group II-) sensitive inhibition of EPSPs following sustained activity at LC synapses. The LC spines are innervated by presynaptic terminals expressing receptors for both L-AP4 (group III, not mGluR4) and t-ACPD (group II) mGluRs. In the scenario presented here, the receptors are localized to different sites on the presynaptic axon: Group III mGluRs are close to (possibly in) the synaptic cleft and are activated by the accumulated glutamate in the cleft, thus reducing subsequent release of glutamate. The group II mGluRs are also found to presynaptically depress EPSPs following bath application of specific agonists but, from the work presented here, are not found to participate in the activity-dependent inhibition of EPSPs. Thus in the current scenario, this receptor is located on the presynaptic terminal, but away from the synaptic cleft, making it less susceptible to activation following accumulation of synaptically-released glutamate.

highly selective AMPA receptor antagonist (GYKI 53655) to isolate a new kainate-dependent synaptic current in hippocampus with kinetic slower than AMPA (Castillo et al., 1997; Vigne and Collingridge, 1997). The amplitude of this synaptic current was dependent on previous stimulations and rarely observed following single EPSPs. The kinetics of the kainate synaptic currents were markedly slower than that of AMPA receptors and could last more than 500 ms depending on their amplitudes. Another possibility which could explain the slow repolarizing phase of the test EPSP was provided by recent work in hippocampus describing a mGluR-dependent calcium-activated nonselective cationic current (I_{CAN}) (Congar et al., 1997). This current is also dependent on the number and frequency of stimuli and was dependent on the activation of a postsynaptic group I mGluR. I_{CAN} displayed slow kinetics of activation and lasted for up to 1 sec. As it is mGluR mediated, I_{CAN} was blocked by GTPγS and was dependent on intracellular Ca^{2+} concentrations. As such, I_{CAN} is similar to the $mGluR_{EPSP}$ characterized in the cerebellum by Batchelor and collaborators (Batchelor et al., 1994; Batchelor and Garthwaite, 1997). However, I_{CAN} and $mGluR_{EPSP}$ are less likely candidates for explaining the delayed repolarization observed in my preparation as they appear to require strong stimuli and would likely not be observed under conditions where only C and T EPSPs were evoked. Furthermore, these would have likely been antagonized by MCPG, although higher concentrations of this antagonist may have been required (1 mM of (+)-MCPG (1 mM) was used to block I_{CAN} and $mGluR_{EPSP}$).

4.5. INTERPRETATION AND SIGNIFICANCE

A schematic representation of the synapse, which could explain results reported in this study is presented in figure 46. The L-AP4-sensitive receptor was placed away from the site of release of neurotransmitters, so not to be activated following relatively low frequency of release. However, it was placed close enough to be able to be activated during high-frequency synaptic transmission and spillover of neurotransmitters. Conversely, the *t*-ACPD-sensitive receptor was placed far enough away from the release site not to be activated during high-frequency synaptic transmission. In this schematic, application of exogenous agonists would result in the activation of either receptor. Therefore, from this work, it is proposed that only the group III mGluR is an autoreceptor in LC. The function of group II in LC remains unknown. However, mGluRs have been found away from the synapse (e.g., on axon and dendrite shafts) and could possibly be targets for other synapses (Martin et al., 1992; Petralia et al., 1996a; Shigemoto et al., 1996). Furthermore, extrasynaptic receptors have been recently proposed to alter the propagation of action potentials (Schrader and Tasker, 1997). However, such a scheme in LC is purely speculative. Note that the Glu transporters were omitted from the schematic. These have been shown on neurons and glia which envelops the terminals (Rothstein et al., 1994). High density of transporters has been shown associated with the postsynaptic terminal (Rothstein et al., 1994). Other mGluRs (5 and 7) have also been included and have been discussed earlier.

4.6. SUMMARY AND CONCLUSION

In this thesis, I have presented compelling evidence that two different presynaptic mGluRs can inhibit excitatory synaptic transmission to LC. Experiments aimed at determining the identities, signal transduction and function of these receptors have been described which supports the claims that a group II and a group III mGluR are involved. The fact that the actions of these receptors are not additive, with the information on coupling and transduction group II and III mGluRs indicate that these receptors share a common pathway downstream of the receptor. Futhermore, unlike previously suggested, this signal transduction pathway is independent of the adenylyl cyclase/cAMP system. While this apparent receptor/function redundancy remains a puzzling concept, additional experiments now demonstrate that under specific conditions only one of these receptors can be activated endogenously. Together, these novel observations suggest that, while two pharmacologically distinct mGluRs, but sharing similar functions, can be activated in my preparation by specific agonist, these are likely not activated under the same conditions, and thus could serve distinct physiological functions. Finally, in depth characterization of the group III mGluR in LC has failed to clearly delineate the identity of this receptor. Hence, the possibility exists that this is an uncharacterized L-AP4-sensitive receptor.

Overall, results presented and discussed in this document provide evidence for new mechanisms involved in short-term plasticity of LC, depressing excitatory synaptic transmission during high frequency activity of the synapse. Such a mechanism could become very useful under pathological conditions preceding and leading to excitotoxicity. Keeping

in mind the central role of the LC/NA system in the CNS, a feedback mechanism such as the one described here would provide a useful mean to control NA outflow throughout the neuraxis.

These findings have been presented, in part, in several international meetings such as the annual meeting of the *Society for Neuroscience* (Dubé and Marshall, 1995, 1996, 1997, Abstracts), the *International Meeting on Excitatory Amino Acids* (New-Hamshire 1997) and the second *International Meeting on Metabotropic Glutamate Receptors* which was held in Taormina, Italy in September 1996. This latter abstract has in fact won one of the Graduate Student Awards. Furthermore part of these findings have been incorporated into a manuscript which was recently published in *Neuroscience* (Dubé and Marshall, 1997). Within the next few months, I anticipate to submit four additional manuscripts which will include results from the signal transduction studies, results from the ADD studies, and results from the mice studies (short communication) respectively. Finally, the fourth manuscript will be describing the actions of $GABA_B$ receptor on excitatory synaptic transmssion.

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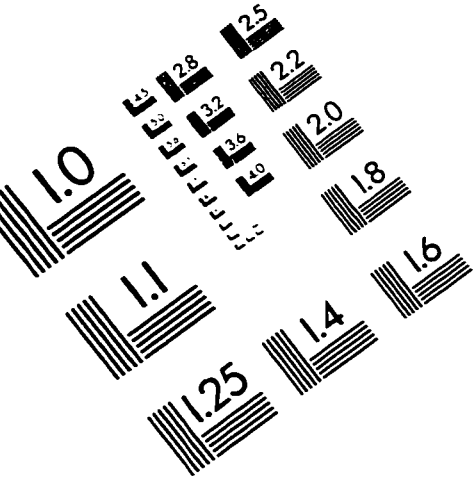
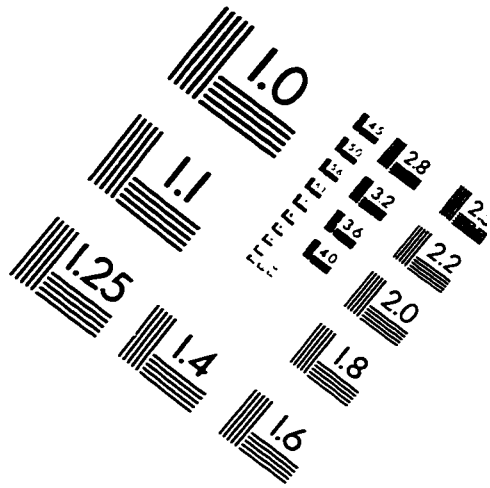
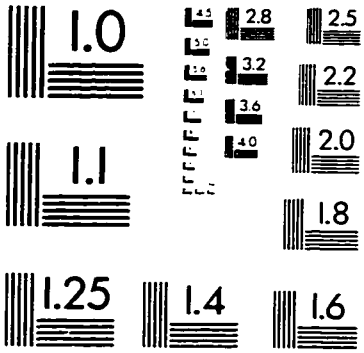
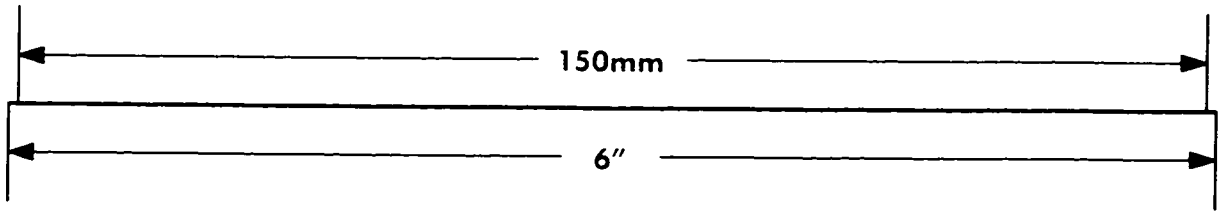
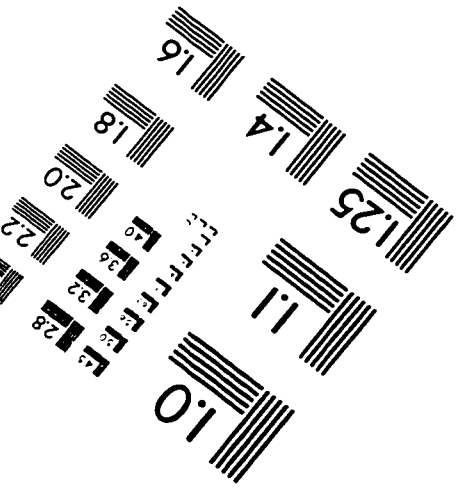
IMAGE EVALUATION TEST TARGET (QA-3)

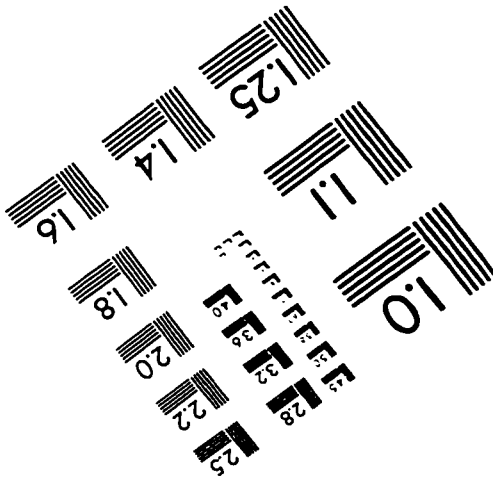












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