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An *In Vitro* Investigation Of The Effects Of Excitatory Amino Acids And Serotonin On Mesencephalic Trigeminal Neurons

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Abstract

The mesencephalic trigeminal nucleus (MeV) is composed entirely of primary sensory neurons which innervate jaw closure muscle-spindles and periodontal mechanoreceptors. Unlike peripherally located primary afferents, such as those found in the dorsal root ganglia and the trigeminal ganglia, the unique central location of the somata of MeV somatosensory and proprioceptive units makes them an accessible target for synaptic input from other parts of the central nervous system (CNS). In fact, numerous studies have revealed prominent innervation of MeV somata by nerve terminals containing a variety of putative neurotransmitter substances including γ-aminobutyric acid (GABA), histamine, dopamine, serotonin, noradrenaline, and glutamate. However, when tested electrophysiologically, none of these substances, except GABA, has been observed to elicit changes in the resting properties of MeV neurons. In the present investigation we have performed intracellular recordings from MeV neurons in an *in vitro* brain slice preparation to reevaluate the effects of two likely candidate neurotransmitters, glutamate and serotonin, on MeV neurons.

Recorded neurons were observed to depolarize in response to exogenously applied glutamate, N-methyl-D-aspartic acid (NMDA), kainate (KA), and (R,S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). The agonists generally evoked larger responses than glutamate, and exhibited a long duration desensitization requiring approximately ten minutes for full recovery. Some cross-desensitization between the glutamate agonists was also observed. MeV neurons exhibit voltage-dependent

subthreshold high-frequency oscillatory activity that may be activated during excitatory amino acid (EAA)-induced depolarizations to enhance their excitability.

Exogenously applied serotonin was observed to cause small depolarization of MeV neurons accompanied by substantial reductions in input resistance. The response was determined to result from activation of the inward rectifier, I_h. Applications of forskolin, 8-bromo-cAMP, and IBMX, substances known to elevate intacellular adenosine 3',5'-cyclic monophosphate (cAMP), were observed to mimic the serotonin response suggesting that the second messenger system mediating the serotonin response involves activation of adenylyl cyclase leading to an increase in intracellular cAMP. The serotonin response could be reproduced with the serotonin agonist, 5-carboxamidotryptamine (5-CT), but not by 8--hydroxy-2-(di-N-propylamino)-tetralin (8-OH-DPAT), and could be antagonized by ketanserin but not by methiothepin. It is speculated that serotonin input contributes to the excitability level of MeV neurons by regulating the degree of activation of I_h.

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This thesis is dedicated to

the memory of my Grandfather,

Kenneth C. Summers (1916-1994)

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5-CT	5-Carboxamidotryptamine
5-HT	5-Hydroxytryptamine (Serotonin)
5-MeOT	5-Methoxytryptamine
8-OH-DPAT	8–hydroxy-2-(di-N-propylamino)-tetralin
AC	Adenylyl cyclase
ACh	Acetylcholine
ACSF	Artificial cerebrospinal fluid
ADA	Adenosine deaminase
AHP	Afterhyperpolarization
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazole propionate
AP	Action potential
Asp	Aspartate
B/ GRP	Bombesin/ gastrin releasing peptide
cAMP	Adenosine 3',5'-cyclic monophosphate
CCK	Cholecystokinin
cDNA	Complimentary deoxyribonucleic acid
Chat	Choline acetyltransferase
CMA	Cortical masticatory area
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPP	(±)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid

DA	Dopamine
DOB	1-(4-bromo-2,5-dimethoxyphenyl)-2-aminopropane
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
Dom	Domoate
DOM	1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane
DRG	Dorsal root ganglia
EAA	Excitatory amino acid
enk	Enkephalin
GABA	γ-Aminobutyrate
GAD	Glutamate decarboxylase
Glu	Glutamate
Gly	Glycine
HDC	Histidine decarboxylase
Hist	Histamine
HRP	Horseradish peroxidase
I	Current
$I_{q/lv\,q}$	Inward rectifier current
IBMX	3-Isobutyl-1-methylxanthine
IR	Immunoreactive
KA	Kainate
LC	Locus Coeruleus
LSD	d-Lysergic acid diethylamide

MeV	Mesencephalic trigeminal nucleus
MoV	Motor trigeminal nucleus
NA	Noradrenaline
NMDA	N-Methyl-D-aspartate
NPY	Neuropeptide Y
PB	Phosphate buffered
PBS	Phosphate buffered saline
PCRt	Parvocellular (lateral) reticular formation
PI	Phosphatidylinositol
PLC	Phospholipase C
Quis	Quisqualate
SP	Substance P
SuV	Supratrigeminal nucleus
TG	Trigeminal ganglia
TH	Tyrosine hydroxylase
trans-PDC	L-*trans*-pyrrolidine-2,4-dicarboxylic acid
TTX	Tetrodotoxin
TVV	*Truncas vagalis ventralis*
V	Voltage
Vc	Subnucleus caudalis of the spinal trigeminal nucleus
Vidm	Dorsomedial part of the subnucleus interpolaris of the spinal trigeminal nucleus

Vint	Intertrigeminal nucleus
VIP	Vasoactive intestinal polypeptide
Vjux	Juxta-trigemnial region
Vodm	Dorsomedial part of the subnucleus oralis of the spinal trigeminal nucleus
Vpdm	Dorsomedial part of the principal sensory trigeminal nucleus

1 Introduction

The neuronal cell bodies of axons innervating masticatory muscle spindles and low threshold periodontal mechanoreceptors form a thin column of cells known as the mesencephalic trigeminal nucleus (MeV) which spans the mesencephalon and rostral portion of the pons. This location is unique among proprioceptive and somatosensory afferents, as the somata of other such cells are situated within ganglia found outside of the central nervous system, such as dorsal root ganglia (DRG) or trigeminal ganglia (TG). Another unique feature of MeV cells is the regular occurrence of synaptic contacts on their somata that has been observed in many different species including the mouse and cat (Hinrichsen and Larramendi, 1970), the hamster (Alley, 1973), the skate (Witkovsky and Roberts, 1976), and the rat (Liem et al., 1991, 1992). In contrast, only one group has observed a small number of synapses from cells of unknown origin on cat DRG cell somata (Kayahara et al., 1981, 1984).

These distinctive features of MeV neurons prompt consideration of whether sensory signals conducted from the periphery could be modulated at the level of the primary afferent cell soma instead of at the terminals where modulation is believed to occur in DRG (for reviews see Rudomin, 1990 and Watson, 1992). Modulation of MeV cell activity in response to cortical masticatory area stimulation, area postrema stimulation, and vagal afferent stimulation has been reported, and although the modulatory mechanism was undetermined, it was speculated that synaptic input onto MeV cell bodies was the most logical explanation for the observed modulation (Kolta et

al., 1990; Manni et al., 1980; Pettorossi, 1983).

Numerous studies employing a variety of techniques have revealed inputs from many different central nuclei impinging upon cells of the MeV, and immunocytochemichal studies have revealed prominent innervation of MeV cells by nerve terminals containing a variety of putative neurotransmitters. Together, these findings further support the notion that MeV cell activity is under the influence of various synaptic inputs at the level of the neuron soma. The lack of correlative electrophysiological effects of neurotransmitters on MeV cells, however, is contra-indicatory to such a notion. Most tests so far have failed to convincingly demonstrate that MeV cells respond electrophysiologically to any of the neurotransmitters found within terminals synapsing on their cell bodies (De Montigny and Lund, 1980; Henderson et al., 1982, Regenold et al., 1988). One exception is a report that MeV neurons depolarize in response to γ-aminobutyric acid (GABA) application (Marshall et al., 1994; Hayar et al. in press).

1.1 The Mesencephalic Trigeminal Nucleus

The MeV consists of a narrow crescent-shaped band of cells that lies along the optic tectum, having a rostrocaudal extension that varies in the evolutionary scale of vertebrates (Fernandez and Doel, 1984). In the rat, the approximately 1000-1600 neurons of the MeV form a thin rostrocaudally oriented column of about 4 mm spanning the entire mesencephalon and rostral portion of pons, with the midbrain portion lining the periaqueductal gray and the pontine portion forming a triangular structure just beneath

and lateral to the floor of the fourth ventricle clinched between the locus coeruleus medially and the parabrachial nucleus laterally (Rokx and van Willigen, 1988).

Generally, two morphologically distinct cell types have been described in the MeV: spherical or ovoid pseudo-unipolar cells of 30-65 μm making up the majority of the population, and multipolar cells or interneurons of 30-50 μm comprising the remainder (Ramón y Cajal, 1909; Hinrichson and Larramendi, 1969; Walberg, 1984; Nomura and Mizuno, 1985; Faccioli et al., 1985; Nomura et al., 1985; Liem et al., 1991; Luo et al., 1991). The pseudo-unipolar cells are homologous to the large mechanosensitive neurons of the DRG and TG and hence are often considered as their central counterparts. Ultrastructural studies have indicated several differences between MeV neurons and their peripheral counterparts aside from location. When viewed under light microscopy or electron microscopy, MeV somal surfaces appear to be studded with small spines endowed with synaptic contacts and exhibit close apposition that is characteristic of electrical coupling (Hinrichsen and Larramendi, 1969, 1970; Nomura et al., 1985; Liem et al., 1991). Electrophysiological studies have further confirmed that cells of the MeV are electrically coupled (Hinrichson, 1970; Baker and Llinás, 1971).

Extracellular and intracellular recordings performed throughout the entire rostrocaudal extent of the MeV during adequate stimulation of either periodontal mechanoreceptors or muscle spindles has demonstrated an organized arrangement within the MeV of a variety of species (Corbin and Harrison, 1940; Jerge, 1963; Passatore et al., 1979a; Passatore et al., 1983). In general, muscle spindle afferents are found

throughout the entire rostrocaudal extent of the MeV with no somatotopic arrangement between afferents innervating different muscles, while periodontal mechanoreceptive units are localized in the caudal half of the nucleus where the majority of MeV cells are packed. This differential distribution of these two distinct groups of MeV neurons has been confirmed using various tracers (Alvarado-Mallart et al., 1975; Jacquin et al., 1983; Gottlieb et al.,1984; Nomura and Mizuno, 1985; Rokx and van Willigen, 1988; Shigenaga et al., 1988a,b, 1989, 1990).

1.1.1 Function of the Mesencephalic Trigeminal Nucleus

The resemblance of MeV cells to the unipolar primary sensory cells of the cerebrospinal ganglia was noted by many investigators in the early part of this century (Allen, 1919; Clark, 1926; Weinberg, 1928; Schneider, 1928; Sheinen, 1930). Clark demonstrated the close similarity of the large cells of the MeV and those of the spinal ganglia which Warrington and Griffith (1904) demonstrated to be connected to muscle spindles. Because of this resemblance, and because of the peripheral distribution of the majority of the MeV fibres to the masticator branches of the trigeminal nerve, the consensus of opinion was that the MeV comprised the origin of primary sensory fibres mediating muscle sensibility from the muscles of mastication (Johnston, 1909; May and Horsely, 1910; Allen, 1919; Thelander, 1924). It was further noted that MeV peripherally projecting fibres also pass into the superior alveolar, inferior alveolar and palatine branches of the trigeminal nerve which supply deep sensation to the teeth, gums, and hard palate and thus may subserve mechanoreceptive functions in these areas (Corbin,

1940).

The systematic studies of Corbin and Harrison (1940) did much to clarify the function of the nucleus. Using extracellular recording techniques in conjunction with various stimuli in the cat, they were able to demonstrate that the nucleus is activated by jaw-opening movements and hence stretch of the masticator muscles, as well as by pressure stimulation of areas in the mouth. Careful histological examination of the areas recorded from revealed that responses to these stimuli were elicited only when the recording leads were within the ipsilateral MeV and not within surrounding neural structures; however, responses in the MeV have subsequently been observed during stimulation of contralateral muscle spindle afferents and periodontal mechanoreceptor afferents (Passatore et al., 1983). It was also demonstrated that the jaw-jerk reflex is abolished by lesions of the nucleus (Corbin and Harrison, 1942). Since the majority of MeV centrally projecting fibres were known to innervate the trigeminal motor nucleus (MoV) (Ramon y Cajal, 1896; and later confirmed by Hugelin and Bonvallet, 1956 and Nakamura et al., 1967) it was surmised that the MeV constitutes the afferent limbs of masticator reflex arcs, thereby coordinating and controlling chewing movements.

Jerge (1963) confirmed the results of Corbin and Harrison (1940) and further elaborated on the structures innervated by the MeV. Again using extracellular recordings, two general classes of cells were found within the nucleus: 1) those innervating muscle spindles of jaw elevator muscles and 2) those innervating pressure receptors of the periodontal membrane of the teeth. Periodontal mechanoreceptive units were found to be of two types: 1) those innervating the periodontal membrane of a

single tooth and 2) those innervating the same structure of two or more adjacent teeth. MeV neurons innervating the hard palate as described by Corbin and Harrison (1940) were not observed by Jerge. It was speculated that in Corbin and Harrison's work receptors of the teeth were being activated by the application of pressure to adjacent areas on the palate, or that stimulation to the palate had elicited jaw-opening reflexes thereby activating spindle units, both of which would lead to a false conclusion that activation of the cells in the nucleus had occurred due to stimulation of receptors in the palate. In both of these early studies the periodontal mechanoreceptors displayed decided directionality in their sensitivity to pressure stimulation. Units were observed to discharge by pressure in any direction up to 90^0 from the direction of minimum threshold, while throughout the remaining 180^0 of the tooth's circumference the unit was inexcitable even at high intensities of stimulation. Such directionality has been further confirmed by Linden and Scott (1989). The muscles represented in the MeV in general are those involved with jaw-closing in most species. Four muscles, the masseter, temporalis, medial pterygoid, and lateral pterygoid, are usually considered together as the principal muscles of mastication. The masseter, temporalis, and medial pterygoid are oriented to execute jaw-closing movements while action of the lateral pterygoid is associated with jaw-opening movements. In both of the early studies regarding MeV function so far mentioned (both performed in the cat) muscle-spindle units were found to project peripherally to the masseter, temporalis, and medial pterygoid with no projections to the lateral pterygoid or any other muscles of the head or buccal regions. These projections have been confirmed by groups using intra-axonal injection of

horseradish peroxidase (HRP) (Gottlieb et al., 1984; Shigenaga et al., 1988a). However, it appears that muscle representation within the MeV is species dependent since in the guinea pig the lateral pterygoid has been shown to be represented within the nucleus (Nozaki et al., 1985) and in the rat, suprahyoidal muscles such as the mylohyoid muscle as well as the anterior belly of the digastricus muscle have been observed to be represented within the nucleus (Jacquin et al., 1983; Yasuda et al., 1995). While several workers have implicated the MeV in extraocular eye-muscle proprioception (Freeman, 1925; Cooper et al., 1953; Fillenz, 1955; Weinberg, 1928) results from Jerge's work and Corbin's and Henderson's work presented convincing evidence against this function. One group has claimed that neurons of the MeV also innervate Golgi tendon organs (Smith, 1969); however, Jerge reported that none of the units recorded from in his work gave responses characteristic of Golgi tendon afferents. Furthermore, in experiments performed on rabbits using succinylcholine to differentiate between muscle spindle and tendon organ afferents, no evidence was found for representation of tendon organs within the MeV (Passatore et al., 1983).

Electrical stimulation of discrete zones of the MeV while recording movement or tension in the lower jaw has indicated that stimulation of areas containing primarily spindle afferents (i.e. more rostral portions of the MeV) induces jaw-closing while only jaw-opening movements are obtained by stimulation of areas containing primarily periodontal mechanoreceptor afferents (more caudal portions of the MeV) (Passatore et al., 1983). In fact it was observed that the jaw-closing response decreased in magnitude until it dissapeared and became a jaw-opening response as the location of stimulation

progressed from the rostral to the caudal portions of the nucleus in good agreement with the organization of the nucleus. These findings provided evidence that a group of neurons within the MeV, which contains prevalently neurons subserving the jaw-closing reflex, participate in the jaw-opening reflex, and are in good agreement with observations by Pfaffman (1939) that mechanical stimulation of the maxillary nerve, teeth, gums, and hard palate cause jaw-opening. Thus, it appears that the total MeV inflow of sensory information is capable of controlling and coordinating movements of the lower jaw through reflex activation and inhibition of MoV cells, permitting a forceful bite without damage to the structures involved in mastication (teeth, gums, and palate).

1.1.2 Efferent Projections of the Mesencephalic Trigeminal Nucleus

Although the central projections of the MeV have been investigated with a variety of methods including intracellular labelling, transganglionic tracing, single-unit recording, autoradiography, and retrograde HRP tracing, diverse and discrepant results have been reported in a variety of species. The projecting targets about which most investigators agree are the MoV, the supra-trigeminal nucleus (SuV), the intertrigeminal nucleus (Vint), the dorsomedial part of the subnucleus oralis of the spinal trigeminal nucleus (Vodm), the lateral reticular formation of the medulla (PCRt), and cervical segments of the spinal cord (Goldstein and Jacobs, 1969; Mizuno and Sauerland, 1970; Herdman, 1980; Matesz, 1981; Matsushita et al., 1981; Dacey, 1982; Ruggiero et al., 1982; Walberg et al., 1984; Appenteng et al., 1985; Nomura and Mizuno, 1985; Peng et al., 1986;

Sirkin and Feng, 1987; Shigenaga et al., 1988a,b; Wang and Li, 1988; Luo and Li, 1991; Lingenhohl and Friauf, 1991; Luo et al., 1991). However projections of the MeV to the juxta-trigeminal region (Vjux), the rostral part of the nucleus ambiguus, nucleus of the solitary tract, facial nucleus, dorsal motor nucleus of the vagus, hypoglossal nucleus, and cerebellum have also been described (Pearson, 1949; Cupedo, 1965; Mizuno and Sauerland, 1970; Matesz, 1981; Nomura and Mizuno, 1985; Peng et al., 1986; Sirkin and Feng, 1987; Shigenaga et al., 1988a,b; Luo and Li, 1991; Luo et al., 1991; Billig et al., 1995). Other authors, such as Ruggiero et al. (1982), have disagreed with these reports based on their experiments in which these projections were not observed. Aside from the above mentioned projections, Herdman (1980) observed labeled fibres in the locus coeruleus (LC), the inferior olivary nucleus, the reticular formation of the midbrain, the oculomotor nucleus, the trochlear nucleus, the rhomboid nucleus of the thalamus, and the globus pallidus, after injecting tritiated leucine into the MeV. Of Herdman's discrepant findings, only projections to the occulomotor and trochlear nuclei have been confirmed (Luo et al., 1991); this investigation also reported newly discovered projections to the accessory facial nucleus and accessory abducens nucleus. In all reports mentioned, projections of the MeV were ipsilateral.

In a recent study concerning MeV projections, Luo and Dessem (1995) have intracellularly labelled muscle spindle afferents with biotinamide which has superior staining properties to other intracellular labeling substances. Generally, their findings are in agreement with previous reports of ipsilateral MeV projections to the MoV, SuV, PCRt, Vodm, and the cerebellum. Newly discovered projections to the dorsomedial part

of the principal sensory trigeminal nucleus (Vpdm), the subnucleus caudalis of the spinal trigeminal nucleus (Vc), and the dorsomedial part of the subnucleus interpolaris of the spinal trigeminal nucleus (Vidm) were also reported. Previously reported projections to the accessory abducens nucleus, facial nucleus, hypoglossal nucleus, nucleus of the solitary tract, and the spinal cord were not confirmed by Luo and Dessem (1995), possibly because these regions are related to periodontal afferent projections. In this same study trigeminothalamic neurons were identified by means of retrograde transport of HRP from the ventroposteromedial nucleus of the thalamus. It was observed that trigeminothalamic neurons in SuV, Vpdm, Vidm, and PCRt were associated with axon collaterals and boutons from muscle spindle afferents, suggesting a powerful input from the MeV to some trigeminothalamic neurons. Thus, it was suggested that muscle length and velocity feed back from spindle afferents to the thalamus, implicating these pathways in the transmission of trigeminal proprioceptive information to the cerebral cortex.

In a follow-up study, Luo and Dessem (1996) provided morphological evidence for synaptic feedback between MeV muscle spindle afferents. Muscle spindle afferents were retrogradely labeled following HRP injections into the jaw elevator muscles of rats. Subsequently, spindle afferent axons were intracellularly stained with biotinamide. Upon examination it was observed that some jaw-muscle spindle afferents make synapses with other cells of the same type. Thus, efferent projections of the MeV also include projections from one cell within the nucleus to another cell of the same type within the nucleus, at least in the case of muscle spindle afferents.

1.1.3 Afferent Projections to the Mesencephalic Trigeminal Nucleus

While a large body of information concerning efferent projections of the MeV is available, little attention has been paid to afferent projections onto the nucleus. To date the most thoroughly investigated projections onto the MeV have been those originating from the brainstem reticular formation. Projections to the MeV from the PCRt have been demonstrated with the use of the anterograde tracers *Phaseolus vulgaris* leuko agglutinin and HRP (Ruggiero et al., 1982; Rokx et al., 1988; Minkels et al.,1991; Ter Horst et al.,1991). Both ipsilateral and contralateral projections were identified with the labeled fibres and synaptic boutons being restricted primarily to the caudal third of the MeV. Electrophysiological investigations have confirmed the existence of projections from the PCRt to both the ipsi- and contralateral MeV (Juch and Rokx, 1988; Minkels et al., 1991). From these studies it is concluded that the PCRt has some modulating effect on the set points of MeV neurons and thereby on the relay of afferent information from receptors in the oral region to the central nervous system (CNS).

Using autoradiographical methods, projections to the MeV have also been reported to arise from the amygdala (Hopkins and Holstege, 1978; Kretteck and Price, 1978). Upon comparison of their data with that of Kretteck and Price (1978), Ruggiero et al. (1982) discovered those levels of the MeV innervated by the amygdala corresponded to a source of trigeminoreticular projections. This, along with the fact that the amygdala is known to project directly to the PCRt at levels that correspond to the terminal projection sites of MeV neurons onto the PCRt (Ruggiero et al., 1979), suggests that the MeV may potentially serve as an integral link between the amygdala and the

PCRt (Ruggiero et al., 1982).

Anterograde and retrograde neuroanatomical tract tracing methods have further demonstrated efferents projecting to the MeV arising from the medial and posterior hypothalamus, the periaqueductal gray, the substantia nigra, the ventral tegmental area, and the raphé nuclei (dorsalis, pontis, and magnus) (Nagy et al., 1986; Yamomoto et al., 1988; Copray et al., 1990a; Copray et al., 1991; Lazarov and Chouchkov, 1995a). Projections from the area postrema to the MeV have been documented based on axon terminal degeneration studies and electrophysiological investigations (Manni et al., 1982). Finally, as mentioned in the previous section, MeV muscle spindle afferents have been observed to synapse with other MeV muscle spindle afferents and as such the MeV can be considered as another afferent projection system to cells contained therein (Luo and Dessem, 1996).

1.1.4 Neurotransmitter Substances Within the Mesencephalic Trigeminal Nucleus

A wide variety of putative neurotransmitters have been observed in fibres contained within the MeV. Nagy et al. (1986) reported the presence of a dense adenosine deaminase-immunoreactive (ADA-IR) plexus which engulfed the majority of MeV cells in the rat. The origin of these ADA-IR fibres was traced to the posterior hypothalamic magnocellular nuclei and periaqueductal gray. Subsequent ultrastructural examination of the connections of the ADA-IR fibres with MeV cells revealed that membrane specializations often occurred at points where MeV cell somata were

contacted by the ADA-IR terminals (Yamamoto et al., 1988). It is known that ADA, histidine decarboxylase (HDC), and glutamate decarboxylase (GAD) frequently coexist in neurons of the posterior hypothalamic magnocellular nuclei, hence, candidate neurotransmitters for the ADA-IR fibres observed to synapse with MeV cells include not only adenosine or ATP but also histamine (Hist), and GABA.

Immunocytochemical staining for HDC has revealed that MeV neurons are surrounded by a large number of HDC-IR fibres and immuno-electron microscopy has demonstrated the presence of synaptic contacts between HDC-IR fibres and MeV cell somata (Inagaki et al.,1987), thus, strengthening the hypothesis that Hist may act as a neurotransmitter in the MeV. Immunostaining for GABA in rat and cat MeVs has revealed the presence of both GABAergic fibres surrounding MeV neurons and actual GABAergic neuronal somata (probably interneurons) within the nucleus (Copray et al., 1990b; Lazarov and Chouchkov, 1995b) further implicating GABA as a neurotransmitter within the MeV.

A light microscopic study in young adult cats has demonstrated that MeV neurons are surrounded by axonal varicosities immunoreactive for enkephalin (enk), substance P (SP), and serotonin (5-HT) (Tashiro et al., 1989). Under oil immersion viewing conditions some of the immunoreactive varicosities appeared to be in direct contact with profiles of MeV neurons, mostly with the cell somata, and rarely on neuronal processes. A more comprehensive immunohistochemical study in the rat using antibodies to 17 major neurotransmitters confirmed the presence of enk-, SP-, and 5-HT-IR fibres making synaptic contact with MeV cell somata and further revealed fibres

immunoreactive for GABA, dopamine (DA), noradrenaline (NA), glutamate (Glu), cholecystokinin (CCK), vasoactive intestinal peptide (VIP), bombesin/gastrin releasing peptide (B/GRP), and neuropeptide Y (NPY) in fibres making synaptic contact with cell somata of the MeV (Copray et al., 1990b). It was postulated that the presence of SP, CCK, VIP, enk, B/GRP, and NPY in fibres contacting MeV cells reflected the coexistence of these peptides with one or more of the other main neurotransmitters observed. In follow-up studies the dopaminergic afferents to the MeV were confirmed and found to originate from the substantia nigra, the ventral tegmental area, and the medial hypothalamus (Copray et al., 1990a) while the serotonergic afferents were also confirmed and found to originate from the dorsal raphe nucleus (Copray et al., 1991). Support for noradrenergic innervation of cells of the MeV has recently been granted by the observation of tyrosine hydroxylase-immunoreactive (TH-IR) fibres making contact with cells of the nucleus (Lazarov and Chouchkov, 1995b). Aside from all of the above mentioned putative neurotransmitters within the MeV, one group (Henderson and Sherriff, 1991) has proposed a cholinergic innervation of MeV cells based on their findings that choline acetyltransferase immunoreactive (Chat-IR) fibres are associated with MeV neurons of the rat and ferret.

More detailed ultrastructural investigation of the 5-HT-containing fibres in the MeV of the cat has revealed direct synaptic contacts between 5-HT-containing terminals and perikarya of MeV neurons (Lazarov and Chouchkov, 1995a). The majority of 5-HT-labeled structures were synaptic terminals in which the immunoreactive material was located within small, round and clear vesicles, as well as, small, granular vesicles of

diameters from 50 to 80 nm and a few large dense-cored vesicles up to 150 nm in diameter. Autoradiographical studies in the rabbit using various radiolabelled 5-HT ligands have demonstrated ketanserine binding on MeV neuronal somata indicating the presence of 5-HT_2 receptors on these cells (Kolta et al., 1993). Thus, not only do cells of the MeV appear to receive 5-HT innervation but they also seem to have the appropriate receptors to respond to this neurotransmitter, and hence 5-HT is considered to be an important neurotransmitter within the MeV.

While reports of glutamatergic innervation of MeV neurons are scarce (only briefly mentioned in Copray et al., 1990b) it may be fair to assume that at least some of the synapses contacting MeV neuronal somata are glutamatergic, since the majority of synapses in the mammalian CNS utilize Glu as their neurotransmitter. The existence of synaptic contacts between MeV neurons is also suggestive of glutamatergic innervation since MeV neurons are believed to utilize Glu as a neurotransmitter (Chandler, 1989; Copray et al., 1990b; Grimwood et al., 1992). Immunolocalization studies revealing Glu receptor subunit expression within the MeV further lends support to the notion that Glu acts as a neurotransmitter within the nucleus (Petralia et al.,1994a,b,c; Petralia and Wenthold, 1992).

1.1.5 Responsiveness of Mesencephalic Trigeminal Neurons to Neurotransmitter Substances

Despite the reports of axon varicosities and terminals immunoreactive for the above mentioned neurotransmitters making synaptic contact with neurons of the MeV,

there is little evidence to date indicating that MeV neurons respond electrophysiologically to any neurotransmitter substances. In fact, De Montigny and Lund (1980) reported that while they performed *in vivo* extracellular recordings from the rat MeV, iontophoretic application of several neurotransmitter substances including kainic acid (KA), Glu, aspartate (Asp), acetylcholine (Ach), GABA, SP, glycine (Gly), DA, Hist, and NA failed to alter the discharge patterns of cells of the MeV. The lack of effect of KA is consistent with observations that neurons of the MeV are selectively resistant to KA-induced lesions (Colonnier et al., 1979), and the lack of effect of SP confirms previous observations that extracellularly recorded MeV neurons are totally unresponsive to this and related substances (Guyenet and Aghajanian, 1977). Furthermore, *in vitro* intracellular recordings from MeV neurons in slice preparations have failed to demonstrate responsiveness of these cells to superfusion of Glu (Henderson et al., 1982) as well as various purinoceptor agonists also applied by superfusion (Regenold et al., 1988).

Only two studies have reported observing synaptic activity while recording from MeV neurons (Roberts and Witkovsky, 1975; Chieng et al., 1993). The first study was performed in decerebrate dogfish, where following direct activation of MeV neurons by stimulation to the trigeminal nerve, slow-rising long lasting epsps which occasionally gave rise to spike discharge could be observed with a latency of around 12 ms following the direct activation. Further characterization of the observed synaptic activity indicated that it most likely arose from the principal trigeminal sensory nucleus but the neurotransmitter(s) involved was not identified nor were attempts made to block the

synaptic activity with traditional blockers such as tetrodotoxin (TTX). In the other study, application of the Australian funnel-web spider toxin, versutoxin, to recorded rat MeV neurons was observed to produce a modest increase in the integrated membrane potential activity as well as to increase 'synaptic' events recorded in the cells without effects on evoked action potentials or current-voltage relations of the cells. Again, however, blockade of the observed 'synaptic' events with TTX was not tested nor were antagonists used to determine the neurotransmitter(s) involved.

Most recently, and contrary to previous findings, Hayar et al. (in press) reported that intracellularly recorded MeV neurons in a rat brain-stem slice preparation respond electrophysiologically to local application of GABA. In this investigation, GABA was observed to induce prominent depolarizations with decreases in input resistance in a manner similar to that which has been observed in DRG (Feltz and Raminsky, 1974; Gallagher et al., 1978; White, 1990) and TG neurons (Puil and Spigelman, 1988). The responses were demonstrated to be direct as they were unaffected by manipulations known to block synaptic activity, and they were shown to be mediated through $GABA_A$ receptors, subunits of which were demonstrated to be localized within the MeV in the same investigation. The failure of previous studies to demonstrate GABA responses in MeV cells was attributed to technical limitations, rapid receptor desensitization, and active GABA uptake.

1.1.6 Modulation of Mesencephalic Trigeminal Nucleus Electrical Activity

The central location of the MeV as well as the numerous afferent projections to

the nucleus suggest that the activity of the primary afferents located therein are under the influence of other centrally located structures. Indeed, despite the lack of observed neurotransmitter effects upon mesencephalic trigeminal cells, modulation of the electrical activity of cells within the MeV by other CNS areas has been documented in mammals (Manni et al., 1977; Passatore et al., 1979b; Manni et al., 1980; Manni et al., 1982; Pettorossi, 1983; Kolta et al., 1990) and in birds (Manni et al., 1978). Investigations concerned with the influence of the cortical masticatory area (CMA) on the MeV in the rabbit have demonstrated that stimulation of the CMA can modify the discharge frequency of muscle spindle afferents while length and tension of the masticatory muscles are kept constant (Passatore et al., 1979b; Manni et al., 1980; Kolta et al., 1990). Generally, CMA effects on MeV units were restricted to approximately 40% of the muscle spindle afferents and consisted of phasic inhibitions, phasic excitations, or mixed responses of both inhibition and excitation occuring with long latencies between stimulation of the CMA and the observed effects upon the afferents. In two cases (Passatore et al., 1979b; Manni et al., 1980), CMA effects upon spindle afferents were attributed to activation of reticular pathways, stimulation of which has been observed to modify jaw muscle spindle afferent activity (Manni et al., 1977). The attribution of CMA effects on MeV neurons to activation of reticular pathways is further supported by the long latency of the observed effects (long period of time for polysynaptic pathways to be activated) and also by the anatomical and physiological relationships known to exist between the cerebral cortex and the brain stem reticular formation (Rossi and Zanchetti, 1957) and between the reticular formation and the MeV (see afferent

projections to the MeV).

Modulation of jaw-muscle spindle afferents has also been observed in studies involving the area postrema (Manni et al., 1982). It was observed that area postrema microstimulation influenced about half of the ipsilateral MeV units recorded. The effect consisted of a decrease in unit discharge rate by 10-25% when units were made to increase their firing rate by jaw lowering, with no effect observed when the mouth was held closed. Furthermore, MeV excitability was demonstrated to be reduced following stimulation of the ipsilateral area postrema and the masseteric reflex was shown to be inhibited. Effects similar to those observed after area postrema stimulation have also been observed by gastric vagal afferent stimulation (Pettorossi, 1983). Conditioning electrical stimulations of *truncas vagalis ventralis* (TVV) were reported to reduce the excitability of MeV spindle units and to inhibit the masseteric reflex. Subsequent mechanical stimulation of the gastric receptors by means of gastric distension also inhibited the masseteric reflex and inhibited the discharge frequency of proprioceptive MeV units by up to 50%. Since vagal fibres have been reported to terminate in the area postrema (Kalia and Mesulam, 1980), but not in the MeV, it was postulated that the inhibitory effects of vagal afferents on the MeV are actually mediated through the area postrema and that this reflex inhibition may be an important phenomenon in emesis, during which time jaw closing reflexes should not occur.

In all cases of observed modulation of MeV activity, the modulatory influences were attributed to activation of synaptic pathways impinging upon MeV neuronal somata since precautions were undertaken to eliminate complicating factors such as

sympathetic or peripheral fusimotor effects.

1.2 Excitatory Amino Acids

The excitatory amino acids (EAAs), for example Glu and Asp, exert strong excitatory actions on the vast majority of mammalian central neurons. Early biochemical studies revealed a central role for Glu in neural function (Weil-Malherbe, 1936) and subsequent electrophysiological studies demonstrated that single neurons of the mammalian brain are excited by Glu and other naturally occuring EAAs (reviewed in Mayer and Westbrook, 1987). Today Glu satisfies, to a large extent, the main criteria for classification as a neurotransmitter (Fonnum, 1984). Indeed, it is now commonly believed that Glu is the principal mediator of fast excitatory synaptic transmission within vertebrate CNSs.

1.2.1 Excitatory Amino Acid Receptors in the Mammalian Central Nervous System

Receptors for Glu (GluRs) can be divided into two major families: ionotropic receptors, which are ligand-gated cation channels and are the major concern of the present investigation, and metabotropic receptors, which are G-protein coupled receptors and shall not be considered for the purposes of this study. Ligand-gated ionotropic GluRs are traditionally classified according to preferred agonists (Monaghan et al., 1989; Watkins et al., 1990). There has been general agreement about the subdivision into N-methyl-D-aspartate (NMDA) and non-NMDA receptors. Typically non-NMDA receptors

are further subdivided into those activated by α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) (formerly defined by the less specific agonist, quisqualate), and those activated by KA. Complicating the classification of non-NMDA ionotropic GluRs are recombinant expression studies that have demonstrated the ability of KA to activate AMPA receptors (Hollmann et al., 1989; Barnard and Henley, 1990; Keinanen et al., 1990) and of AMPA to activate KA receptors (Huettner, 1990). Nevertheless, KA-binding sites that differ from high-affinity AMPA binding have been identified by ligand-binding approaches (Young and Fagg, 1990). A classical high-affinity site for KA is the CA3 area of the hippocampal formation, neurons of which have been demonstrated to be relatively vulnerable to KA compared to those of other regions (Meldrum and Garthwaite, 1990). Also, an apparently pure population of KA receptors has been described in peripherally located neurons of DRG (Huettner, 1990). Furthermore, recent molecular cloning of ionotropic GluRs does in fact imply the existence of three distinct GluR families based on sequence similarity and/or the ability of functional co-assembly (for review see Sommer and Seeburg, 1992; Hollmann and Heinemann, 1994; Schoepfer et al., 1994; Jorgensen et al., 1995).

1.2.1.1 AMPA Receptors

Following the first isolation of a cDNA clone by expression cloning coding for a subunit of a Glu-gated ion channel (Hollmann et al., 1989), three closely related genes were identified, referred to as either GluRA-D (Keinanen et al., 1990) or GluR1-4 (Boulter et al., 1990). These subunits are of similar size and share approximately 70% amino acid

sequence identity. The deduced amino acid sequences predict subunits with approximately 900 amino acids with four hydrophobic membrane-associated domains, an extracellularly located N-terminus, an intracellularly located C-terminus, and a large extracellularly located loop between the third and fourth membrane-associated domains (Bettler and Mulle, 1995). Recombinant expression of the GluR1-4 receptor subunits in either mammalian cells (Keinanen et al., 1990) or in *Xenopus* oocytes (Boulter et al., 1990; Nakanishi et al., 1990; Sakimura et al., 1990; Dawson et al., 1990; Lambolez et al., 1991) clearly indicated that this receptor class exhibits high-affinity AMPA-binding sites that closely resemble those characterized in brain membranes. As no other known subunits have been found to code for binding sites with such high affinity for AMPA, the GluR1-4 subunits are believed to comprise the AMPA subfamily of GluRs.

Analysis of the molecular properties of AMPA receptor subunits has revealed that each of the four subunits can be expressed as two splice variants termed 'flip' and 'flop' in which a short segment preceeding the fourth transmembrane domain can be encoded by two different exons that are used in a mutually exclusive manner (Sommer et al., 1990). Functionally, flip and flop channel subunits differentially affect the properties of Glu-activated currents. Upon fast application, Glu elicits currents at AMPA receptor channels which exhibit a fast rise time and then decay to a plateau value in the continued presence of the agonist. This plateau is more pronounced with flip- than with flop-containing receptors.

Another important molecular determinant of AMPA receptor channel properties resides in a single amino acid position within the second transmembrane segment. In

GluR1, 3, and 4 this position is occupied by a glutamine (Q) residue whereas in GluR2 this position is occupied by an arginine residue (R) (Keinanen et al., 1990) as a result of an RNA editing process (Sommer et al., 1991). Channels assembled from GluR1, 3, and 4 (homomeric and heteromeric) display doubly rectifying current-voltage (I-V) relationships (Boulter et al., 1990) and are permeable to divalent cations (Mg^{2+}, Ca^{2+}) (Hollmann et al., 1991) while channels incorporating the GluR2 subunit (homomeric or heteromeric) exhibit near-linear I-V relations and drastically reduced divalent cation permeability (Hollmann et al., 1991; Verdoorn et al., 1991). Thus, it appears that the GluR2 subunit dominates channel properties. It follows then, that since native AMPA receptors found on neurons are generally impermeable to Ca^{2+}, they contain the GluR2 subunit. In contrast, AMPA receptors expressed on Bergmann glia in the cerebellum show high Ca^{2+} permeability and are thus most likely not to contain GluR2 subunits (Muller et al., 1992; Burnashev et al., 1992).

The GluR1-4 subunits each form homomeric ion channels when expressed in oocytes or in transfected cells. Agonist potencies follow the order quisqualate (QA) > domoate (Dom) ~ AMPA > Glu > KA, with AMPA, Glu and QA acting as partial agonists while KA and Dom appear to be full agonists eliciting maximal responses (Hollmann et al., 1989; Nakanishi et al., 1990; Sakimura et al., 1990). Although AMPA is the most potent specific agonist, KA does elicit the largest responses; both agonists act at the same site but the KA (and Dom) steady state currents are larger because these agonists do not desensitize the receptors, whereas Glu, QA, and AMPA currents are smaller due to fast desensitization.

1.2.1.2 Kainate Receptors

The first molecular evidence for the existence of high-affinity KA receptors was provided by cloning of cDNAs for KA-binding proteins from frog and chick brains (Wada et al., 1989; Gregor et al., 1989). Subsequently, using low-stringency hybridization screening with GluR1-4 cDNA probes, low affinity (GluR5-7) and high affinity (KA-1 and KA-2) KA receptor subunits were cloned (Bettler et al., 1990; Egeberj et al., 1991; Werner et al., 1991; Bettler et al., 1992; Herb et al., 1992). GluR5-7 are of approximately the same size as GluR1-4, whereas KA1-2 subunits are slightly larger. GluR5-7 share 75-80% amino acid sequence identity with each other, but only 40% with GluR1-4, setting them apart as a different subfamily of receptor subunits. KA-1/2 also share high amino acid sequence homology with each other (70%), but only about 37% with GluR1-4 and 43% with GluR5-7.

GluR5-7 appear to share the typical overall structure of GluR1-4, each having four putative membrane-associated domains with presumably extracellularly located N-termini, intracellularly located C-termini and a large putatively extracellular domain between the third and fourth membrane associated segments (Bettler and Mulle, 1995). The same editing mechanism acting on the codon for the Q/R site in the second transmembrane domain of GluR2 has also been found to operate in the homologous site in GluR5 and GluR6 but not in GluR7 (Sommer et al., 1992; Bettler et al., 1992; Lomeli et al., 1992). In contrast to GluR2, however, the editing of GluR5 and GluR6 is incomplete so that both edited (R) and non-edited (Q) forms coexist. As for GluR2, editing changes the amplitude of the response, the divalent cation permeability, and the

rectification properties of ion channels formed with these subunits (Sommer et al., 1992). Two additional editing sites have recently been reported for the first transmembrane domain of GluR6 (Kohler et al., 1993) in which an isoleucine (I) and a tyrosine (Y) which are encoded in the gene, are edited to become valine (V) and cysteine (C) respectively. Again the edited receptors differ in their ion permeability properties suggesting that the first transmembrane domain is also involved in determining properties of the ion channel portion of the receptor.

KA1 and KA2 share the same structural features as GluR1-7. The mechanisms active in other non-NMDA GluR for creating receptor diversity do not appear to be active in this subfamily of GluRs since there are no indications of RNA editing at the Q/R site in the second transmembrane domain which contains a Q in both KA1 and 2 subunits, nor have any splice variants been reported. Expression of GluR5(Q) (unedited) in oocytes has been reported to generate functional channels that give only tiny responses to Glu (Bettler et al., 1990) while expression of the same subunits in transfected cell lines was reported to result in larger responses that could be activated by Dom > KA > Glu > AMPA (Sommer et al., 1992). Homomeric GluR6(R) (edited) channels expressed in oocytes are reported to respond to Dom > KA > QA > Glu but, interestingly, not to AMPA (Egebjerb et al., 1991). Contrary to what has been reported for receptors composed of GluR1-4 subunits, both Glu and KA have been observed to desensitize GluR5 and GluR6 receptors completely with no observable steady state currents upon prolonged application of the agonists (Sommer et al., 1992). GluR7 does not appear to form functional homomeric channels in oocytes or transfected cell lines

(Bettler et al., 1992; Lomeli et al., 1992). Coexpression of GluR5-7 with GluR1-4 does not lead to any detectable differences in the responses compared to the respective single subunits indicating that GluR5-7 probably do not form heteromeric complexes with GluR1-4 (Bettler et al., 1990; Sommer et al., 1992; Bettler et al., 1992).

No electrophysiological responses have been detected when either KA1 or KA2 were expressed as homomeric proteins in oocytes or in transfected cell lines (Werner et al., 1991; Sakimura et al., 1992; Herb et al., 1992; Kamboj et al., 1992); however, radioligand-binding studies to membranes of transfected cell lines did demonstrate the presence of high-affinity binding sites for KA > QA > Dom > Glu while AMPA binding was undetectable (Werner et al., 1991; Herb et al., 1992; Kamboj et al., 1992). When the KA1 subunit is coexpressed with GluR1-4, no changes in functional properties were observed and, thus, heteromeric complexes involving the two subclasses are presumed not to be formed (Werner et al., 1991). Coexpression of KA1 with GluR5-7 has not been reported, however, coexpression of KA2 with GluR5 and GluR6 produces receptors with functional properties not present in the respective homomeric receptors (Sakimura et al., 1992; Herb et al., 1992). The most impressive functional difference between homomers and heteromers is the responsiveness of the heteromeric GluR6/KA2 receptors to AMPA (not seen with GluR6 homomers).

1.2.1.3 N-Methyl-D-Aspartate Receptors

The first subunit cDNA coding for rat NMDA gateable GluRs was isolated through expression cloning by Nakanishi's group (Moriyoshi et al., 1991). Then a second

subfamily of NMDA receptor subunits (NMDAR2A-D) was revealed by the use of low-stringency hybridization screening combined with the use of primers designed to conserved regions between NMDAR1 and other GluRs (Monyer et al., 1992; Meguro et al., 1992; Kutsuwada et al., 1992; Ikeda et al., 1992). The two NMDAR subfamilies display low but significant homology (22-29% amino acid sequence identity) to other GluR subunits and only 26-27% amino acid sequence identity with each other; however, the NMDAR2A-D subunits share considerable homology with each other (42-56% amino acid identity). Despite the low overall homology with non-NMDA receptors, NMDAR subunits are believed to share the same transmembrane topology as non-NMDA receptor subunits with four membrane-associated domains, an extracellularly located N-terminus, an intracellularly located C-terminus, and a large putatively extracellularly located loop between the third and fourth membrane-associated domains (Mori and Mishina, 1995).

Recombinant homomeric NMDAR1 channels expressed in oocytes respond to Glu, NMDA, QA , ibotenate, and Asp but not to AMPA or KA and as for native NMDA receptors, these recombinant receptors require the presence of Gly as a co-agonist (Nakanishi et al., 1992; Moriyoshi et al., 1991; Yamazaki et al., 1992). Also consistent with native NMDA receptors, homomeric NMDAR1 receptors display a prominent voltage-dependent Mg^{2+} block such that responses are not elicited at resting potentials negative to -20mV in the presence of Mg^{2+} (Moriyoshi et al., 1991; Nakanishi et al., 1992). Furthermore, the homomeric channel was demonstrated to display high Ca^{2+} permeability similar to native NMDARs (Yamazaki et al., 1992). Thus, it has been demonstrated that the functional properties reported for NMDA receptors from studies

in other systems, such as tissue slices or cell cultures (for review see Mayer and Westbrook, 1987; Honoré, 1989), can all be found to reside within homomeric NMDAR1 receptors indicating that all the different sites responsible for interaction with various agonists, antagonists, or modulators, reside on the same protein. However, in all of the investigations current amplitudes at homomeric NMDAR1 receptors are reported to be fairly small, suggesting that other subunits are likely necessary to give the receptor it's full *in vivo* efficacy.

None of the NMDAR2 subunits assembles into functional ion channels when expressed as homomeric receptors in oocytes or transfected cells (Meguro et al., 1992; Monyer et al., 1992; Kutsuwada et al., 1992; Ikeda et al., 1992). Coexpression of NMDAR2A with either NMDAR2B or NMDAR2C, or of NMDAR2B with NMDAR2C also did not produce functional channels (Monyer et al., 1992). However, when coexpressed with NMDAR1, each of the four NMDAR2 subunits has been found to coassemble with NMDAR1 into functional heteromeric channels with properties that differ from those of homomeric NMDAR1 receptors (Meguro et al., 1992; Kutsuwada et al., 1992). Most notably, current amplitudes for NMDAR1/NMDAR2D, NMDAR1/NMDAR2C, NMDAR1/NMDAR2A, and NMDAR1/NMDAR2B are ~5-, ~20-, ~40-, and ~60-fold larger respectively than NMDAR1 homomeric receptor currents. On the other hand, the typical native NMDAR properties still remain in the heteromeric complexes (ie. Gly as a co-agonist, voltage-dependent Mg^{2+} blockade, Ca^{2+} permeability, etc.) although they are usually somewhat altered.

1.3 Serotonin (5-hydroxytryptamine)

For many years investigators had known of a blood-borne chemical that produced vasoconstriction which was termed serotonin (a 'serum' factor that affected blood vessel 'tonus') and of a substance present in the gut that increased intestinal motility which they termed enteramine. In the mid-twentieth century, the single compound 5-HT that produced both of these effects was isolated and synthesized, and its molecular structure was elucidated (Rapport et al., 1948; Rapport, 1949; Hamlin and Fisher, 1951). Shortly thereafter, 5-HT was found to be present in vast quantities in the mammalian CNS, leading to the proposal of 5-HT as a neurotransmitter (Twarog and Page, 1953; Amin et al. 1954). Since this proposal, 5-HT has gained wide acceptance as a neurotransmitter substance within the mammalian brain and has been the focus of many investigations because of its presumed involvement in major psychoses.

1.3.1 The Mammalian Brain Serotonin Nuclei

The 5-HT cell bodies in the brain are located in the brain stem on or near the midline and can be divided into two superior and inferior groups based on the early developmental appearance of mesencephalic and myelencephalic groups. The superior group consists of four main nuclei: the caudal linear nucleus, the median raphé nucleus and its laterally displaced cells in the nucleus pontis centralis oralis, the dorsal raphe nucleus, and the lateral B9 neurons lying just dorsal to the medial lemniscus. The inferior group consists of five main nuclei: the nucleus raphe obscurus, the nucleus raphe pallidus, the nucleus raphe magnus, neurons in the ventrolateral medulla including the

lateral paragigantocellular nucleus and the intermediate reticular nuclei, and the area postrema. These serotonergic neurons have extensive projections, both rostrally and caudally, from the superior and inferior groups, respectively (for review see Jacobs and Azmitia, 1992).

1.3.2 Serotonin Receptors

The Serotonin Club Receptor Nomenclature Committee recently proposed a new nomenclature scheme for 5-HT receptors which requires three fundamental properties of a receptor to be described to ensure correct classification: its operational (drug related), transductional (receptor coupling), and structural (primary amino acid sequence) characteristics (Humphrey et al., 1993). When applied to the currently recognized 5-HT receptors, these criteria indicate the existence of up to seven receptor classes: 5-HT_1 through 5-HT_7 families (for reviews see Martin and Humphrey, 1994; Boess and Martin, 1994; Peroutka, 1994).

1.3.2.1 5-HT_1 Receptors

At least five 5-HT_1 receptor subtypes are now recognized (5-HT_{1A} through 5-HT_{1F} excluding 5-HT_{1C} which has been relocated to the 5-HT_2 receptor class) (for review see Boess and Martin, 1994). All are seven transmembrane domain, G-protein coupled receptors encoded by intronless genes, comprising between 365 and 422 amino acids and displaying overall sequence homology of about 40% (Kobilka et al., 1987; Libert et al., 1989; Albert et al., 1990; Hamblin and Metcalf, 1991; Weinshank et al., 1992; Levy et al.,

1992; Zgombick et al., 1992; McAllister et al., 1992). These receptors are preferentially linked to the inhibition of adenylyl cyclase (AC), though other transduction mechanisms are reported.

1.3.2.1.1 5-HT_{1A} Receptors

In many species including man, 5-HT_{1A} receptors are located predominantly in brain regions concerned with mood and anxiety (the limbic sytem) and to a lesser extent in areas governing temperature, feeding and locomotion. Metergoline, d-lysergic acid diethylamide (LSD) and the indoles (eg. 5-carboxamidotryptamine (5-CT)) are potent but non-selective 5-HT_{1A} receptor agonists. Numerous selective agonists nevertheless have been described, most being piperazine derivatives (buspirone, flesinoxan, etc.) or benzodioxanes (e.g. spiroxatrine), but the aminotetraline 8-hydroxy-2-(di-N-propylamino)-tetralin (8-OH-DPAT) remains the most useful. 5-HT_{1A} receptor activation has been variously associated with stimulation of AC (Sijbesma et al., 1991), inhibition of AC (Fargin et al., 1989; Albert et al., 1990; Liu and Albert, 1991; Fowler et al., 1992; Banerjee et al., 1993), stimulation of phosphatidylinositol (PI) turnover (Fargin et al., 1989; Raymond et al., 1989; Raymond et al., 1992; Mangel et al., 1993), opening of K^+ channels (Karschin et al., 1991), and increases in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) (Liu and Albert, 1991; Raymond et al., 1992; Mangel et al., 1993). The variety of observed effects of activation of 5-HT_{1A} receptors indicates that the outcome of receptor activation is most likely dependent upon the second messenger machinery available to interact with the activated receptor. However, by far the most commonly observed effect of 5-HT_{1A}

receptor activation is the inhibition of AC activity, suggesting that this is the preferential coupling of the receptor.

1.3.2.1.2 5-HT_{1B} Receptors

5-HT_{1B} receptors appear to be specific to rats and some other rodents. Their precise roles in central regulation remain unknown but highest densities are found in the basal ganglia, substantia nigra, and cortex where they function either as autoreceptors on serotonergic nerve terminals or as hetero-receptors modulating the release ACh and Glu (Pazos and Palacios, 1986; Engel et al., 1986; Maura and Raiteri, 1986; Raiteri et al., 1986). The peptide ergots (eg. cyanopindolol) and the indoles (eg. 5-CT) are potent agonists at 5-HT_{1B} receptors, but exhibit similar potencies at 5-HT_{1A} receptors (Martin and Humphrey, 1994). The indole analog, CP93,129, appears to be the first really selective 5-HT_{1B} receptor agonist (Macor et al., 1990). 5-HT_{1B} receptors, like 5-HT_{1A} receptors, are preferentially linked to inhibition of AC; however, in hippocampal serotonergic nerve terminals, receptor function is unaffected by pertussis toxin, cholera toxin, or N-ethyl-maleimide implying that the receptor coupling does not involve G_i, G_s, or G_o proteins (Blier, 1991).

1.3.2.1.3 5-HT_{1D} Receptors

Genes encoding two 5-HT_{1D} receptor subtypes (5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$) have been described (Libert et al., 1989; Hamblin and Metcalf, 1991; Weinshank et al., 1992; Levy et al., 1992; Jin et al., 1992; Hamblin et al., 1992; Demchyshyn et al., 1992; Mochizuki et

al., 1992; Veldman and Bienkowski, 1992). It is now evident from molecular comparison that the 5-$HT_{1D\beta}$ subtype is a non-rodent homologue of the 5-HT_{1B} receptor (Adham et al., 1992), although the two display completely different pharmacological profiles. Like the 5-HT_{1B} subtype, 5-HT_{1D} receptors function as autoreceptors on nerve terminals and as hetero-receptors functioning to modulate the release of Glu and ACh (Raiteri et al., 1986; Harel-Dupas et al., 1991). No selective 5-HT_{1D} receptor agonists or antagonists are currently available. 5-CT, RU24969, sumatriptan, and L-694,247 are potent agonists but show limited selectivity over other 5-HT_1 receptors. Although metergoline and methiothepin are typically used as antagonists, these too show poor selectivity. Both 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors are negatively coupled to AC (Hamblin and Metcalf, 1991; Zgombick et al., 1991; Veldman and Bienkowski, 1992; Weinshank et al., 1992). However, stimulation of cAMP formation, stimulation of PI turnover, and elevation of $[Ca^{2+}]_i$ have all been reported in response to receptor activation (Maenhaut et al., 1991; Van Sande et al., 1993; Zgombick et al., 1993).

1.3.2.1.4 5-HT_{1E} Receptors

The absence of any useful selective ligands has precluded identification of specific functional roles for the 5-HT_{1E} receptor, although the human receptor has now been cloned (McAllister et al., 1992; Zgombick et al., 1992). Expression of the recombinant receptor in various cell lines has revealed that high affinity [^{3}H]5-HT binding is displaced by 5-CT and ergotamine with relatively low affinity. Activation of the expressed receptor causes inhibition of forskolin-stimulated cAMP formation indicating

a negative coupling to AC (McAllister et al., 1992; Zgombick et al., 1992; Gudermann et al., 1993).

1.3.2.1.5 5-HT_{1F} Receptors

Low stringency screening of a mouse brain cDNA library with a mouse 5-HT_{1B} receptor-derived probe revealed a close relative of the 5-HT_{1E} receptor that was subsequently identified in the human brain (Amlaiky et al., 1992; Adham et al., 1993a); because of its unique pharmacological profile, it was designated as the fifth 5-HT_1 receptor subtype (5-HT_{1F}). Specifically, the receptor exhibits high affinty for 5-HT, sumatriptan, and the non-peptide ergots (methylergonovine and methysergide), with comparatively low affinity for 5-CT. Expression studies have revealed that stimulation of the receptor mediates primarily inhibition of AC but also stimulates PI turnover and rapidly elevates $[Ca^{2+}]_i$ (Adham et al., 1993b).

1.3.2.2 5-HT_2 Receptors

The 5-HT_2 receptor class now comprises three distinct receptor subtypes: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} (formerly 5-HT_{1C}). Each is a seven transmembrane domain, G-protein-coupled receptor similar in molecular size and displaying about 60% sequence homology. 5-HT_2 receptors possess both introns and exons and couple preferentially to phospholipase C (PLC) leading to stimulation of PI turnover.

A wide range of 5-HT_2 receptor antagonists are available and several are useful radiolabeled probes (eg. spiperone, ketanserine, mesulergine, etc.); unfortunately, all of

these antagonists display poor selectivity with regard to the three 5-HT_2 receptor subtypes. Likewise there are no subtype-specific 5-HT_2 agonists. α-Methyl-5-HT and the phenylalkylmines (DOI, DOB, DOM) are most often used as 5-HT_2 agonists but are equally active at all three subtypes of receptors. Receptor stimulation of PI turnover and rises in $[Ca^{2+}]_i$ have been demonstrated in studies using both native and recombinant 5-HT_2 receptors (Hoyer, 1988; Hoyer et al., 1989; Lubbert et al., 1987; Pritchet et al., 1988; Julius et al., 1988; Julius et al., 1990; Kursar et al., 1992; Stam et al., 1992; Loric et al., 1992; Wainscott et al. 1993). Whether or not the Ca^{2+} mobilization is secondary to the PI metabolism is as yet undetermined.

1.3.2.3 5-HT_3 Receptors

5-HT_3 receptors are ligand-gated cation channels. Cloning of the murine (Hope et al., 1993; Werner et al., 1993) and rat (Johnson and Heineman, 1992; Isenberg et al., 1993) 5-HT_3 receptors has revealed that several features of the ligand-gated ion channel superfamily are preserved in the amino acid sequence, including the typical four hydrophobic regions predicted to be membrane-spanning domains, a large amino terminal domain and short carboxyl-terminus that are presumed to be extracellular. In fact the 5-HT_3 receptor shows significant amino acid sequence identity with the α-subunit of the *Torpedo californica* nicotinic ACh receptor (27%) and the α7 neuronal nicotinic ACh receptor (30%). There is no direct evidence for the existence of 5-HT_3 receptor subtypes, but profound species differences in ligand binding affinities and electrophysiological characteristics have been reported (Peters et al., 1992; Fozard, 1992).

In the periphery, 5-HT_3 receptors are located exclusively on neurons and elicit depolarizations which result in transmitter release from parasympathetic, sympathetic, sensory, and enteric neurons (Fozard, 1984). In the CNS, 5-HT_3 receptors are relatively sparse in comparison to other 5-HT receptors, and function to modulate transmitter release (Costall et al., 1987; Blandina et al., 1989; Alhaider et al., 1991; Galzin and Langer, 1991).

Numerous selective 5-HT_3 receptor antagonists are available, the most comprehensively studied being MDL72222, tropisetron, ondansetron, and granisetron (Glennon and Dukat, 1991). The potency and high selectivity of these antagonists makes them invaluable for defining and classifying 5-HT_3 receptors. There are no equally selective 5-HT_3 receptor agonists, but 2-methyl-5-HT and the arylbiguanides (e.g. phenylbyguanide) are most frequently used. Activation of both native and recombinant 5-HT_3 receptors leads to a rapid inward current that quickly desensitizes upon prolonged application; the channel discriminates poorly between Na^+ and K^+ and allows passage of large organic cations such as Tris (Peters et al., 1992; Maricq et al., 1991).

1.3.2.4 5-HT_4 Receptors

Originally, the 5-HT_4 receptor was characterized in the CNS as a 5-HT receptor that was positively coupled to AC and that displayed a unique pharmacological profile (Dumuis et al., 1988; Bockaert al., 1990). Specifically, the benzamide SC-53-116 and the substituted benzamides such as metoclopramide, zacopride, cisapride, and renzapride are all potent agonists and a number of antagonists such as SDZ 205,557, RS-23597,190,

SC-53606, DAU 6258, and GR 113808 have all been reported to be potent and selective (Bockaert et al., 1990; Turconi et al., 1991; Flynn et al., 1992; Grossman et al., 1993). Recently two different cDNAs (presumably splice variants) encoding 5-HT_4 receptors have been isolated from the rat brain providing concrete evidence for the existence of this family of 5-HT receptors (Gerald et al., 1995). Expression of the cloned receptors in COS-7 cells confirmed that the 5-HT_4 receptors are positively coupled to AC and revealed pharmacological profiles similar to those previously described for native 5-HT_4 receptors (Gerald et al., 1995).

1.3.2.5 5-HT_5 Receptors

Two subtypes of 5-HT_5 receptors have been cloned: 5-HT_{5A} from mouse, rat, and human (Plassat et al., 1992; Erlander et al., 1993; Rees et al., 1994), and 5-HT_{5B} from mouse and rat (Matthes et al., 1993; Erlander et al. 1993; Wisden et al., 1993). The primary amino acid sequences of the proteins possess all of the features of a seven transmembrane segment, G-protein coupled receptor. When expressed in various cell lines the 5-HT_5 receptors display high affinity for LSD, ergot derivatives, methysergide, methiothepin, 5-CT, 8-OH-DPAT, and sumatriptan, thus displaying a pharmacological profile similar to that of 5-HT_1 receptors. However, in contrast to the other G-protein coupled 5-HT receptors, no effects on PI metabolism or AC activity have been observed in relation to 5-HT_5 receptors when expressed in COS-7 or NIH-3T3 cells (Plassat et al., 1992; Erlander et al., 1993) although, in the case of the 5-HT_{5B} receptor, a fraction of binding sites observed in COS-1 cells were displaced by GTP analogues suggesting that

the receptor does in fact couple to G-proteins in these cells (Wisden et al., 1993).

1.3.2.6 5-HT_6 Receptors

The 5-HT_6 receptor has been cloned from the rat and expressed in HEK-293 and COS-7 cells (Monsma et al., 1993; Ruat et al., 1993). The primary structure revealed that the protein belonged to the seven transmembrane, G-coupled receptor superfamily. This class of 5-HT receptors was demonstrated to display high affinity binding sites for LSD, 5-methoxytryptamine (5-MeOT), methiothepin, and 5-CT, but the most striking pharmacological feature of the 5-HT_6 receptor is its high affinity for antipsychotic drugs such as loxapine and clozapine as well as tricyclic antidepressants such as amoxipine and clomipramine (Monsma et al., 1993). The expressed 5-HT_6 receptors were shown to be positively coupled to AC. The first evidence for the *in vivo* existence of the 5-HT_6 receptor has come from studies of pig caudate putamen membranes where 5-HT receptor agonists induced a concentration-dependent stimulation of AC activity with a rank order of potency very similar to that of the expressed receptor (5-HT > 5-MeOT > 5-CT > sumatriptan) (Schoeffter and Waeber, 1994).

1.3.2.7 5-HT_7 Receptors

The 5-HT_7 receptor has been cloned from human, rat, mouse, guinea-pig, and *Xenopus laevis* (Bard et al., 1993; Lovenberg et al., 1993; Meyerhof et al., 1993; Shen et al., 1993; Plassat et al., 1993; Tsou et al., 1994; Nelson et al., 1995). Amino acid sequence analysis has demonstrated the receptor to belong to the superfamily of G protein-

coupled receptors. All of the cloned 5-HT_7 receptors display high affinity for the 5-HT_1 agonists 5-CT and 8-OH-DPAT and the 5-HT_2 antagonists mesulergine and ritanserin as well as several of the less selective 5-HT receptor ligands such as methiothepin and metergoline. Like the 5-HT_6 receptor, the 5-HT_7 receptor also has high affinity for several typical and atypical antipsychotic drugs (clozapine, spiperone, loxapine etc.) and the tricyclic antidepressant amitriptyline. Because of the high affinity of the 5-HT_7 receptor for 5-HT_1 agonists and since the 5-HT_7 receptor has been demonstrated to be positively coupled to AC, it is believed that this receptor might correspond to previously reported 5-HT_1-like receptors that are positively coupled to adenylyl cyclase (Shenker et al., 1985; Markstein et al., 1986; Fayolle et al., 1988; Prosser et al., 1993).

1.4 Objectives and Hypotheses

An overwhelming amount of evidence exists suggesting that the somata of MeV neurons are under the influence of various synaptic inputs. This evidence includes the existence of synaptic contacts containing a variety of putative neurotransmitter and neuromodulator substances impinging upon MeV neuronal somata, indications that mesencephalic trigeminal cells possess the receptors necessary to respond to some of these neurotransmitter substances, projections from various centrally located nuclei to cells of the MeV, and demonstrations that the activity of MeV neurons can be modulated by other nervous system structures. In spite of this evidence, most studies have failed to demonstrate responsiveness of mesencephalic trigeminal neurons to a wide variety of neurotransmitters. However, the recent report that MeV neurons are capable of

responding to GABA (Hayar et al., in press) suggests that previous investigations of MeV neurotransmitter responsiveness were flawed in their methodologies, and that MeV neurons are in fact capable of responding electrophysiologically to neurotransmitter substances. Based on these findings, it was the intention of this study to reinvestigate the effects of two well-known neurotransmitters upon MeV neurons.

Specifically, the effects of EAAs (Glu and its agonists) and 5-HT on mesencephalic trigeminal neurons have been reevaluated. These two substances were chosen because of the large body of evidence implicating them as neurotransmitters to which MeV neurons may respond (see section on neurotransmitters in the MeV); furthermore, they have well-documented effects on DRG neurons, cells that represent the peripheral counterparts of MeV neurons (Agrawal and Evans, 1986; Puil and Spigelman, 1988; Lovinger and Weight, 1988; Huettner, 1990; Wong and Mayer, 1993; Molokanova and Tamarova, 1995). It was the hypothesis of this investigation that mesencephalic trigeminal neurons would be observed to respond to Glu and to 5-HT.

1.4.1 Specific Objectives

1) Imperative to the investigation is the ability to obtain a preparation containing the neurons to be investigated and perform stable electrophysiological recordings from them. Thus, the first objective was to obtain a brain-stem slice preparation containing the MeV and maintain it *in vitro* for intracellular recordings during which cells could be identified based on their electrophysiological characteristics.

2) To investigate the actions of EAAs on MeV neurons and to characterize any

effects observed.

3) To investigate the actions of 5-HT on MeV neurons and characterize any effects observed.

2 Materials and Methods

Intracellular recordings of mesencephalic trigeminal neurons in a rat brain slice preparation have been performed. Electrophysiological characterization and intracellular staining of neurons was performed to ensure that recorded cells displayed characteristics previously reported for MeV neurons. Furthermore, various drugs have been applied by pressure or superfused to recorded cells to determine in what manner they might alter the electrophysiological properties of MeV neurons.

2.1 Surgery

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) weighing 50 to 150 g were used for the study. The animals were anaesthetized by exposure to 2.5% halothane in an oxygen-rich chamber for 3-5 min. Immediately after removal from the anaesthetic, the animals were surgically decapitated at the atlanto-occipital joint following a rostro-caudal cut over the scalp to expose the skull. Following decapitation the skull was peeled back with forceps to expose the brain. After careful removal of the dura mater, a transverse section at a level 2-2.5 mm rostral to the cerebellum was made for insertion of a spatula under the brainstem caudal to the section; the spatula was used to sever the ventral attachments (i.e. cranial nerves) of the brain to the skull. The portion of the brain caudal to the section (hereafter referred to as the brain) was then carefully lifted out of the cranial cavity and placed into a petri dish filled with cold (0-4^0 C) oxygenated artificial cerebrospinal fluid (ACSF).

The importance of keeping the tissue cold during the dissection process cannot be stressed enough. This process minimizes anoxic damage to the tissue and improves the texture of the tissue for sectioning of the brain into the block and for slicing the block. For this reason ice-cold ACSF was frequently poured over the brain and tissue block throughout the dissecting process.

2.2 Brain Slice Preparation

The brain was transferred from the petri dish to a flat cool surface on which there was a piece of filter paper moistened with cool ACSF. Using razor blades, a block of tissue (approximately 6x5x5 mm) containing the mesencephalon and pons was separated from the rest of the brain. The tissue block was glued with cyanoacrylate (Crazy Glue) to a plexiglass stage cover with the rostral surface uppermost and dorsal surface resting against a plexiglass block cushioned with an agar slab. Following attachment of the stage cover (with tissue block glued on) to a vessel filled with cold oxygenated ACSF that rests on the track of a Vibroslice unit (Campden Instruments Ltd., England), transverse brain stem slices of 300-350 μm in thickness were taken. The blade of the Vibroslice unit was set on maximum swinging amplitude and highest vibration frequency while the advancement of the blade into the tissue block was set on the lowest speed. Determination of the level of slicing was in most cases made with the naked eye, and in some cases, with the aid of a dissecting microscope (Carl Zeiss, Jena Instruments Ltd., Germany) to identify the level of the MeV. Usually, two or three slices were obtained at the level of the MeV from one rat. Generally the most caudal MeV

containing slice was used for experimental puposes because this is the area with the highest density of MeV neurons. The selected slice was then transferred to the slice recording chamber. The whole dissection, from decapitation to acquisition of the slice, was usually performed within 10-12 min.

After placing the slice in the recording chamber, gold electron microscope grids were placed over the area of the MeV on each side of the slice to stabilize the tissue in the areas where recordings would be made. These grids were held in place by two small platinum bars. A period of at least one hour was then allowed for the slice to recover before any attempts at recording were made.

2.3 Slice Chamber and Perfusate

The slice recording chamber consists of a cylindrical piece of plexiglass with a circular hollow on top, inside of which was a circular step that forms a resting support for an O-ring covered on its top side with a nylon mesh support for the brain slice. On the side of the plexiglass chamber there is a tunnel, to allow for perfusion, leading into the circular hollow at a level above where the O-ring rests. Another tunnel leads from the bottom of the hollow to the back of the recording chamber where another hollow exists to allow for drainage of excess perfusate. A vacuum line removes the excess perfusate from the top of the second hollow and allows for control of the depth of perfusate within the recording chamber. The recording chamber rests upon the stage of a dissecting microscope which is illuminated from the underside; hence, providing transillumination of anything within the recording chamber to be viewed in the

microscope from above.

After a slice was obtained, it was placed into the recording chamber on top of a piece of lens paper that rested on the nylon mesh support of the O-ring and was superfused with ACSF at a rate of 1.5 ml/min. The ACSF was prepared on the day of the experiment by dilution of a stock solution concentrated 5-fold with deionized, double-distilled water. The ACSF contained (in mM): NaCl 118.0, KCl 3.0, $CaCl_2$ 2.5, $MgSO_4$ 0.8, NaH_2PO_4 1.0, $NaHCO_3$ 20.0 (30 for the cold dissection ACSF), and D-glucose 10.0. The stock solution contained all of the above mentioned ingredients except for $NaHCO_3$ and D-glucose which were added to the diluted stock the day of the experiment. In some experiments a modified ACSF was used in which no $MgSO_4$ was included since NMDA responses are blocked by Mg^{2+} ions at resting potentials (see Introduction section 1.2.1.3); the Mg^{2+}-free ACSF contained (in mM): NaCl 119.84, KCl 3.0, $NaHPO_4$ 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 20.0, and D-glucose 10.0. Osmolarity was calculated to be in the range of 290-300 mOsm. The ACSF was stored in reservoirs placed above the level of the recording chamber and connected to the recording chamber via a perfusion line made of tygon tubing. Flow from the reservoirs to the recording chamber was gravity induced and controlled with a flow regulator.

While in the reservoirs the ACSF was continuously heated and oxygenated. The reservoirs housing the ACSF were jacketed such that the ACSF could be warmed by warm water (35^0 C) circulating through the outer layer of the jar. In order to prevent the ACSF from losing heat on the way from the reservoir to the recording chamber, a portion of the perfusion line was run through a plastic jacket which was also warmed

by circulating warm water (35^{0}C). With the use of these methods the ACSF in the slice chamber was adequately maintained at 32±0.5^0 C and pH 7.4±0.05.

2.4 Electrophysiological Recordings

Intracellular recording electrodes, with tip resistances of 35-110 MΩs when filled with 2 M KAc, were pulled from glass fibre-filled, borosilicate glass pipettes (1.2 mm outer diameter, 0.7 mm inner diameter, World Precision Instruments, Fla., U.S.A.) using a Brown-Flaming puller (Sutter Instruments Co., Cal., U.S.A.). KAc-filled electrodes were connected to an HS-2 headstage (gain = 0.1H) of an Axoclamp 2A amplifier (Axon Instruments Inc. Cal., U.S.A.) by an electrode holder (1.2 mm, E.H. Wright, Conn., U.S.A) through a silver chloride-coated silver wire (0.255 mm in diameter). The electrodes were manually manipulated with a macromanipulator (Narashige, Japan) into the ACSF contained within the recording chamber above the slice in the region of the MeV while being viewed under the dissecting microcope (10-40X). The active bridge circuit was completed by a reference electrode containing 2 M KAc trapped in a 3-4% agar bridge connecting the excess perfusate in the second hollow to the headstage.

Potential differences (eg. intracellular potentials) measured at the recording electrode tip were amplified (Axoclamp 2A) and displayed by conventional methods (oscilloscope) and recorded on a chart recorder (Gould 2400). Constant current pulses of small amplitude (-0.2 nA) generated by a Grass stimulator connected to the amplifier were routinely passed through the recording electrode for balancing (i.e. determine the resistance of the active bridge circuit) and to detect any apparent increases in resistance

when the electrode encountered the tissue and structures within the tissue. Also, while the recording electrode sat in the ACSF just above the surface of the slice the capacitance of the electrode was neutralized with the aid of the capacitance neutralization feature of the amplifier, and the potential difference read across the bridge circuit was set to 0 mV.

Movement of the recording electrode following placement in the area of the MeV above the slice was accomplished with an hydraulic micromanipulator (Newport, Cal., U.S.A.). Plugging of the electrode tip as it was lowered into the tissue could be dealt with by 'ringing' the electrode (ie. cleaning the tip by passing a high frequency current pulse through it) with a remote buzzer attached to the amplifier. This ringing is also useful for disrupting the membranes of cells encountered by the tip of the electrode and, thus, useful for penetrating cells for intracellular recordings. Upon impalement of a cell, a hyperpolarizing current of -0.5 to -1.0 nA was appplied to the intracellular recording electrode in order to help the cell stabilize, and the bridge circuit was usually rebalanced. Once the cell stabilized, the hyperpolarizing current was usually reduced or removed. Upon stabilization, current injection protocols for generating voltage-current (V-I) relations and evoking action potentials were performed with and recorded on a personal computer using pClamp 5.5 or 6.0 (Axon Instruments Inc, Cal., U.S.A.).

2.5 Identification of MeV Neurons

Recorded cells were identified as mesencephalic trigeminal cells based on their anatomical location and their electrophysiological characteristics. Frequently, the slices used contained the caudal third of the MeV at the level of the LC which in the

transverse brain slice is clearly identifiable at 10-40X magnification with transillumination as a bright spot at the lateral edge of and ventral to the IVth ventricle. In such cases cells of the MeV could be reliably found along the lateral border of the LC. In order to confirm that the recorded cells displayed morphology consistent with that previously reported for MeV neurons, some recorded cells were intracellularly stained with neurobiotin (Vector Laboratories Inc., Cal., U.S.A.).

2.5.1 Intracellularly Stained Neurons

Recording electrodes used for intracellular staining of neurons were filled with 2% neurobiotin in KCl (2 M) and had tip resistances of 40-90 MΩ. Following electrophysiological characterization of impaled cells and various drug tests, some neurons were injected with iontophoretically ejected neurobiotin via depolarizing rectangular current pulses (3.3 Hz, 150 ms duration, +1.0-2.0 nA) for at least 10 minutes. Following injection, the electrode was immediately removed from the cell and the slice was allowed to remain in the bath and perfused for at least 45 minutes to allow time for the neurobiotin to diffuse throughout the injected cell. Slices were then fixed by placing them in Falcon cell culture dishes (Becton Dickinson and Co., N.J. U.S.A.) filled with Lana's fixative. Following overnight fixation, the slices were rinsed and stored at 4^0C in 10-18% sucrose phosphate buffer (PB) (10 mM).

For visualization of injected cells, the slices were first agitated in 0.3% Triton X-100 (BDH Chemicals, Toronto, Ont., Can.) phosphate buffered saline (PBS) (10 mM) overnight at 4^0C in a shaker. The slices were then incubated in a 1:2000 dilution of CY3-

streptavidin : 0.1% Triton X-100 10 mM PB for 2 hours, after which the slices underwent three rinses in 10 mM PB of 15-30 min. each. The tissue was then cleared by successive agitations in 20% glycerol PB (1 hr), 50% glycerol PB (2 hrs), and 80% glycerol PB (18 hrs), following which they were mounted in 80% glycerol and coverslipped. Slides were viewed and pictures were taken under a fluorescent microscope (Zeiss MC100, Carl Zeiss Inc., Germany) with a Texas Red filter (excitation = 590 nm, emission = 615 nm).

2.6 Drug Applications

All drugs used in the present investigation were prepared from stocks diluted in ACSF and pH-adjusted to 7.4±0.1 with HCl and NaOH.

2.6.1 Pressure Application

Pressure electrodes (of tip diameters from 5-10 μm) pulled from the same type of pipettes used for pulling intracellular recording electrodes were pulled on another program of the Brown-Flaming puller. Drug-containing pressure electrodes were placed about 50-100 μm above the surface of the slice aimed in the vicinity of the recording electrode while recording from an impaled cell. Pressure ejection of drugs from the pipettes was performed and controlled using a multi-channel Picospritzer (General Valve Corp., N.J., U.S.A.) connected to a nitrogen tank for a pressure source. Pressures used ranged from 10-20 psi lasting from 5 to 40 ms, depending upon the characteristics of the electrode being used. Drugs applied by pressure were: GABA (Sigma), Glu (Sigma), NMDA (Sigma), KA (Sigma), AMPA (Toronto Research Chemicals Inc.), 5-HT (Sigma),

5-CT (RBI), 8-OH-DPAT (RBI), DOI (RBI), and α-methyl-5-HT (RBI). In cases where responses to pressure-applied drugs appeared to undergo desensitization, the pressure electrode was distanced from the recording electrode in between applications but always replaced to the same position for application.

2.6.2 Superfused Applications

For superfusion of drugs, stock solutions of drugs were diluted in warmed and oxygenated ACSF contained in an alternate resevoir. The drugs were applied to the slice chamber by switching the perfusion solution to this second resevoir by means of a three-way tap. Using this process there was a time lag for the drugs to reach the recording chamber of 1.5 min. from the time of switching the flow of ACSF from the primary reservoir to the secondary one. Drugs applied by superfusion were: 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (RBI), (±)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (RBI), cyclothiazide (RBI), L-*trans*-pyrrolidine-2,4-dicarboxylic acid (*trans*-PDC) (Tocris Cookson), CsCl (Sigma), ZD 7288 (Tocris Cookson), 8-Bromo-cAMP (Biomol), 3-isobutyl-1-methylxanthine (IBMX) (Sigma), forskolin (Sigma), H-89 (Biomol), H-7(Biomol), ketanserin (RBI), methiothepin mesylate (RBI), and TTX (Sigma).

2.7 Statistics

Means and standard error of the means (S.E.M.s) were calculated on a personal computer using prism software. Satistical significance of protein kinase inhibition effects on 5-HT responses was assessed using a paired student's t-test.

3 Results

3.1 Electrical Properties of Recorded Mesencephalic Trigeminal Neurons

Electrical properties for which means are reported are based on a representative sample of 20 neurons.

Once impaled, neurons of the MeV displayed an 'injury' potential (>-50 mV) and discharged at rates greater than 150 Hz lasting from 30 s to a couple of minutes. Following a period usually lasting approximately 5 minutes, during which hyperpolarizing current (-0.5 to -1.0 nA) was injected into the impaled cells, recorded cells would recover and reach a stable membrane potential at which no rhythmic activity or spontaneous discharges, and no apparent synaptic potentials could be observed. Once stabilized, recorded neurons of the MeV had resting membrane potentials ranging from -50 to -70 mV (mean = -57.8 ± 1.0 mV) as measured upon exiting the cells. Input resistances (R_{input}) ranging from 11.4 to 39.1 MΩs (mean = 28.4 ± 1.6 MΩs) were determined from the slope of the V-I relations of the neurons at resting potential.

Action potentials (APs) generated with depolarizing current injection (0.5 to 2.4 nA) ranged from 57.1 to 77.6 mV (mean = 64.7 ± 1.7 mV) in amplitude and between 0.384 to 0.592 ms (mean = 0.448 ± 0.057 ms) in duration as measured at threshold (-49.9 to -34.7 mV, mean = 41.2 ± 1.0 mV) with overshoots of 13.1 to 35.1 mV (mean = 23.5 ± 1.3 mV) (Fig. 1A). Prominent after-hyperpolarizations (AHPs) of 3.5 to 16.1 mV (mean = 9.4 ± 0.9 mV) were observed following spike generation. At peri-threshold potentials suprathreshold stimuli often evoked high frequency trains of APs having little or no

change in successive APs within a burst (inset of Fig. 1A). These trains of APs displayed frequencies closely approximating those of sub-threshold potential oscillations observed in recorded neurons (see below). APs could also be elicited at the termination of an hyperpolarizing current pulse (as in Figs. 2A,C and 4B). Such 'off-spikes' are analogous to the anodal-break response observed in TG neurons by Puil and Spigelman (1988). MeV neuron APs could be fully abolished by perfusion of the slice with TTX (0.5 μM) and recovered upon removal of the drug (Fig. 1B).

All recorded neurons of the MeV exhibited varying degrees of inward rectification as evidenced from their 'sagging' voltage responses to intracellular injections of hyperpolarizing current pulses (up to -3.0 nA) (Fig. 2A). This inward rectification of MeV neurons displayed a time- and voltage-dependence. Displacement of the membrane potential reached a peak within 6.0 to 13.2 ms (mean = 9.3 ± 0.4 ms) after the start of the hyperpolarizing pulse and then declined to a steady-state level within 17.1 to 78.0 ms (mean = 38.8 ± 3.4 ms) after the start of the pulse (as determined using a pulse of -2.4 nA for all measurements). The 'sag' in the voltage response displayed larger amplitude and shorter latency with increasingly larger current injections, demonstrating the voltage-dependence of the rectification. This type of inward rectification is similar to the slowly developing mixed cation conductance (Na^+/K^+) termed I_f (cardiac tissue) and I_h or I_q (nervous tissue), originally described in cardiac sino-atrial fibres (Yanagihara and Irisawa, 1980) and subsequently observed in a variety of mammalian central neurons including DRG, TG, and thalamic neurons (Mayer and Westbrook, 1983; Puil and Spigelman, 1988; McCormick and Pape, 1990a). Perfusion of

slices with CsCl (5-10 mM) reversibly antagonized the inward rectification exhibited by MeV neurons (Fig. 2B) while perfusion with the novel specific I_h blocker ZD 7288 (Harris and Constanti, 1995) irreversibly abolished the inward rectification displayed by these neurons (Fig. 2C). The blockade of I_h by ZD 7288 was accompanied by a decrease in the excitability of MeV neurons (Fig. 3). Depolarizing current injections sufficient to generate APs before I_h blockade were incapable of generating APs after ZD 7288 treatment and bursts of APs generated with large stimulations could not be obtained after I_h blockade. The changes in excitability were not associated with any changes in the resting potential of the neurons during ZD 7288 treatment.

Upon termination of hyperpolarizing current pulses that activated I_h, the membrane potential was observed to overshoot the resting level and then return to rest. If the overshoot was large enough to reach threshold, off-spikes would be generated as in Figs. 2A,C and 4B. Involvement of I_h in the overshoot phenomenon is suggested by the antagonism of the overshoot during CsCl and ZD 7288 treatment of recorded neurons (Figs. 2B, C).

The vast majority of recorded neurons displayed voltage-dependent high frequency oscillatory patterns of activity. At normal resting potentials a stable membrane potential could be recorded. However, upon depolarization of neurons by current injection through the recording electrode, subthreshold potential oscillations with frequencies greater than 100 Hz could be observed, with larger depolarizations eliciting an increase in the amplitude of the oscillations (Fig. 4A). The oscillations were evident in most neurons subjected to subthreshold depolarizing current injection but were absent

during injections of current pulses in the hyperpolarizing direction. However, the overshoot observed upon return of the membrane potential to rest following a hyperpolarizing pulse was frequently followed by oscillations of a few millivolts in amplitude similar to those observed with subthreshold depolarizing current pulses (Fig. 4B). The oscillations were also evident following APs generated with suprathreshold current injections.

3.2 Morphology of Intracellularly Stained Neurons

Neurons displaying the above-mentioned electrophysiological characteristics intracellularly stained with neurobiotin appeared spherical or ovoid (25-50 μm in diameter) with a single process emanating from the soma (Fig. 5).

3.3 Effects of EAAs on Mesencephalic Trigeminal Neurons

Where amplitudes for responses elicited by EAAs are reported, a representative sample of at least 20 responses from different neurons was used to calculate those averages.

Pressure application of Glu (20 mM in the pressure pipette) resulted in a depolarization of MeV neurons ranging from 1 to 6mV (mean = 3.1 ± 0.3 mV) and lasting from 8 to 40 s (Fig. 6A). Attempts to enhance the Glu response by perfusing slices with the glutamate uptake inhibitor, *trans*-PDC (up to 500 μM), were without effect on Glu responses (n=5, Fig. 6B). In Mg^{2+}-free ACSF the Glu response was partially blocked by bath application of the competitive, AMPA/KA-specific antagonist CNQX

(20 μM) alone, and the NMDA-specific, competitive antagonist CPP (20 μM) alone, but was antagonized to a greater extent when both antagonists were present together (n=6) indicating that the Glu response consists of both an AMPA/KA (non-NMDA) and an NMDA component (Fig 6A). Complete blockade of Glu response was not observed in any of the tests with both antagonists. Glu responses were observed in 32 of 40 neurons tested.

Tests with specific ionotropic Glu receptor agonists confirmed that MeV cells are capable of responding to EAAs via AMPA/KA and NMDA GluRs. Pressure application of AMPA (10 mM in the pressure pipette) produced depolarizations of 4 to 10 mV (mean = 6.0 ± 0.4 mV) that lasted from 65 to 300 s in recorded neurons (Fig. 7A). KA (10 mM in the pressure pipette) application resulted in depolarizations ranging from 4 to 13 mV (mean = 7.7 ± 0.6 mV) and lasting 45 to 130 s (Fig. 7B). Both the AMPA (n=6) and the KA (n=11) responses could be at least 50% antagonized by perfusion of the slice with CNQX (20 μM) for 5 to 10 minutes and recovered within 10 to 15 minutes after removal of the antagonist (Figs. 7A and B). In slices perfused with Mg^{2+}-free ACSF, recorded neurons responded to pressure application of NMDA (10 mM in the pressure pipette) with a 3 to 10 mV (mean = 6.3 ± 0.4 mV) depolarization lasting 10 to 120 s (Fig. 8). NMDA responsiveness could be reduced by perfusion of the slice with Mg^{2+}-containing ACSF and recovered upon washout of the Mg^{2+} (n=12, Fig. 8 upper trace). The NMDA response could be antagonized by CPP (20 μM) with recovery upon washout of the antagonist (n=14, Fig. 8 lower trace). Glu agonist responses were observed in 166 of 178 neurons tested.

Applications of the ionotropic GluR agonists were frequently observed to depolarize recorded cells sufficiently to reach threshold for AP generation. At high temporal resolution, the APs could be seen to arise from the peaks of high-frequency oscillations like those observed at peri-threshold membrane potentials (Fig. 9). Perfusion of slices with TTX (500 nM) eliminated action potentials from responses which elicited firing and caused a small but consistent reduction in the amplitude of agonist responses; the major portion of the responses, however, remained during TTX treatment, which would be expected to block indirect responses to the drug application (n=5, Fig. 10).

Agonist responses were observed to exhibit desensitization that lasted for prolonged periods of time, as indicated by a decrease in response amplitude from one response to the next (Fig. 11). In an attempt to quantify the degree of desensitization at different time points, responses were elicited with various intervals between the applications, with the first response representing the control response and the second the desensitized response. Because responses were always found to return to control levels if given 10 minutes to recover, at least 10 minutes were allowed to pass before each test was performed. Using this protocol, 50% recovery times (i.e. time required between applications for desensitized responses to reach the same amplitude as control responses) of 2 to 3 min., 3 to 4 min., and 1 to 2 min. were found for AMPA, KA, and NMDA responses, respectively. Figure 11 displays the full time course of recovery for each of the agonists with a representative example of a desensitizing KA response. Each time-point represents the cumulative data of response amplitudes from at least five different neurons. Because measurement of response amplitude was difficult when responses

reached threshold for AP generation (as in Fig. 11D), neurons used to generate these data were hyperpolarized with negative current injection throughout the test so that threshold would not be reached.

Cross-desensitization between AMPA and KA responses was observed (n=4, Fig. 12). In the example shown, both KA and AMPA responses are shown to be consistent with 10 minutes between applications (Fig. 12 upper and middle traces). However, KA applied 5 min. before AMPA is observed to decrease the amplitude of the AMPA response from control levels (compare AMPA response of lower trace with those of the middle trace). Furthermore the AMPA application is shown to decrease the amplitude of a KA response obtained 5 min. after the AMPA application and 10 min. after the previous KA application (Fig. 12 lower trace). Cross-desensitization between NMDA and non-NMDA responses was also apparent (n=4, Fig. 13).

In an attempt to determine whether AMPA and KA were working through the same receptor, tests were performed with the desensitization modulator cyclothiazide which has been reported to block desensitization of AMPA receptors but not KA receptors (Partin et al., 1993; Wong and Mayer, 1993). Perfusion of slices with cyclothiazide (up to 500 μM for periods of up to 15 min.) did not alter AMPA responses, nor did it block desensitization of AMPA responses (n=4, Fig. 14).

3.4 Effects of Serotonin on Mesencephalic Trigeminal Neurons

Basic properties of 5-HT responses are reported for data averaged from 20 responses elicited in 20 different MeV neurons.

Pressure application of 5-HT (10 mM in the pressure pipette) onto recorded MeV neurons produced slowly developing, small depolarizations ranging from 1 to 4 mV (mean = 2.1 ± 0.2 mV) in amplitude (Fig. 15). These depolarizations were associated with a decrease in the chord R_{input} of the neurons ranging from 21 to 38% (mean = 27.5 ± 1.1%) as determined from the measurement of resistance pulses (-0.5 to -1.0 nA) applied to recorded neurons through the recording electrode during 5-HT responses (Fig. 15). The duration of 5-HT responses was from 50 to 130 s. Of the 53 recorded neurons to which 5-HT was applied, 41 responded in the manner described above while the rest were unaffected by the drug. These effects of 5-HT appear to be the result of a direct response of recorded neurons since they appeared unchanged in the presence of TTX (500 nM) (n=3, data not shown).

Similar effects of 5-HT have been reported in prepositus hypoglossi neurons (Bobker and Williams, 1989), thalamic relay neurons (Pape and McCormick, 1989; McCormick and Pape, 1990b) and facial motoneurons (Larkman and Kelly, 1992). In each of these cases, 5-HT responses were found to be the result of activation of I_h and could be antagonized by blockade of I_h with CsCl. Blockade of I_h with superfusion of CsCl (5-10 mM) also antagonized 5-HT responses of MeV neurons in a reversible manner (n=6, Fig. 16A). Since Cs^+ may not be selective for I_h at the high doses needed for blockade, we tested the ability of the novel selective I_h antagonist, ZD 7288, to block 5-HT responses. Superfusion of ZD 7288 (50 μM) was observed to irreversibly block I_h and 5-HT responses (n=5, Fig 16B). Thus, it appears that 5-HT mediates its effects on mesencephalic trigeminal neurons by activating I_h. To ensure that Cs^+ and ZD 7288 were

specific for blockade of 5-HT responses, and did not simply decrease neurotransmitter responsiveness through some general mechanism, GABA applications were given during I_h blockade in two experiments (e.g. Fig. 16A). Neither CsCl or ZD 7288 was observed to antagonize GABA responses during blockade of 5-HT responses by these substances.

Blockade of I_h with CsCl and ZD 7288 resulted in an apparent increase in R_{input} of MeV neurons, as evidenced by the increase in size of the resistance pulses in figures 16A and B after CsCl and ZD 7288 treatment. If it is assumed that the only effect of CsCl and ZD 7288 on MeV neurons is a blockade of I_h, which may not be true for CsCl but is for ZD 7288 (Harris and Constanti, 1995), then the increase in R_{input} associated with these substances would seem to indicate that there is considerable I_h active at resting membrane potentials. Alternatively, it is possible that the resistance pulses used (-0.5 to -1.0 nA) to observe 5-HT responses are themselves activating I_h before the peak of the pulse is reached, so that when I_h is blocked the resistance pulses increase in magnitude. To test the latter possibility, I_h was blocked with ZD 7288 in MeV neurons while monitoring their resistance with small resistance pulses (-0.2 nA) that should not activate I_h (n=3, Fig. 17). In two cases small positive resistance pulses (+0.2 nA) were used to ensure that no I_h activation in response to resistance pulses was occurring (Fig. 17A). From these tests, it is clear that blockade of I_h is not accompanied by a true increase in R_{input} of MeV neurons, and thus, I_h is not active at resting membrane potentials. Further evidence that I_h is not active at rest in MeV neurons is the lack of effect of ZD 7288 on membrane potential. If I_h was active at rest, then blockade of this current would be expected to result in hyperpolarization of recorded neurons, however, no such

hyperpolarizations were observed (Figs. 17A and B).

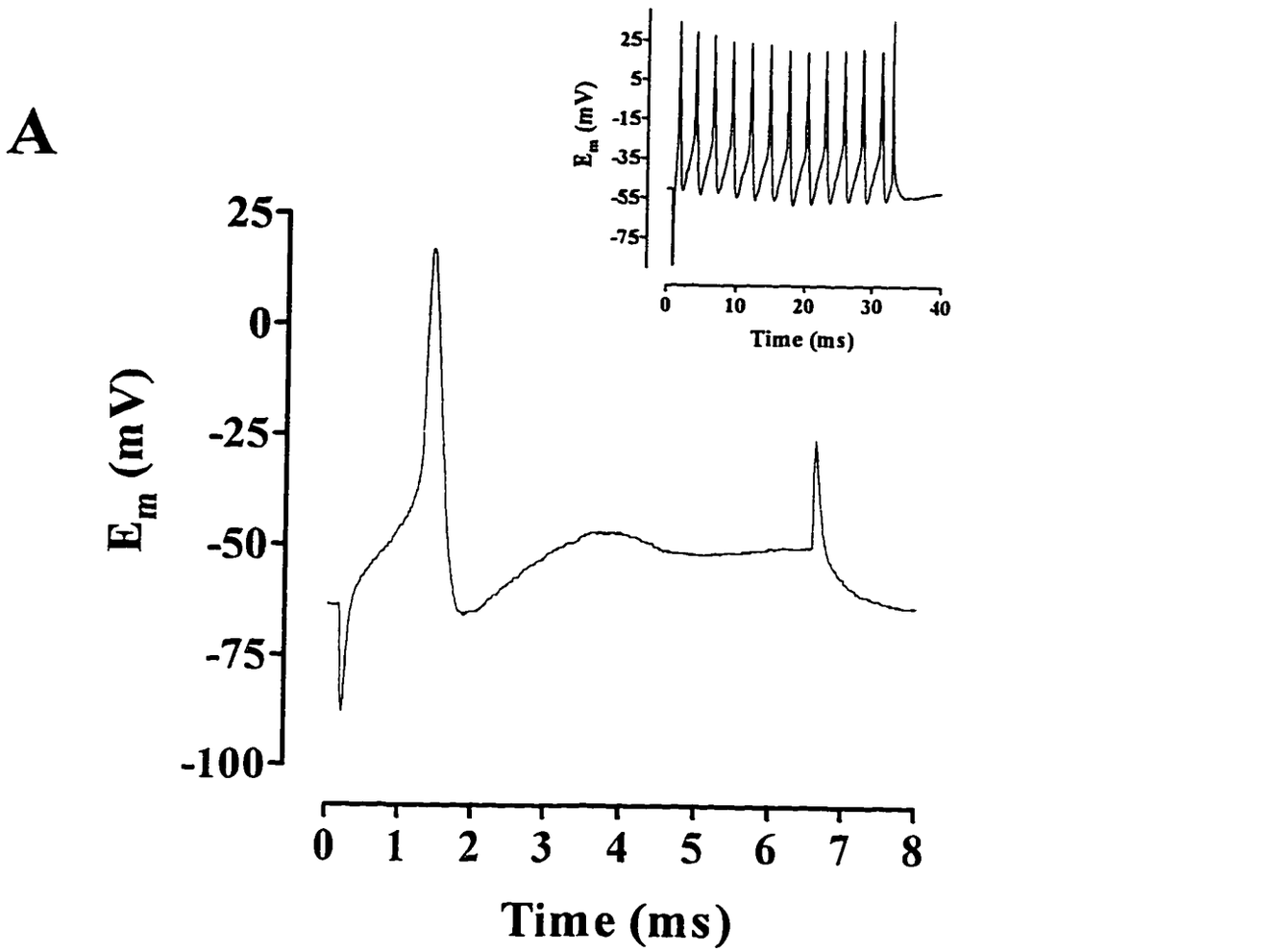
Investigations of the second messenger system involved in the 5-HT response of MeV neurons suggest that 5-HT may activate I_h by stimulating AC, leading to an increase in intracellular cAMP. Superfusion of the AC stimulant, forskolin (20-100 μM) (n=3), the cell-permeable cAMP analog, 8-bromo-cAMP (300-600 μM) (n=5), and the phosphodiesterase inhibitor, IBMX (200-400 μM) (n=3), were all observed to mimic the effects of 5-HT (Figs. 18A,B and C). The effects of forskolin were not readily reversed, while those of 8-bromo-cAMP and IBMX could usually be reversed within a 30min. washout period. 5-HT-induced responses of neurons before 8-bromo-cAMP and IBMX treatment were occluded during responses to these substances but recovered upon washout of the cAMP analog and the phosphodiesterase inhibitor (eg. Fig. 18C). 8-bromo-cAMP effects were antagonized during blockade of I_h (n=3, Fig. 19).

Whether increases in intracellular cAMP activate I_h in a protein kinase A- (PKA) dependent manner in other systems is a matter of some controversy (for review see Pape, 1996). We examined the effects of the specific PKA inhibitor, H-89 (80-100 μM), and in one case the general protein kinase inhibitor, H-7 (200 μM), on 5-HT responses. During protein kinase inhibition, 5-HT-mediated decreases in R_{input} were significantly reduced by up to 49.2% (mean = 31 ± 7.9%; $p<0.01$) (n=5, Fig. 20).

Preliminary pharmacological identification of the 5-HT receptor subtype mediating 5-HT responses in mesencephalic trigeminal neurons was attempted. 5-HT responses were mimicked by the 5-HT receptor agonist, 5-CT (5 mM in the pressure pipette) (n=6, Fig. 21B), but not by 8-OH-DPAT (10 mM in the pressure pipette) (n=4, Fig. 21C). The

5-HT_2 agonists, α-methyl-5-HT (n=1) and DOI (n=2) were also observed to be ineffective in generating responses (data not shown). Reversible antagonism of 5-HT responses was achieved with superfusion of the 5-HT receptor antagonis,t ketanserin (20-80 μM) (n=5, Fig. 22A), whereas responses were unaffected by the antagonist methiothepin (40 μM) (n=2, Fig. 22B).

Figure 1. MeV APs. In **A** a typical AP elicited with a depolarizing current step (1.5 nA) in a recorded MeV neuron is shown. The inset displays a high frequency repetitive firing response to a larger current pulse (2.4 nA) of another neuron with a more depolarized resting potential. In **B** MeV action potentials are demonstrated to be sensitive to TTX (500 nM).


A
25
0
-25
-50
-75
-100
E_m (mV)
0 1 2 3 4 5 6 7 8
Time (ms)
25
5
-15
-35
-55
-75
E_m (mV)
0 10 20 30 40
Time (ms)

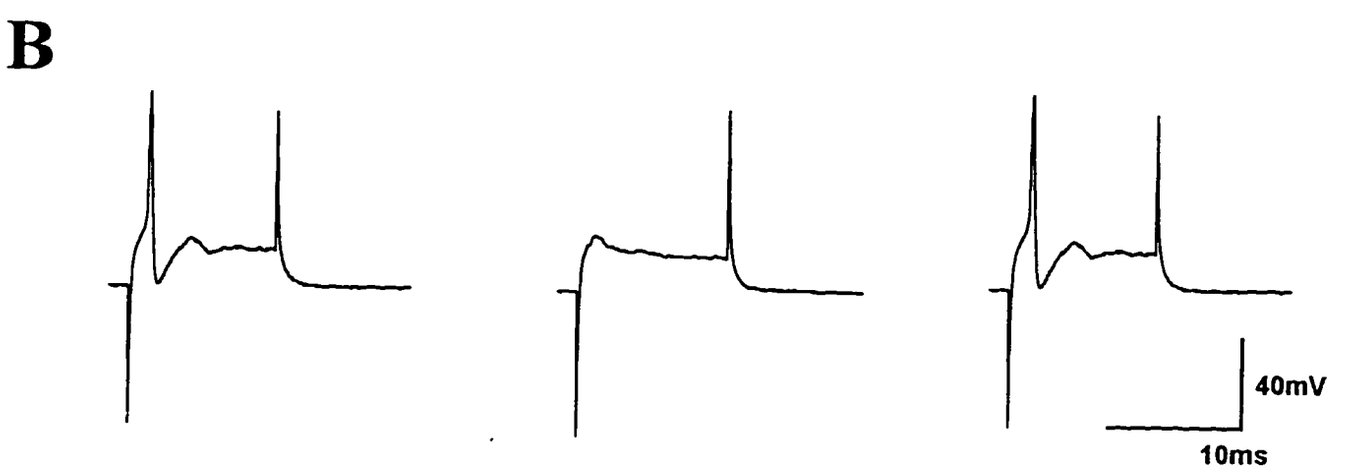

B
40mV
10ms
Control
10min.
on TTX
30min. washout
of TTX

Figure 2. Inward rectification of MeV neurons. **A** Typical response of an MeV neuron to hyperpolarizing current steps (from -2.1 to 0.3 nA by steps of 0.3 nA) with inset displaying the voltage-current relation of the cell at the peak of the voltage response (■) and at the steady-state level of the response (▲). **B** Antagonism of the inward rectification by CsCl (8 mM perfused for 15 min.) with recovery upon washout of the CsCl for 20 min. **C** Antagonism of the inward rectification by ZD 7288 (50 μM perfused for 15 min.) which was irreversible with wash periods of up to 3 hrs. Note overshoot phenomenon apparent at the termination of hyperpolarizing pulses and blockade of the overshoot following CsCl znd ZD 7288 treatment.

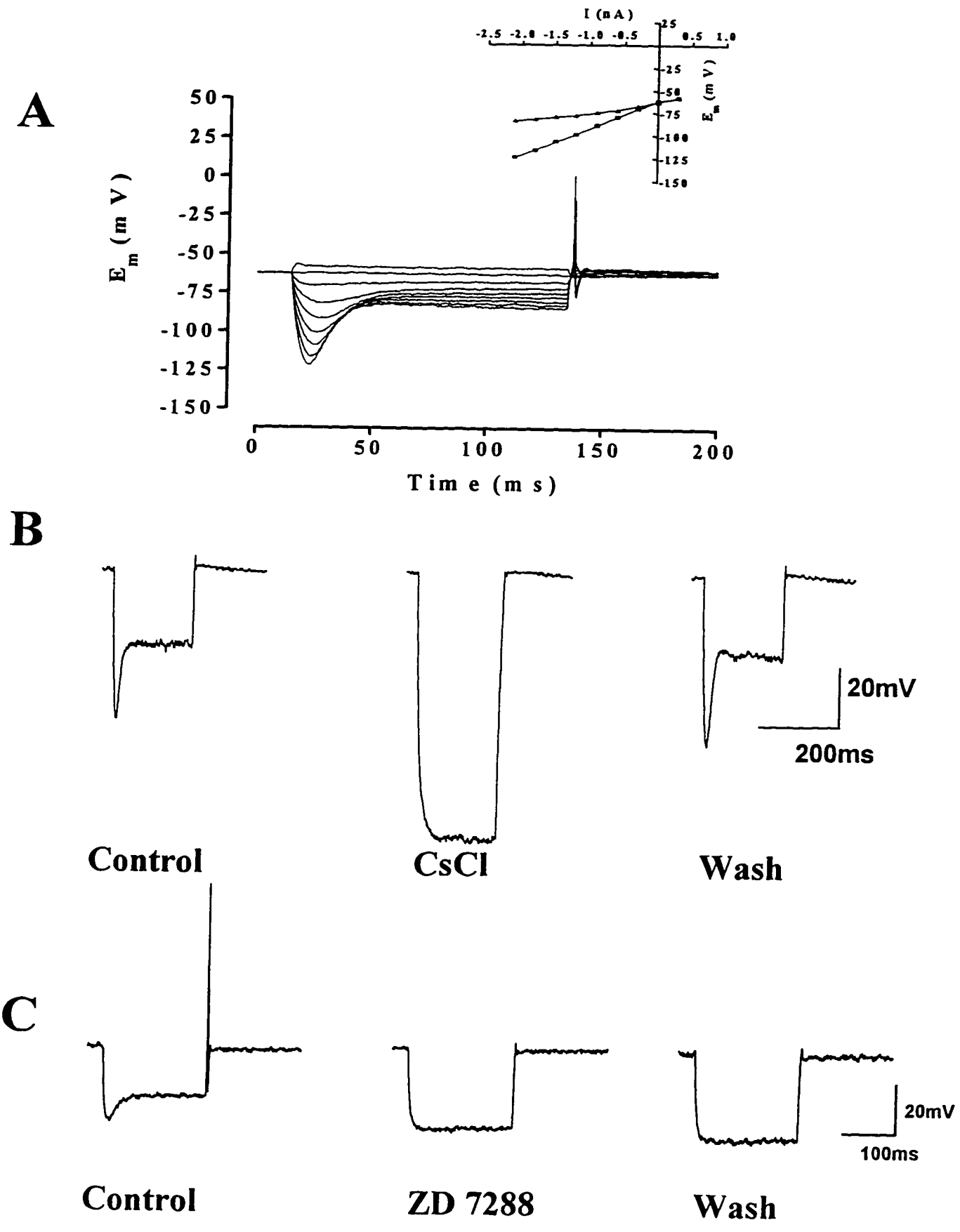

A
I (nA)
-2.5 -2.0 -1.5 -1.0 -0.5 0.5 1.0
25
50
75
100
125
150
E_m (mV)
50
25
0
-25
-50
-75
-100
-125
-150
E_m (mV)
0
50
100
150
200
Time (ms)
B
20mV
200ms
Control
CsCl
Wash
C
20mV
100ms
Control
ZD 7288
Wash

Figure 3. I_h and excitability. MeV neurons exhibit decreased excitability after blockade of I_h with ZD 7288. In the control situation (left column of traces) the neuron is observed to generate an AP in response to a just threshold stimulus (0.85 nA) and to generate a burst of APs to a larger stimulus (2.0 nA). Following blockade of I_h by ZD 7288 the just threshold stimulus was insufficient to cause firing and the neuron was incapable of generating a burst of APs. (Note: the resting potential of the neuron did not change during ZD 7288 treatment.)

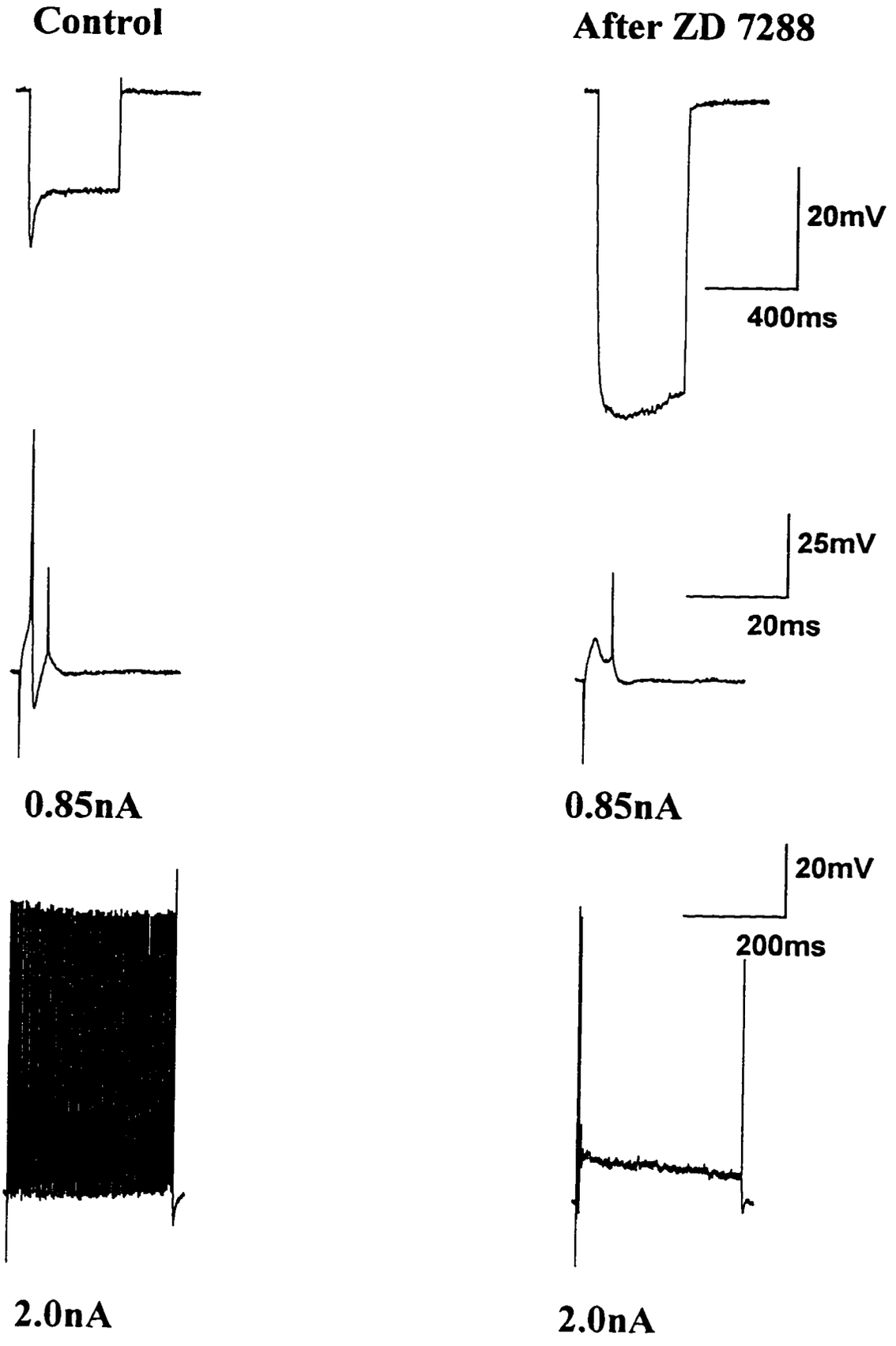
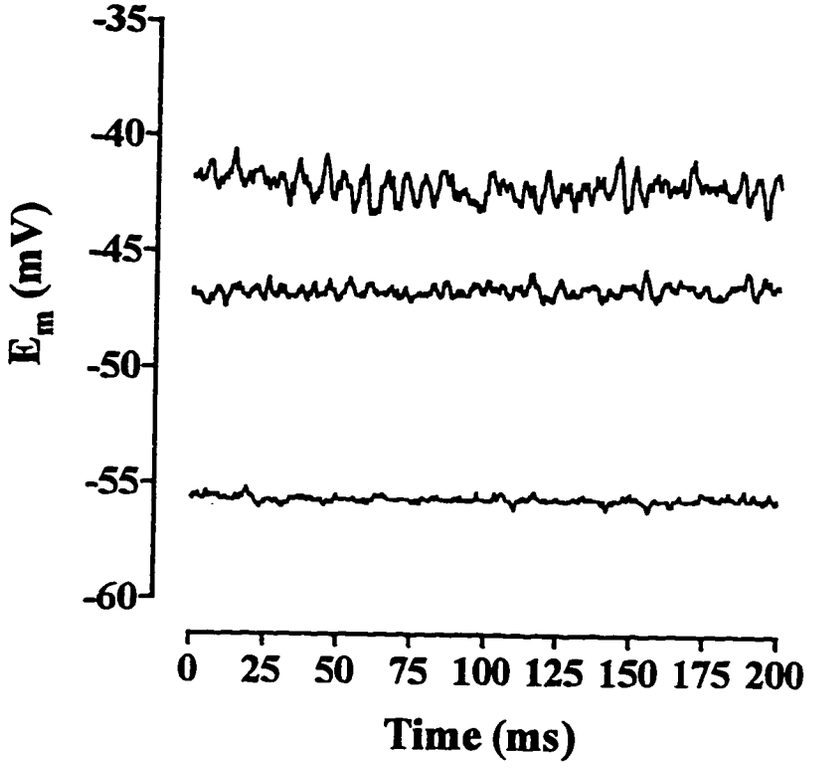

Control
After ZD 7288
20mV
400ms
25mV
20ms
0.85nA
0.85nA
20mV
200ms
2.0nA
2.0nA

Figure 4. Subthreshold potential oscillations of MeV neurons. **A** Recordings from an MeV neuron at resting potential (bottom trace) and more depolarized potentials (middle and top traces), displaying the voltage-dependent oscillations observed in most recorded neurons. The neuron was depolarized by current injection through the recording electrode. **B** Oscillations observed following off-spikes generated with an hyperpolarizing current pulse. Inset displays the oscillations following the second off-spike at higher amplification.

A


-35
-40
-45
-50
-55
-60
E_m (mV)
0 25 50 75 100 125 150 175 200
Time (ms)

B

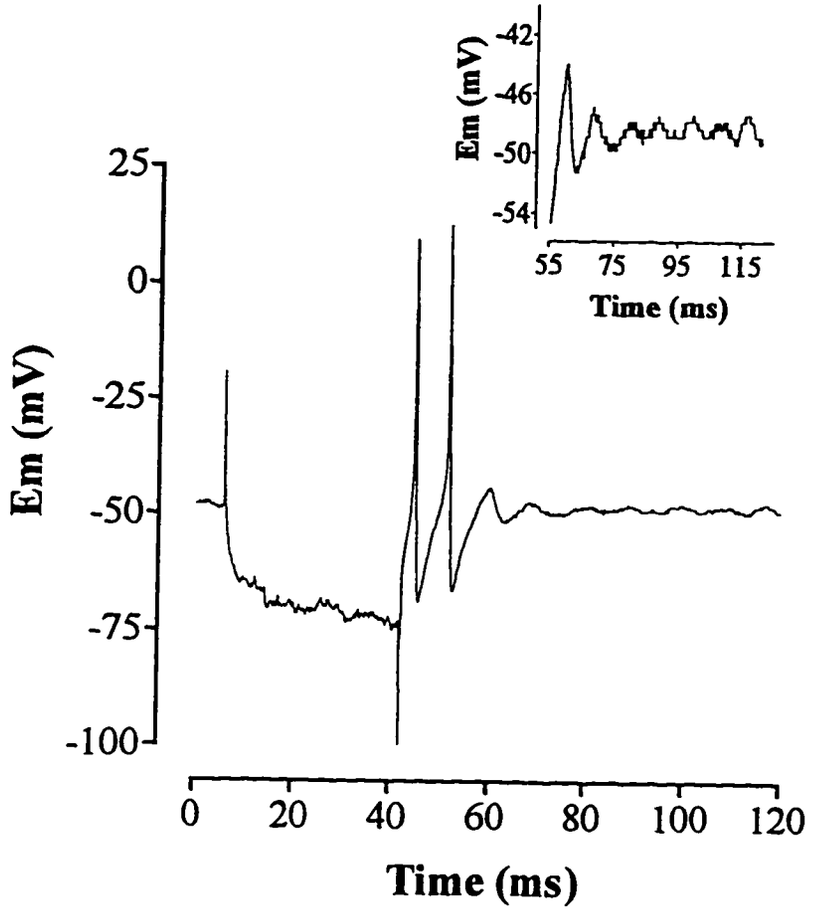

25
0
-25
-50
-75
-100
Em (mV)
0 20 40 60 80 100 120
Time (ms)
-42
-46
-50
-54
Em (mV)
55 75 95 115
Time (ms)

Figure 5. Intracellularly stained MeV neuron. (Scale = 100 μm)

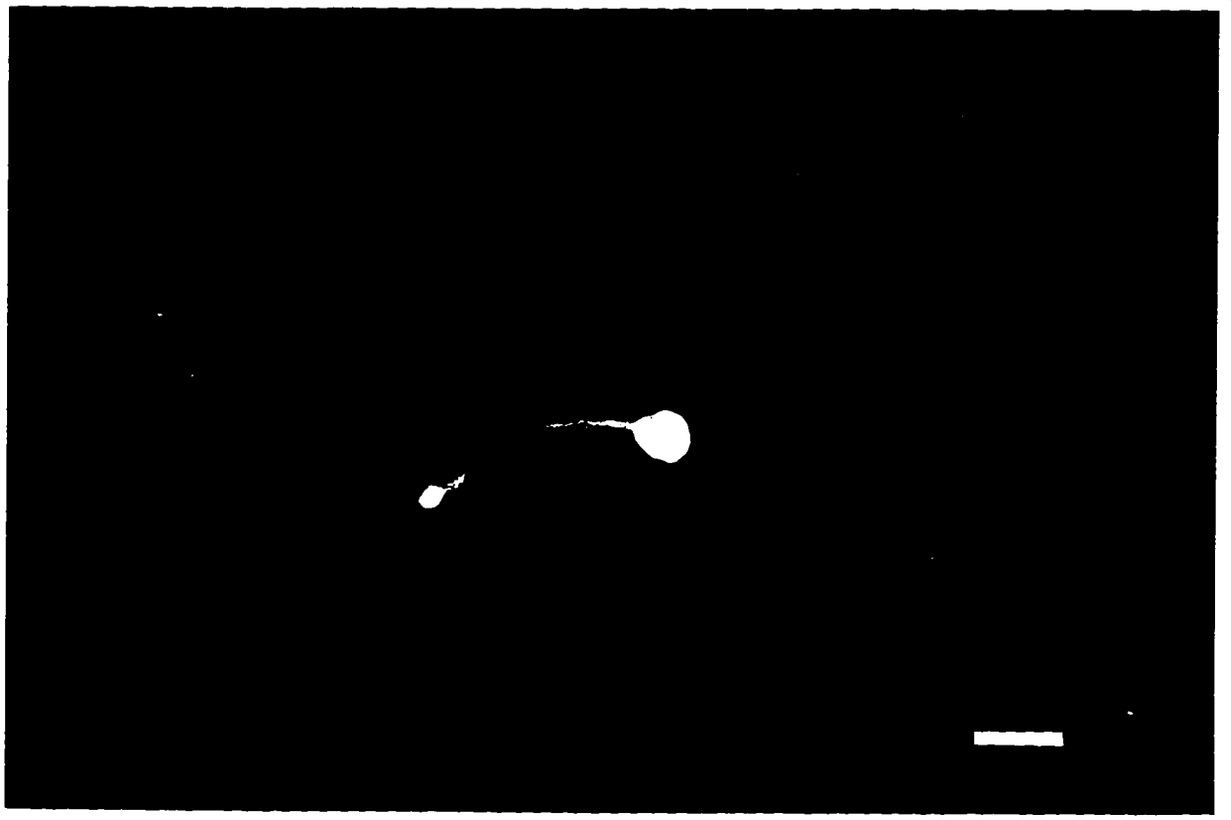


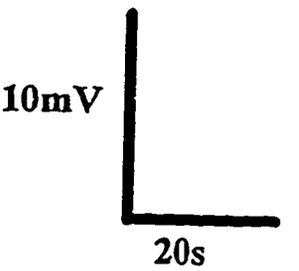
Figure 6. Glu responsiveness of MeV neurons. **A** Typical response of an MeV neuron to pressure application of Glu in Mg^{2+}-free ACSF showing partial antagonism by CNQX (20 μM perfused for 8 min.) and CPP (20 μM perfused for 8 min.) when the antagonists were applied alone, with a greater degree of antagonism occuring when both antagonists were applied simultaneously (each at 20 μM for 8 min.). In each case the Glu response recovered to control levels upon washout of the antagonists (10 min. for each washout) before the next antagonist was tested. **B** The Glu uptake inhibitor *trans*-PDC (500 μM perfused for 20 min.) has no effect on Glu responses.

Glu in Mg^{2+}-free ACSF

Glu on CNQX

Glu on CPP

Glu on CPP and CNQX



Glu control response

Glu on trans-PDC

Glu after wash

Figure 7. AMPA and KA responses of MeV neurons. Representative examples of AMPA (**A**) and KA (**B**) responses (elicited in different neurons). Both the AMPA and the KA responses are antagonized by perfusion of the slices with CNQX (20 μM perfused for 8 min. in **A** and 6 min. in **B**) and recovered upon washout of the antagonist (10 min. washouts for both **A** and **B**). APs are truncated due to frequency response limitations of the chart recorder.

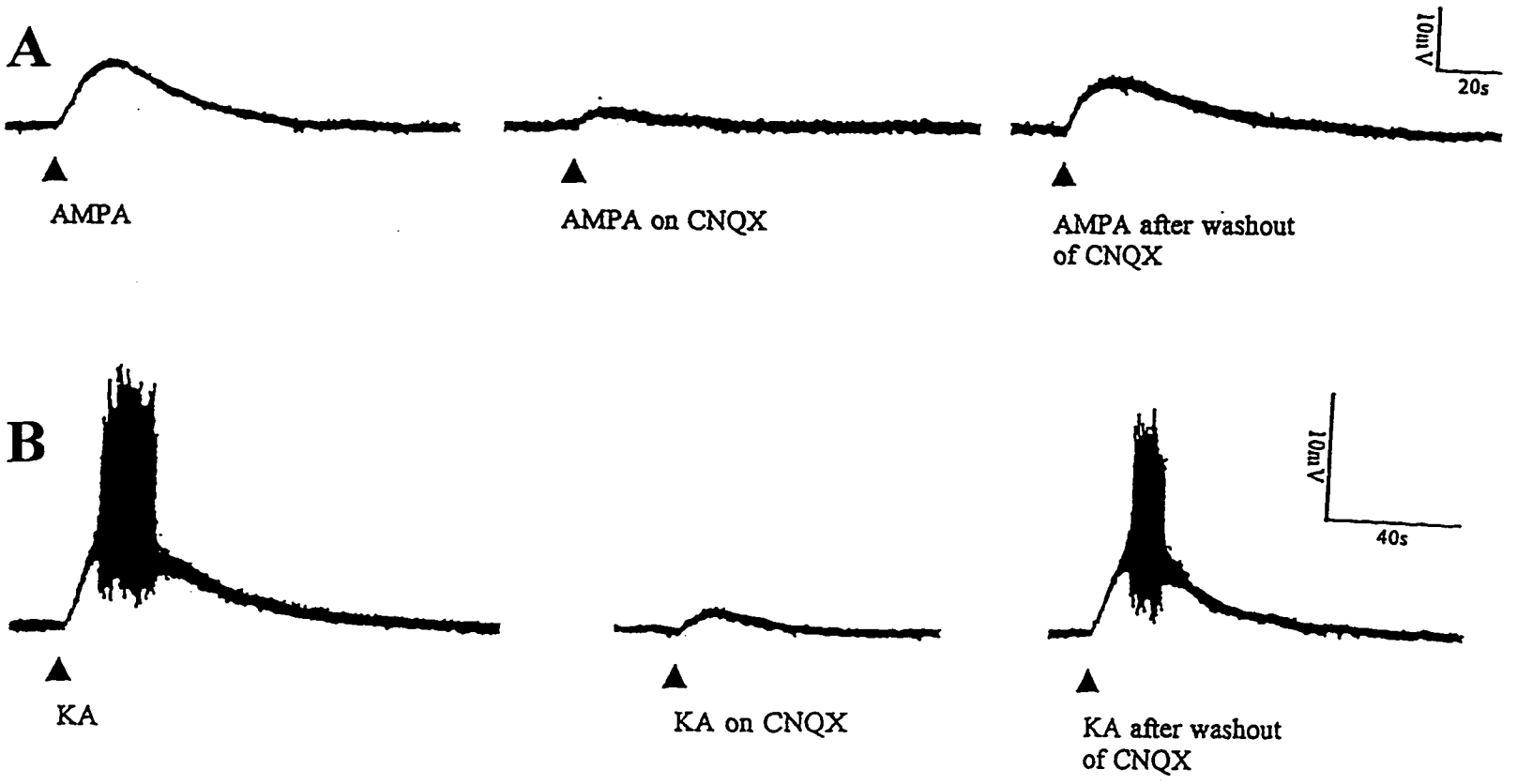
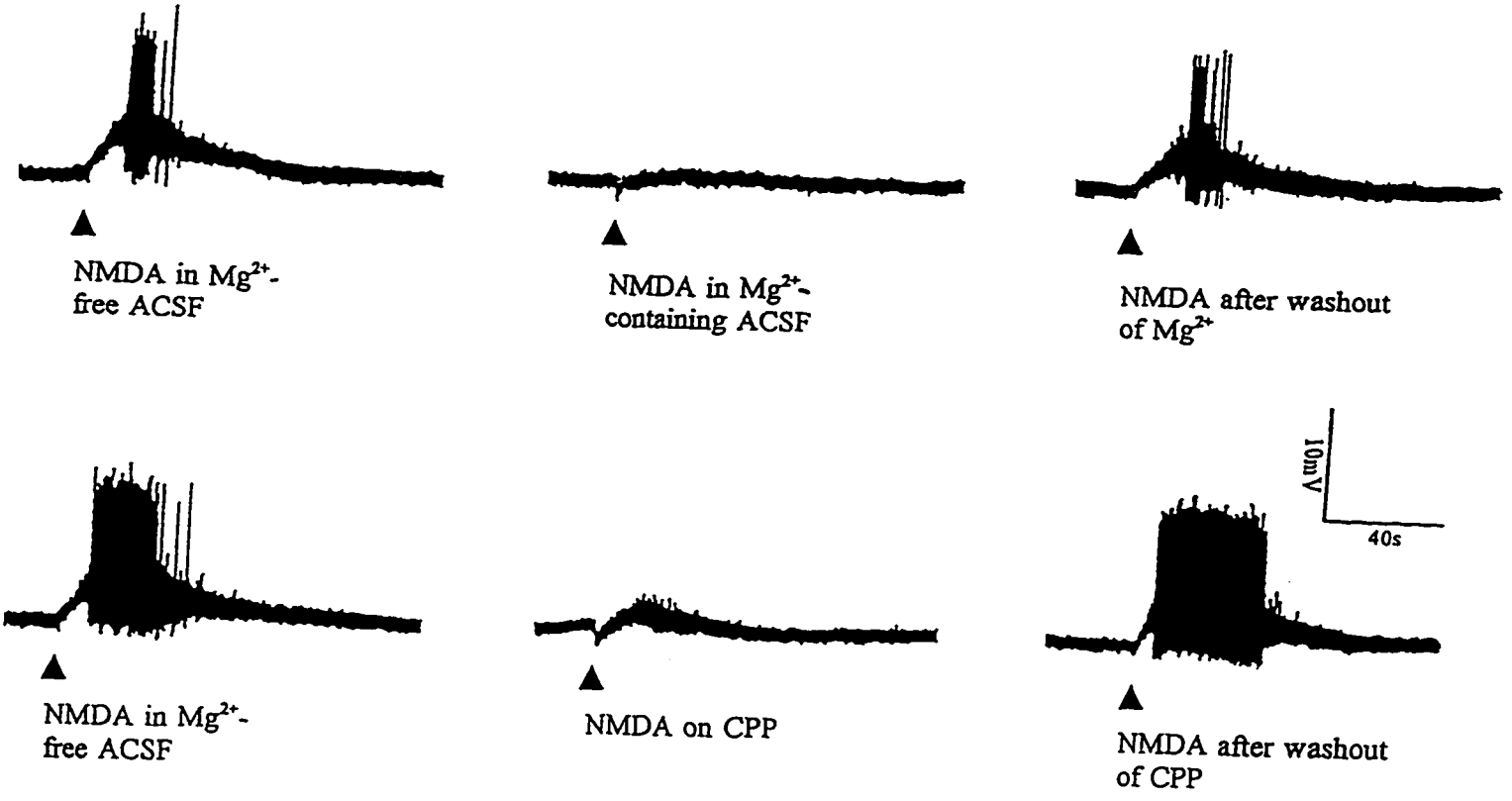

A
10mV
20s
AMPA
AMPA on CNQX
AMPA after washout
of CNQX
B
10mV
40s
KA
KA on CNQX
KA after washout
of CNQX

Figure 8. NMDA responsiveness of MeV neurons. Typical NMDA responses observed in MeV neurons. In the upper trace the NMDA response is demonstrated to be sensitive to Mg^{2+} (1 mM perfused for 10 min.). In the lower trace CPP (20 μM perfused for 4min.) is demonstrated to antagonize the NMDA response. In both cases the response recovered after a 10 min. washout period of the antagonists. AP responses are truncated, as in Fig. 7.



NMDA in Mg^{2+}-free ACSF

NMDA in Mg^{2+}-containing ACSF

NMDA after washout of Mg^{2+}

NMDA in Mg^{2+}-free ACSF

NMDA on CPP

NMDA after washout of CPP

Figure 9. EAA responses can induce oscillations. **A** Full response of an MeV neuron to KA. **B** Portions of the full KA response shown in **A** are displayed at a higher time resolution and gain (with truncated spikes); top trace displays the stable resting membrane potential before drug application, middle trace displays the oscillatory behaviour of the membrane potential during the KA response, and the bottom trace displays the return of the stable membrane potential following the KA response. Note in the middle trace that the frequency of AP firing is about the same as the frequency of the subthreshold oscillations.

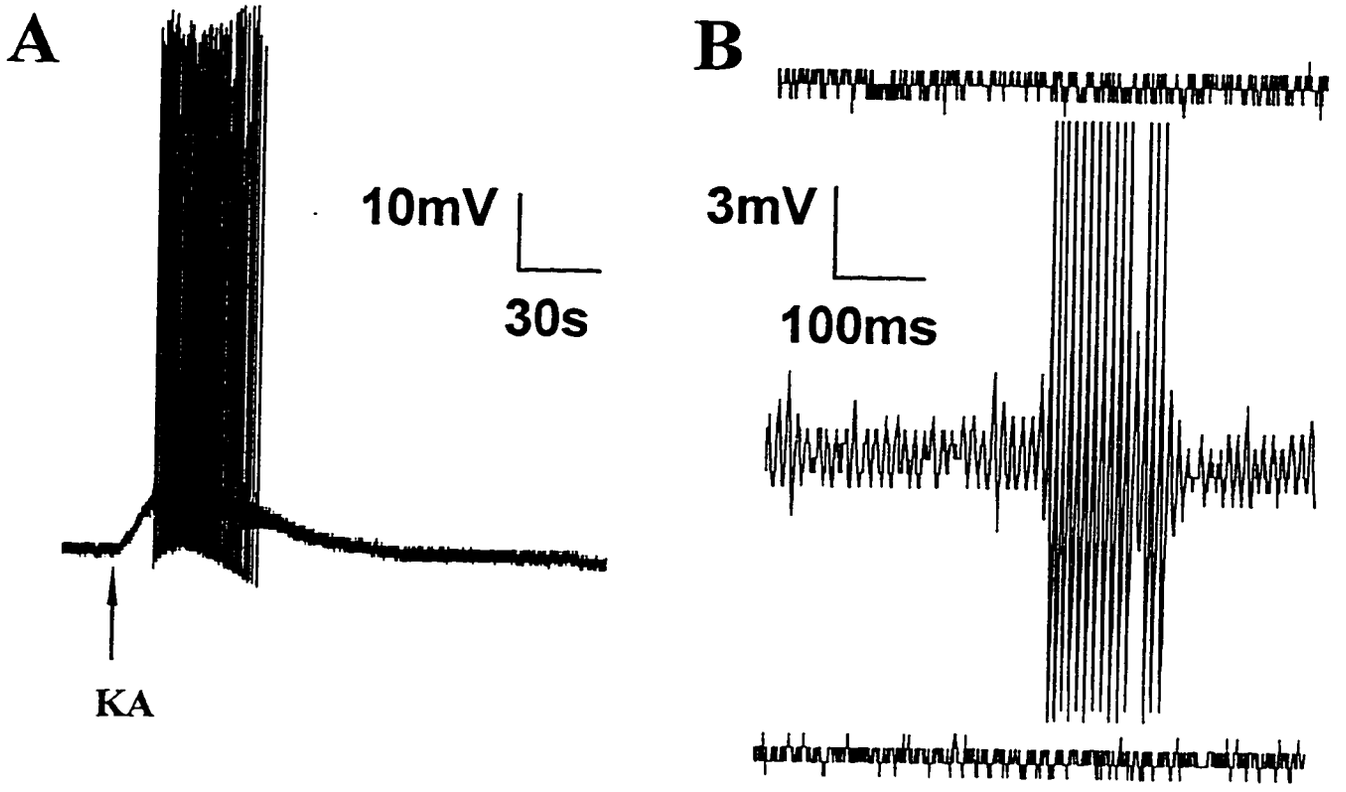

A
10mV
30s
KA
B
3mV
100ms

Figure 10. EAA responses and TTX. Responses of two MeV neurons to KA (**A**) and NMDA (**B**) before (left side, control) and after (right side, TTX) TTX treatment (0.5 μM for 10 min.). Spikes in A are truncated.

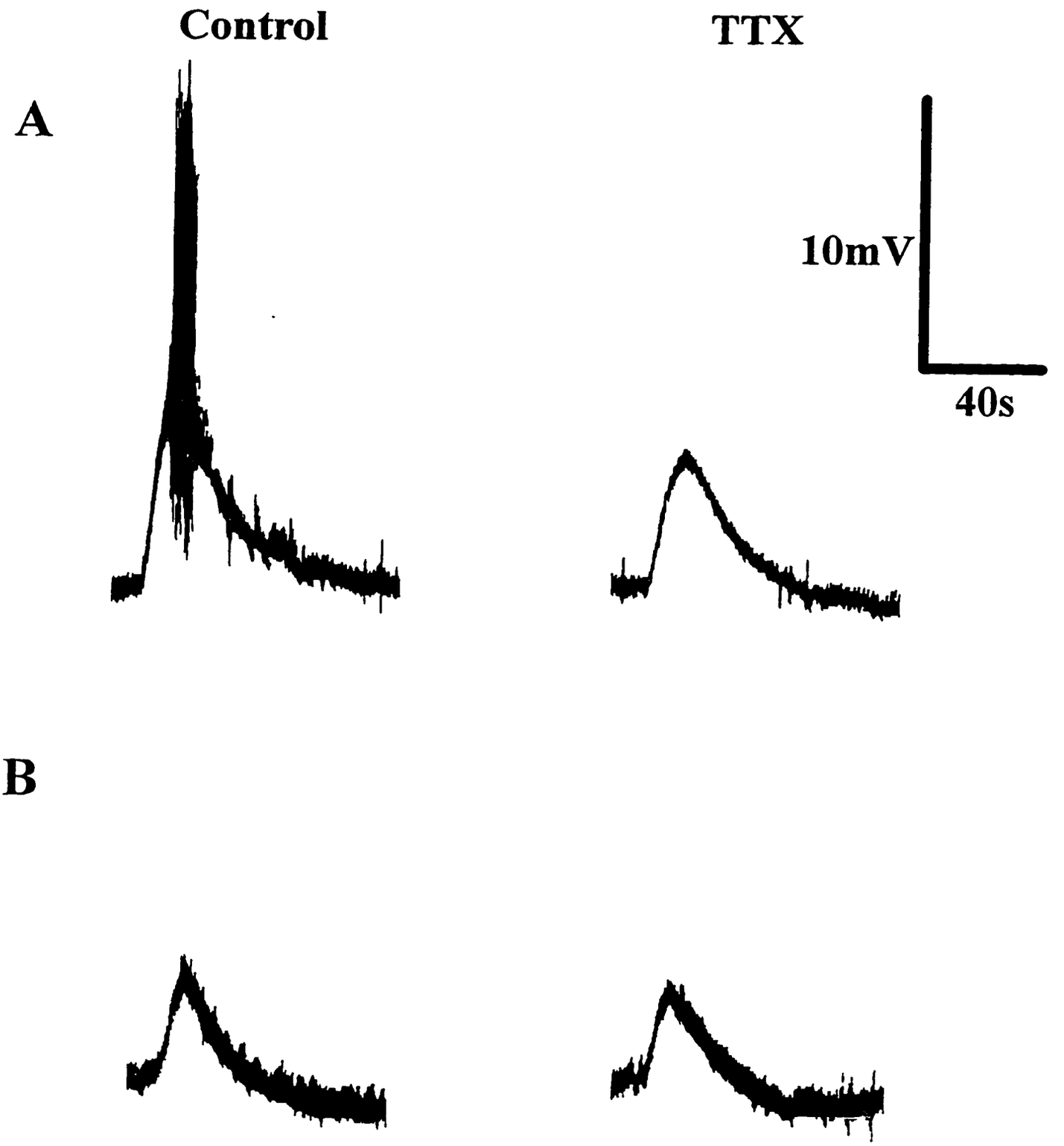
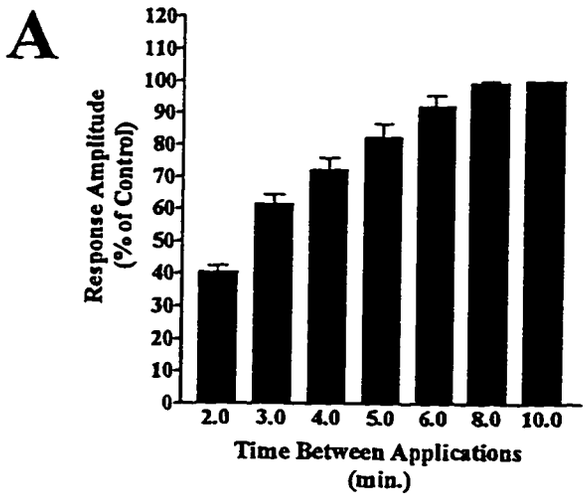
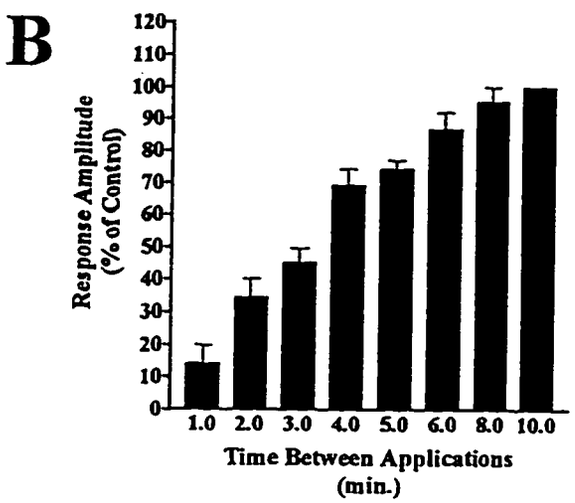
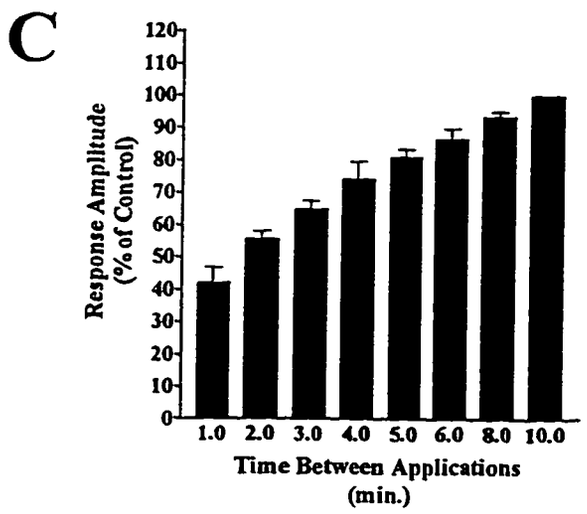

Control
TTX
A
10mV
40s
B

Figure 11. Desensitization of EAA responses. Time courses of desensitization for AMPA (**A**), KA (**B**), and NMDA (**C**) responses. Each time point represents cumulative data from at least five different neurons. For AMPA (**A**) there is no 1 min. time point because AMPA control responses typically did not return to resting potentials within this time period. In **D** an example of the observed desensitization of a KA response in one MeV neuron is shown with the second KA application (test response) occuring 2,4,6,8, and 10 min. after the control responses (first response in each trace) from the top to the bottom trace. There is a time delay of at least 10 min. between test responses and the next control response to allow for recovery of desensitization. AP amplitude is truncated in **D**.


A
Response Amplitude
(% of Control)
120
110
100
90
80
70
60
50
40
30
20
10
0
2.0 3.0 4.0 5.0 6.0 8.0 10.0
Time Between Applications
(min.)


B
Response Amplitude
(% of Control)
120
110
100
90
80
70
60
50
40
30
20
10
0
1.0 2.0 3.0 4.0 5.0 6.0 8.0 10.0
Time Between Applications
(min.)


C
Response Amplitude
(% of Control)
120
110
100
90
80
70
60
50
40
30
20
10
0
1.0 2.0 3.0 4.0 5.0 6.0 8.0 10.0
Time Between Applications
(min.)

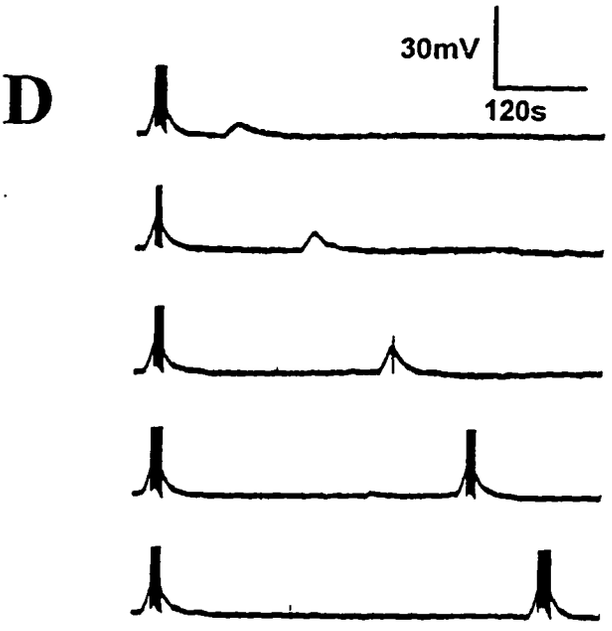

D
30mV
120s

Figure 12. Cross-desensitization of AMPA and KA responses. In the upper and middle traces KA and AMPA responses are consistent when applied at 10 min. intervals. In the bottom trace an AMPA application given 5 min. after a KA application is decreased in amplitude from controls (middle trace) and a KA response is decreased in amplitude from controls (upper trace and fist response of bottom trace) when elicited 5 min. after the AMPA response but 10 min. after the previous KA response.

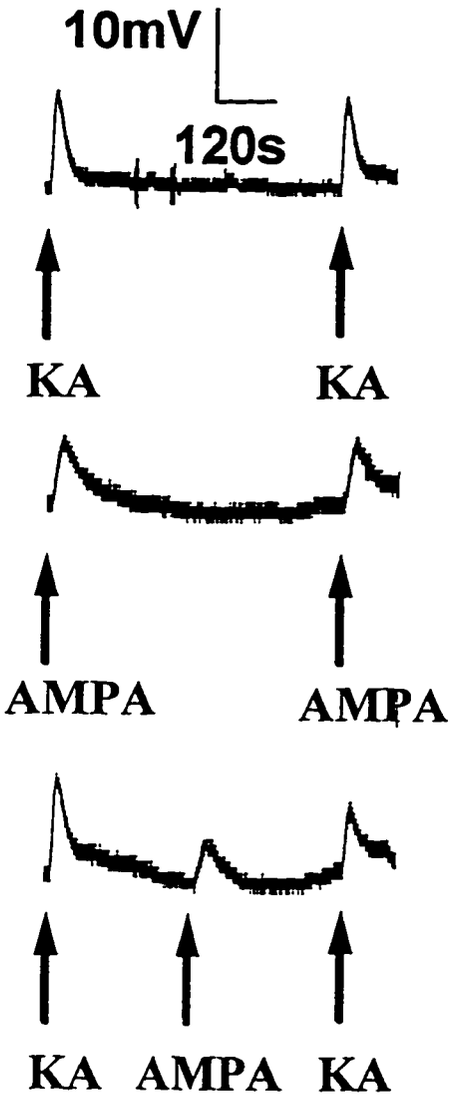

10mV
120s
KA
KA
AMPA
AMPA
KA
AMPA
KA

Figure 13. Cross-desensitization of NMDA and non-NMDA responses. **A** NMDA responses are desensitized if evoked 2 min. after either an NMDA response or a KA response. **B** KA responses are desensitized if evoked 2 min. after either a KA response or an NMDA response.

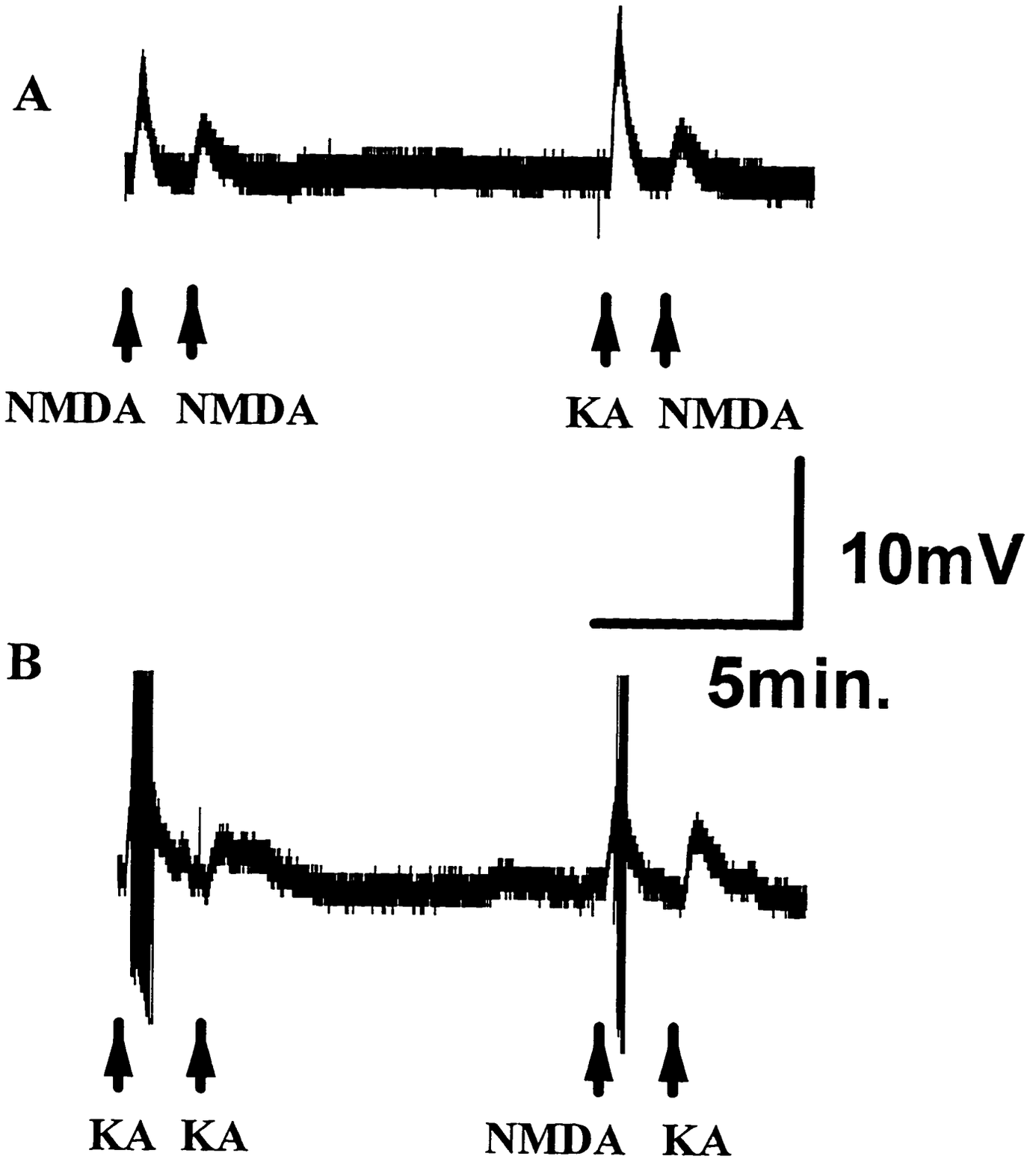

A
NMDA NMDA
KA NMDA
10mV
5min.
B
KA KA
NMDA KA

Figure 14. Cyclothiazide effects on AMPA responses. In the top trace an AMPA response is desensitized when generated two minutes after a control response. In the bottom trace the second AMPA response is still desensitized after treatment of the slice with cyclothiazide (500 μM for 15 min.).

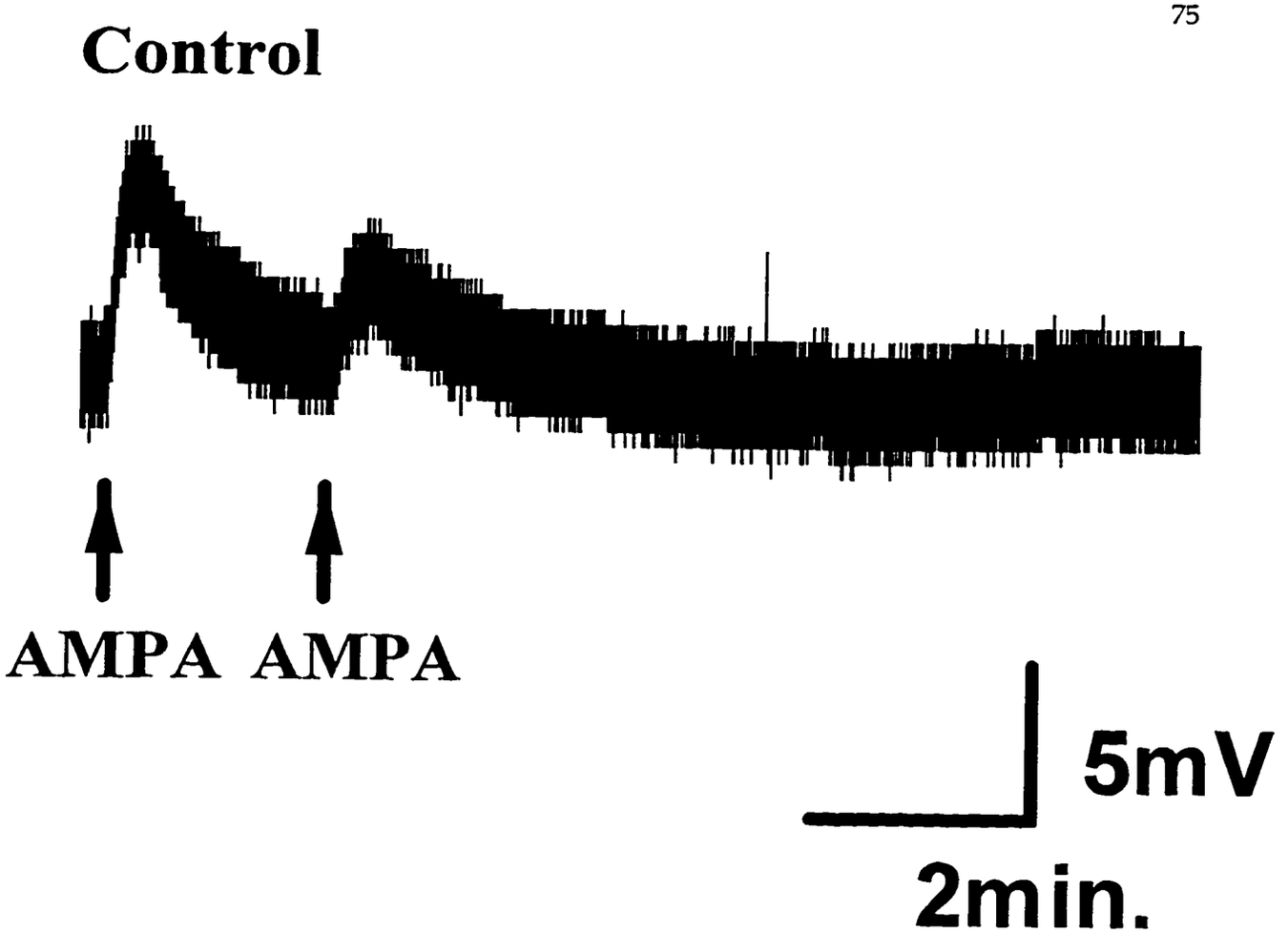

Control
AMPA AMPA
5mV
2min.
After cyclothiazide treatment
AMPA AMPA

Figure 15. Typical 5-HT response of an MeV neuron. 5-HT is demonstrated to cause a small depolarization accompanied by a decrease in neuronal input resistance. The thick black line represents the membrane potential recording of the neuron and the downward deflections are negative current pulses used to monitor the input resistance of the neuron(resistance pulses, -0.5 nA for 40 ms at 0.5 Hz).

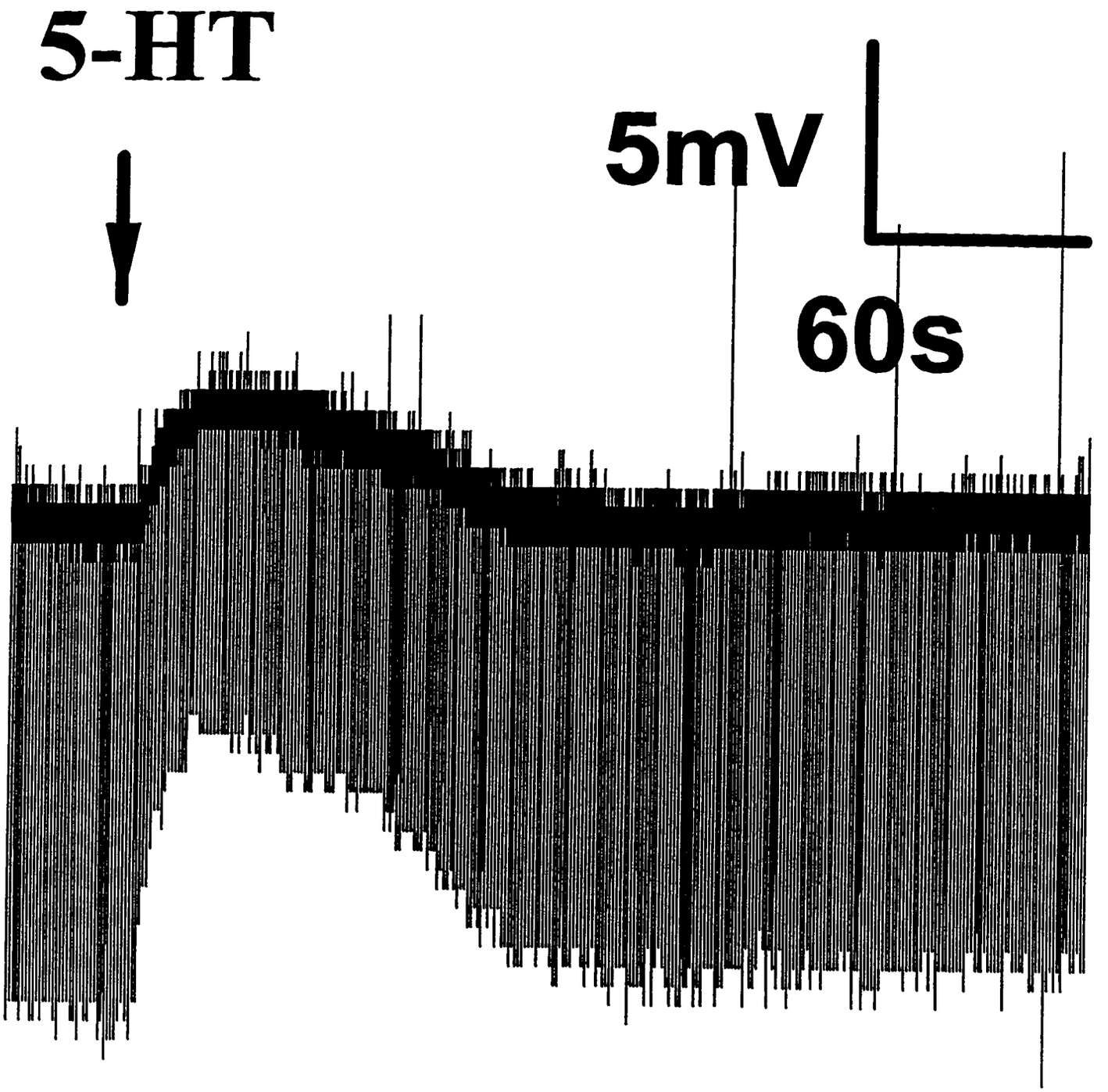

5-HT
5mV
60s

Figure 16. I_h involvement in 5-HT responses. In **A** blockade of I_h with CsCl (5 mM perfused for 8 min.) (bottom traces) antagonizes 5-HT responses but not GABA responses (top traces). Both the I_h and the 5-HT response are recovered upon washout of CsCl (30 min.). In **B** blockade of I_h with ZD 7288 (50 μM perfused for 10 min.) (bottom traces) irreversibly antagonizes 5-HT responses (top traces) even after a 1 hr wash period. Resistance pulses used were -1.0 nA for 40 ms at 0.5 Hz and -0.5 nA for 40 ms at 0.5 Hz for **A** and **B** respectively.

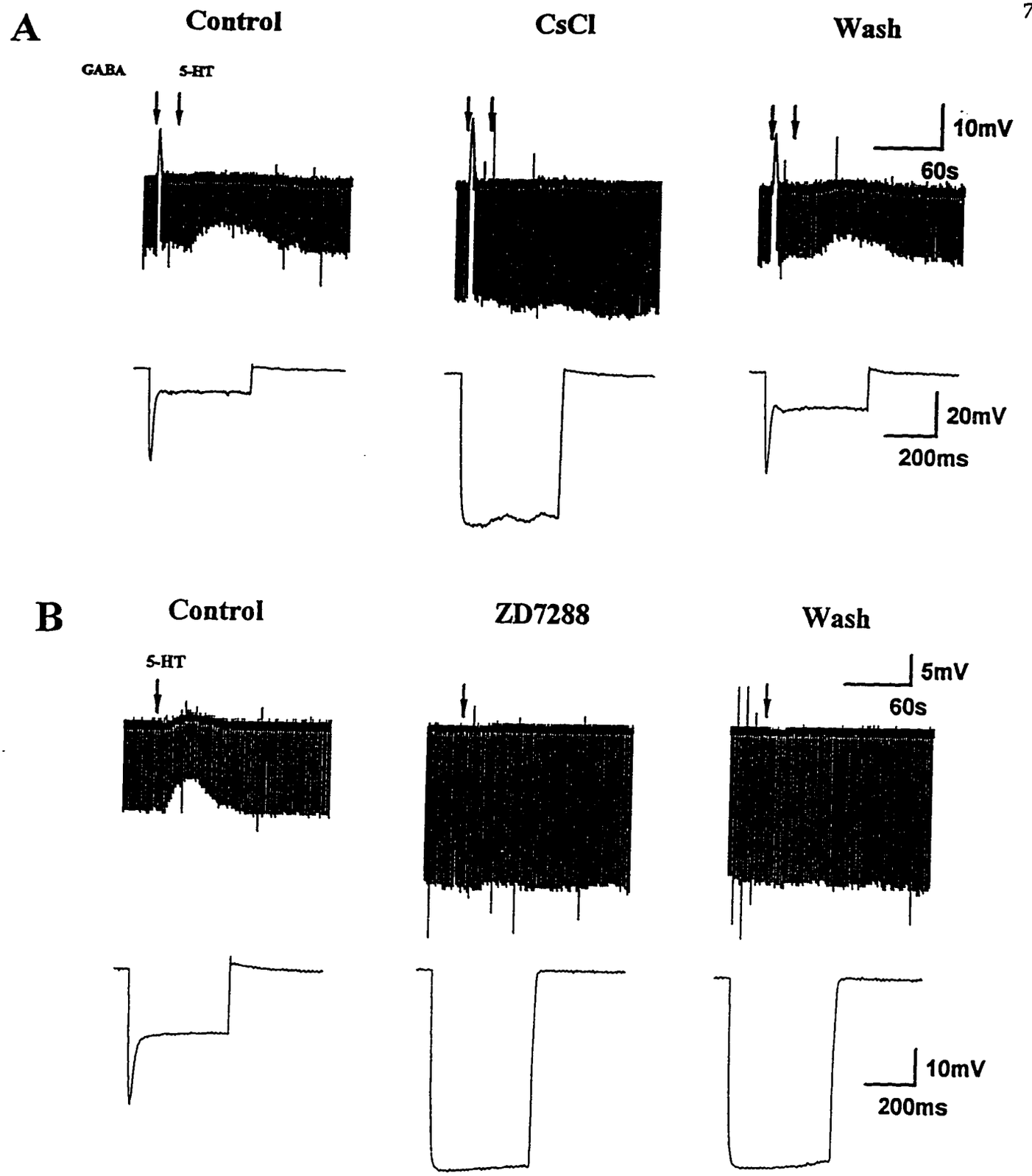
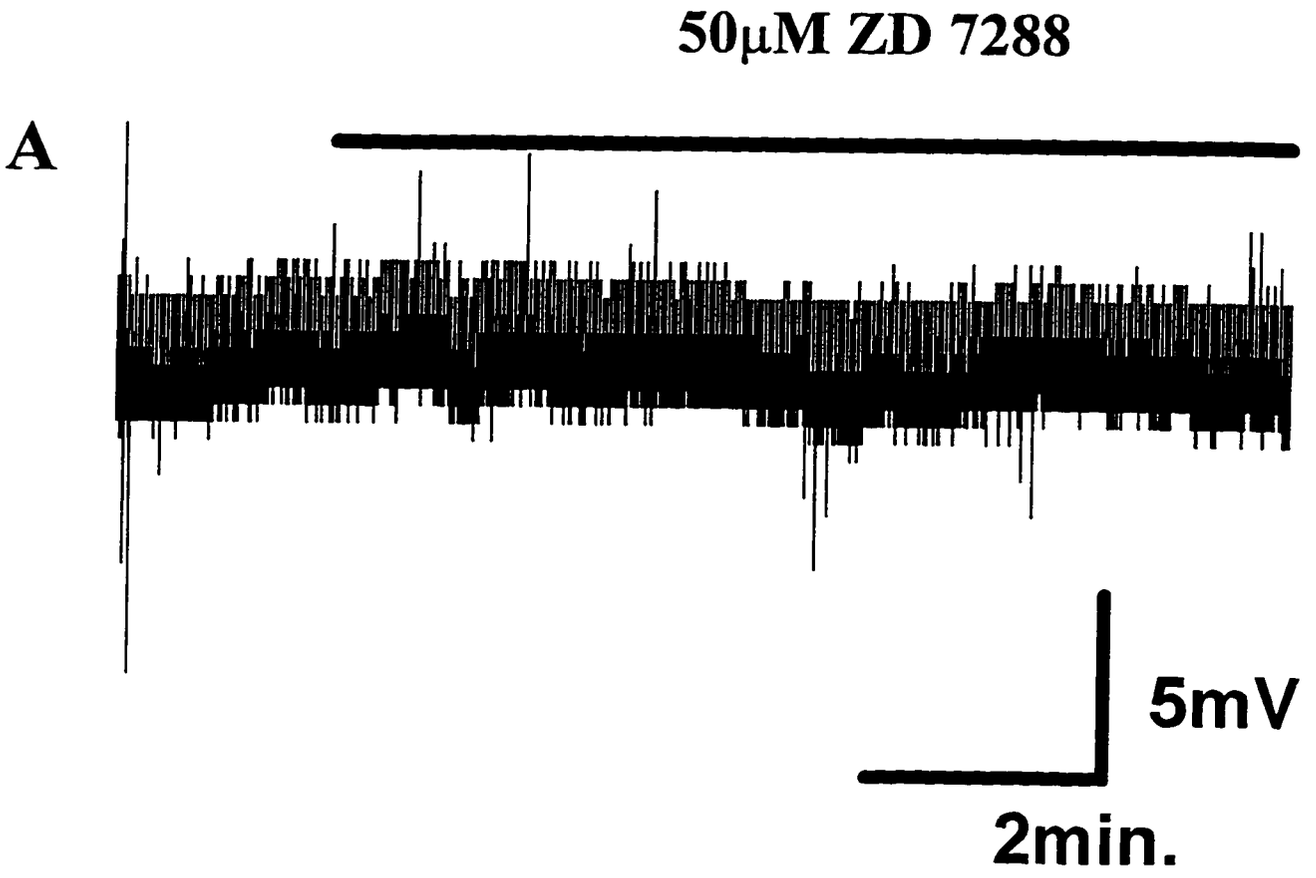

A
Control
CsCl
Wash
GABA
5-HT
10mV
60s
20mV
200ms
B
Control
ZD7288
Wash
5-HT
5mV
60s
10mV
200ms

Figure 17. I_h is not active at rest in MeV neurons. **A** Recording of an MeV neuron during perfusion with 50μM ZD 7288 while monitoring the input resistance of the cell with small posisitive resistance pulses (+0.2 nA for 40 ms at 0.5 Hz). **B** Same as A but with small negative resistance pulses (-0.2 nA for 40 ms at 0.5 Hz). In both **A** and **B** the drug perfusion period is marked by the bar above the recording. Note that the membrane potential does not hyperpolarize and the input resistance does not increase.


50μM ZD 7288
A
5mV
2min.

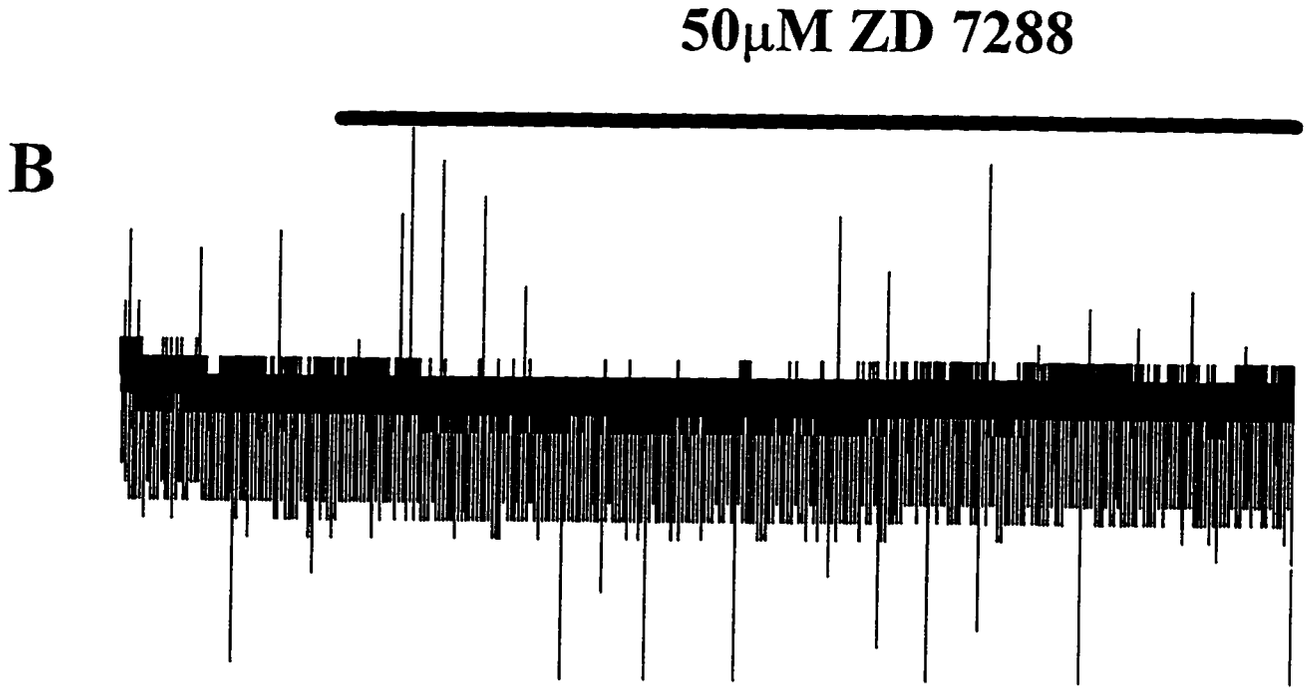

50μM ZD 7288
B

Figure 18. Forskolin, 8-bromo-cAMP, and IBMX mimic 5-HT responses in MeV neurons. Bath applications of the adenylyl cyclase stimulant forskolin (**A**), the cAMP analog 8-bromo-cAMP (**B**), and the phosphodiesterase inhibitor IBMX (**C**) mimmicked 5-HT responses in MeV neurons. Application periods are denoted by the horizontal bars above the recordings. In C 5-HT responses are demonstrated to be occluded during the IBMX effect and recovered as the IBMX response is washed out. In all three records resistance pulses used were -0.5 nA for 40 ms at 0.5 Hz.

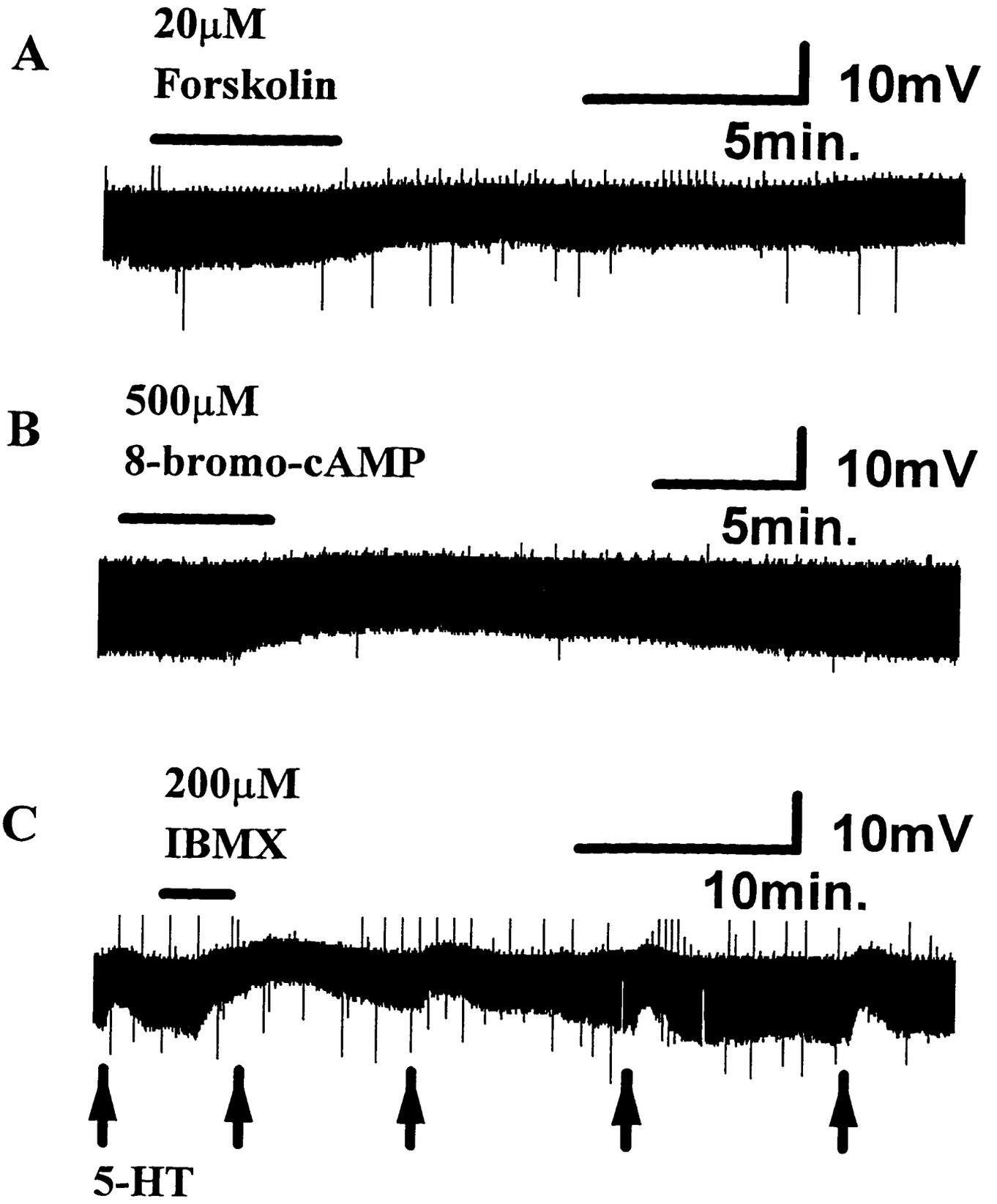

A
20μM
Forskolin
10mV
5min.
B
500μM
8-bromo-cAMP
10mV
5min.
C
200μM
IBMX
10mV
10min.
5-HT

Figure 19. The effect of 8-bromo-cAMP can be blocked by ZD 7288. Upper and lower traces are records from the same neuron before (upper trace) and after ZD 7288 treatment (lower trace). 8-bromo-cAMP applications are marked by the horizontal bar above the records. Resistance pulses used were -0.5 nA for 40 ms at 0.5 Hz.

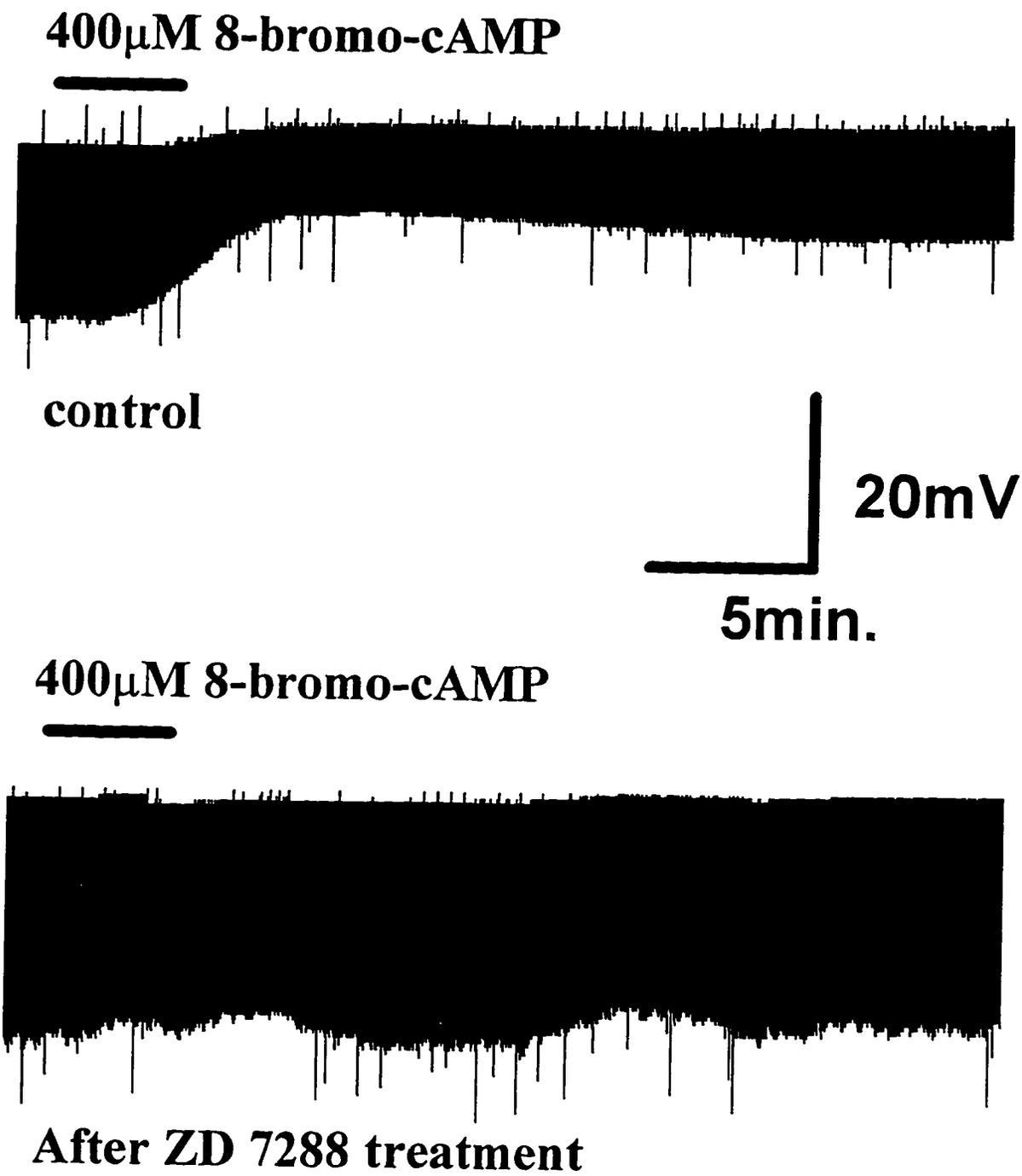

400μM 8-bromo-cAMP
control
20mV
5min.
400μM 8-bromo-cAMP
After ZD 7288 treatment

Figure 20. 5-HT responses of MeV neurons are partially antagonized by protein kinase inhibition. Perfusion of slices with the PKA inhibitor H-89 (80 μM perfused for 10 min.) is shown to partially antagonize 5-HT responsiveness of an MeV neuron. The antagonism is reversible with a 15 min. washout period. Resistance pulses used were -0.5 nA for 40 ms at 0.5 Hz.

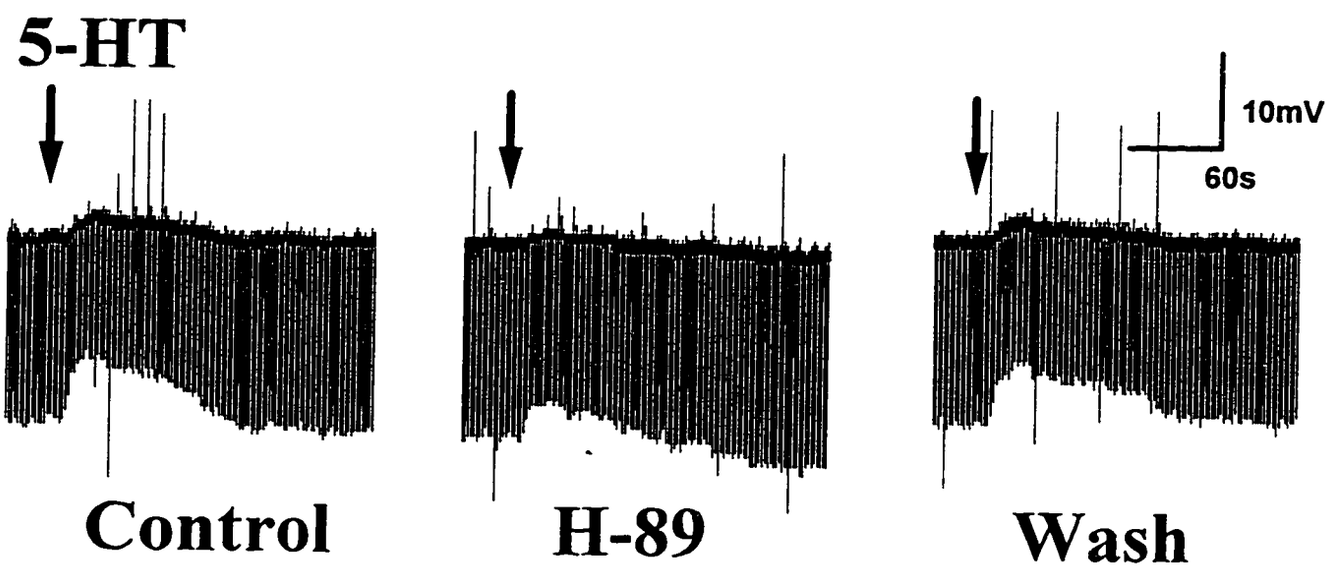

5-HT
10mV
60s
Control
H-89
Wash

Figure 21. 5-HT agonist responses of MeV neurons. The 5-HT response in **A** is demontrated to be mimicked by the 5-HT agonist 5-CT (**B**) but not by the agonist 8-OH-DPAT (**C**). All records taken from the same cell. Resistance pulses used were -0.5 nA for 40 ms at 0.5 Hz.

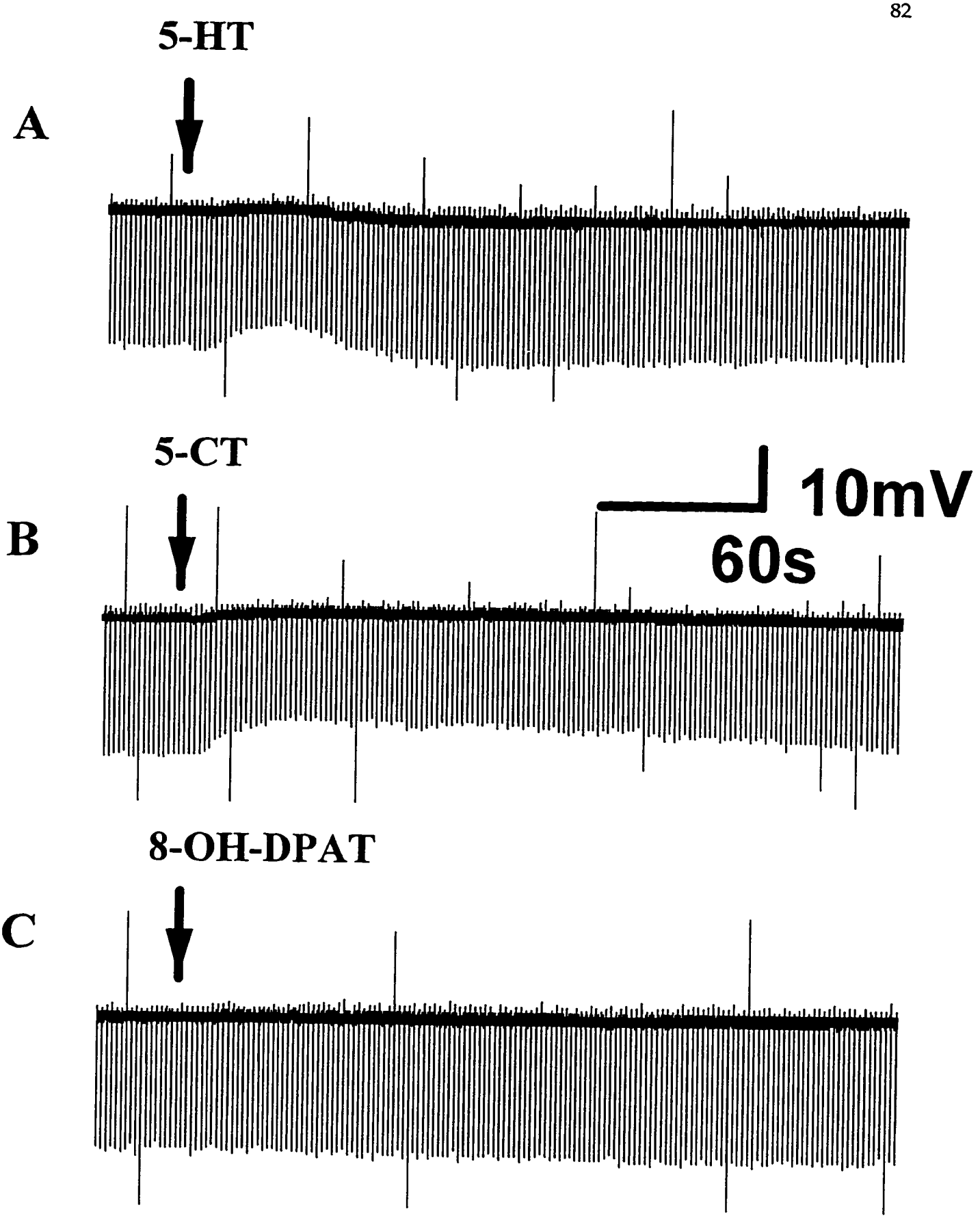
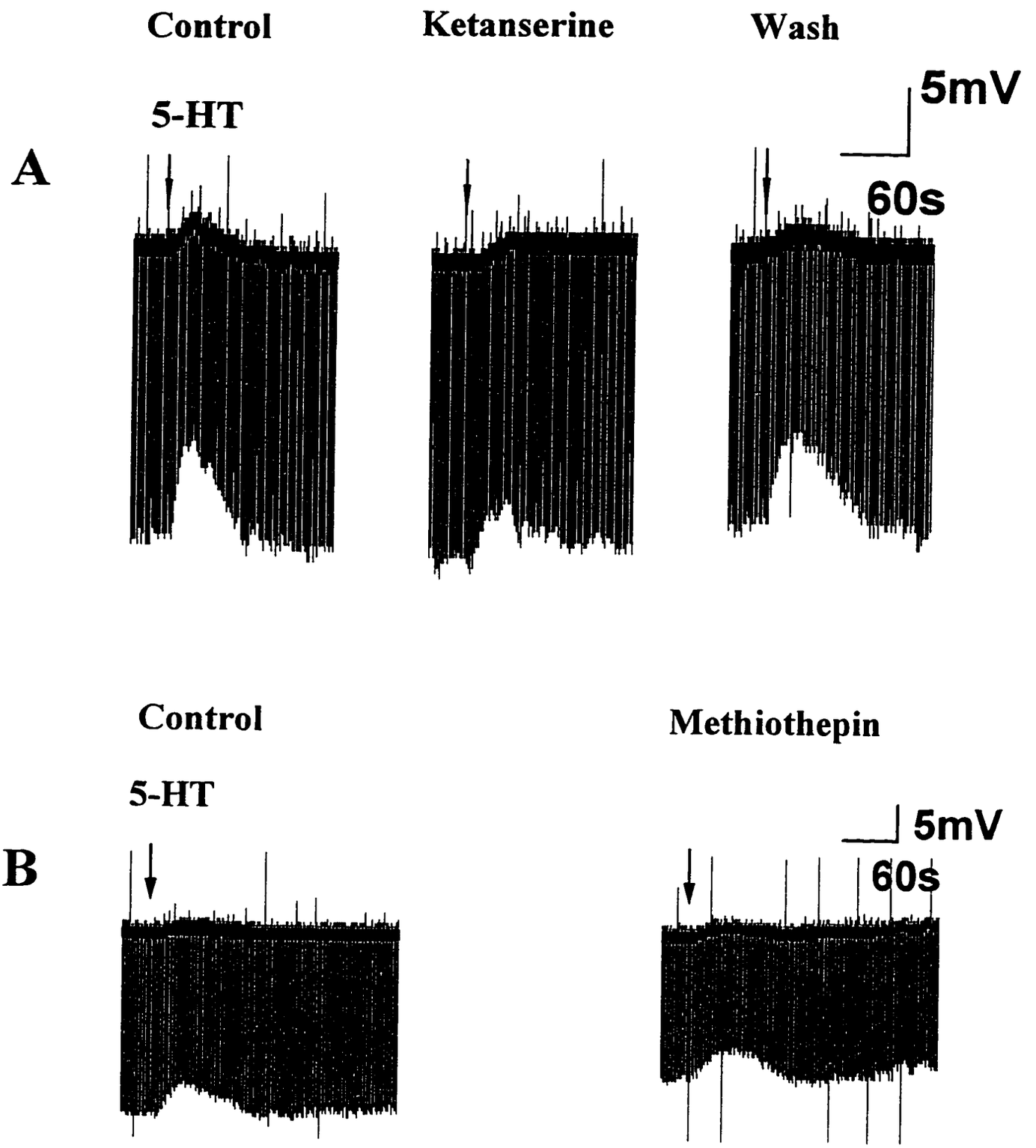

5-HT
A
5-CT
B
10mV
60s
8-OH-DPAT
C

Figure 22. 5-HT antagonists tested in MeV neurons. **A** Partial antagonism of a 5-HT response by ketanserin (50 μM perfused for 5 min.) and recovery upon washout of the antagonist (40 min. washout). **B** The antagonist methiothepin was not effective in antagonizing 5-HT responses (40 μM perfused for 15 min.).


Control
Ketanserine
Wash
5mV
5-HT
A
60s
Control
Methiothepin
5-HT
5mV
B
60s

4 Discussion

4.1 Electrophysiological and Morphological Properties of Mesencephalic Trigeminal Neurons

Recorded cells displayed electrophysiological and morphological properties consistent with those previously reported for neurons of the MeV but not of surrounding nuclei (Henderson et al. 1982; Zheng et al., 1989; Marshall et al., 1995). The cells were large round or ovoid unipolar neurons with resting potentials on the order of -60mV, low input resistances, short duration, TTX-sensitive APs, an inwardly rectifying conductance, and voltage-dependent oscillations. It is therefore concluded that the cells recorded from in this study were indeed MeV neurons.

4.1.1 The Inward Rectifier, I_h

Of particular importance for this study is the property of a time- and voltage-dependent inwardly rectifying conductance, I_h, both for identification purposes and as a mediator of 5-HT responses. I_h differs from the classical inward rectifier in that the classical inward rectifier: (a) is a pure K^+ conductance that has no significant contribution of Na^+; (b) can be antagonized by external Ba^{2+}, in addition to Cs^+; (c) is active mainly negative to the K^+ equilibrium potential; (d) activates within a few milliseconds of an appropriate change in membrane potential; and (e) is ohmic in nature, with its voltage-dependence arising secondarily to voltage-dependent block by internal Mg^{2+} or polyamines (reviewed by Pape, 1996). It is certain that the inward rectification exhibited

by MeV neurons is mediated through I_h channels and not classical inward rectification channels because of the slow time course of activation, and antagonism by the selective I_h blocker, ZD 7288. Furthermore, the inward rectification in MeV neurons has been reported not to be blocked by Ba^{2+} (Zheng et al., 1989; Marshall et al., 1995), but is clearly antagonized by Cs^+, as demonstrated in the present investigation.

Ionic currents thought to be mediated through I_h or I_f channels have been observed in a diverse group of cell types in various species, including cardiac and smooth muscle cells, salivary-gland cells of the leech, and endothelial cells of the blood-brain barrier, as well as in neurons in diverse regions of the vertebrate and invertebrate peripheral and central nervous systems (reviewed by Pape, 1996). However, despite the seemingly widespread expression of these channels, little is known about their function, especially in neurons. One suggested physiological role for I_h concerns a contribution to the resting membrane potential of cells. Typically, in neurons, I_h has been observed to begin to activate in the membrane potential range of -45 to -60mV with half-activation between -75 to -90mV (Mayer and Westbrook, 1983; McCormick and Pape, 1990a; Larkman and Kelly, 1992; Ingram and Williams, 1994). Thus, since typical resting potentials lie within the voltage range of -50 to -100mV, and I_h is reported not to inactivate (McCormick and Pape, 1990a; Tokimasa and Akasu, 1990), a sustained inward I_h current is likely to assist in determining resting potential and R_{input} of some neurons. When applied to resting neurons Cs^+, or specific bradycardic agents known to block I_h, have in some cases resulted in hyperpolarizations with increases in apparent R_{input}, strongly suggesting a contribution of inward currents carried through I_h channels to

resting membrane potentials (McCormick and Pape, 1990a; Maccaferri et al., 1993; Pape, 1994; Travagli and Gillis, 1994). Furthermore, neurons in the basolateral amygdala lacking I_h were found to possess a resting potential substantially closer to the K^+ equilibrium potential compared to their counterparts displaying a strong I_h (Womble and Moises, 1993).

At first it appeared that I_h contributed to the resting potential in MeV neurons because of the apparent increase in R_{input} of the neurons upon treatment with Cs^+ or ZD 7288; however, hyperpolarizations were not observed when cells were treated with these agents. Careful monitoring of the R_{input} during ZD 7288 treatment indicated that the observed increase in R_{input} was actually an artifact of the size of the resistance pulses used, so that when smaller pulses were used, R_{input} was found to remain constant during blockade of I_h, demonstrating that in MeV neurons there does not appear to be any significant contribution of I_h to the resting potential. It should be noted, however, that whether I_h contributes to the resting potential of MeV neurons will of course be dependent upon the resting level of the neuron and the activation range of I_h. Thus, it would be useful to perform voltage-clamp experiments to ascertain the activation range of I_h in MeV neurons to definitively determine if the current could participate in determining the resting potential.

Another possible role for I_h is a contribution to overshoots in membrane potential following hyperpolarizations (i.e. generation of off-spikes). Because the reversal potential of I_h has been found to be positive to the normal resting potential (Mayer and Westbrook, 1983; McCormick and Pape, 1990a; Tokimasa and Akasu, 1990; Kiehn and

Harris-Warwick, 1992; Travagli and Gillis, 1994), hyperpolarizing membrane responses from rest evoke a net Na^+/K^+ current that is inward both during activation (during the hyperpolarization) and deactivation (upon the return from hyperpolarization to rest); the result is a transient depolarizing overshoot of the original potential level possibly leading to AP generation following removal of the hyperpolarizing stimulus. Consistent with previous observations (Mayer and Westbrook, 1983; Solomon and Nerbonne, 1993; Bayliss et al., 1994) blockade of I_h was observed to antagonize the overshoot in MeV neurons demonstrating its contribution to this phenomenon. Even if the overshoots are not large enough to generate off-spikes, they may be able to bring the neuron to a potential level where other ion conductances become activated, for instance those responsible for the observed subthreshold oscillations, positioning the cell in a state of heightened excitability.

It is clear from the present investigation that I_h contributes substantially to the excitability level of MeV neurons. Blockade of I_h with ZD 7288 was observed to increase the magnitude of depolarizing stimuli necessary to generate APs and to inhibit bursting activity of MeV neurons. The decreased excitability exhibited in the presence of ZD 7288 could possibly be the result of a non-selective effect of the compound on another ionic conductance, for instance the voltage activated Na^+ conductance responsible for spike generation. Such an effect, however, seems unlikely as APs could still be elicited during ZD 7288 treatment, and previous investigations have observed no effect of the I_h channel antagonist on spontaneous AP properties in substantia nigra pars compacta neurons at concentrations comparable to those used in the present study (Harris and Costanti, 1995).

I_h has been observed to interact with a low-threshold Ca^{2+} current to control bursting activity, and hence excitability, in a variety of neurons (Crepel and Penit-Soria, 1986; McCormick and Pape, 1990a; Foehring and Waters, 1991; Corotto and Michel, 1994); however, such a low-threshold Ca^{2+} current has not been observed in MeV neurons. It is more likely that I_h contributes to MeV neuronal excitability by limiting the degree of hyperpolarization from activation of K^+ conductances or electrogenic pump activity following AP activity, as has been suggested in other neuronal networks (Baker et al., 1987; Birch et al., 1991; Eng et al., 1990; Gordon et al., 1991). This function of I_h could also be of critical importance in the prevention of conduction failure, especially at regions of reduced safety factors, such as axonal branch points. Such a function is supported by reports that I_h is considerably more pronounced in A- than C-fibers of sensory ganglion neurons and that the frequency-following capability of A-fiber neurons is greater than that of C-fiber neurons (Gallego and Eyzaguirre, 1978; Gorke and Pierau, 1980). While this proposed function of I_h cannot help to explain why its blockade necessitates an increase in stimulus intensity for spike generation, it does provide a possible explanation for the inhibition of bursting activity observed in the present investigation.

4.1.2 Oscillations of Mesencephalic Trigeminal Neurons

The oscillatory properties of MeV neurons at peri-threshold potentials is a notable property of these cells. High-frequency oscillations of this type have been previously reported for MeV neurons (Zheng et al., 1989; Pedroarena et al., 1994; Marshall et al.,

1995). Similar events have been observed in TG cell somata (Puil et al., 1988; Puil and Spigelman, 1988), where they appear to reflect high frequency resonance properties of the cells (Puil et al., 1986; Puil et al., 1987, 1988). The primary involvement of a K^+ conductance in the oscillations of TG neurons is suggested by antagonism of the oscillatory behaviour with the K^+-channel blocker, tetraethylammonium (TEA) and enhancement of the oscillations with the K^+-channel blocker, 4-aminopyridine (4-AP) (Puil et al., 1989). Similarly, a K^+ conductance appears to be intimately involved with MeV neuron oscillations as both TEA and 4-AP are reported to increase their amplitude (Zheng et al., 1989). A Ca^{2+} conductance is also suspected of contributing to MeV neuron oscillations since perfusion with Ca^{2+}-free ACSF has been observed to abolish the oscillations (Zheng et al., 1989). There appears to be no contribution of voltage-gated Na^+ conductances to the oscillations of either TG or MeV neurons since perfusion with TTX has no effect upon the oscillations (Puil and Spigelman, 1988; Zheng et al., 1989). Clearly, further studies are necessary to determine the ionic mechanisms responsible for generation of the voltage-dependent oscillations as well as their function in sensory information processing.

It has been suggested that in TG neurons the oscillatory properties of the neurons may enable the neuronal membrane to act as a filter with particular bandpasses at different membrane potentials (Puil et al., 1987). Polarization of the membrane potential to a different value where resonance (and hence oscillations) occurs would confer upon the neuron the ability to pass and amplify a specific band of frequencies of current input while rejecting input frequencies above and below the resonant bandwidth. A similar

function for the oscillating properties of MeV neurons may be postulated, and in fact, may be more physiologically relevant as the somata of mesencephalic trigeminal neurons receive synaptic inputs (see afferent projections to the MeV), whereas TG neuronal somata have not been observed to receive synaptic inputs (Lieberman, 1976).

4.2 Neurotransmitter Responsiveness of Mesencephalic Trigeminal Neurons

Although there have been many reports describing innervation of neurons within the MeV by axons containing various transmitters, previous investigations have failed to convincingly reveal responsiveness of MeV neurons to any such substances other than GABA, when applied exogenously. In this study it was found that MeV neurons are capable of responding to exogenously applied EAAs and 5-HT.

4.2.1 EAA Responses of Mesencephalic Trigeminal Neurons

Cells responded to Glu with both non-NMDA- and NMDA-mediated components. Applications of selective agonists revealed CNQX-sensitive AMPA- and KA-receptor-mediated responses and NMDA-receptor-mediated responses that were Mg^{2+}- and CPP-sensitive. While agonist-induced responses were slightly diminished in the presence of TTX they still remained evident, suggesting that the responses were primarily elicited by direct effects of the drugs on the recorded cells. These findings are consistent with immuno-localization studies that demonstrate AMPA, KA, and NMDA receptor subunit expression within the MeV (Petralia and Wenthold, 1992; Petralia et al., 1994a,b,c). The

slight reduction in the responses observed in the presence of TTX may indicate that when synaptic transmission is not blocked, the exogenously applied EAAs may depolarize terminals forming synapses with recorded MeV neurons sufficiently to cause release of other neurotransmitter substances (e.g. 5-HT) which can contribute to the observed responses.

Despite responsiveness of recorded MeV neurons to both AMPA and KA, there remains some uncertainty concerning whether these responses were mediated by the same or different receptors, since both agonists have been reported to have actions on both subtypes of receptors (Hollmann et al., 1989; Barnard and Henley, 1990; Huettner, 1990; Keinanen et al., 1990; Nakanishi et al., 1990; Sakimura et al., 1990; Sommer et al., 1992). The observed cross-desensitization between AMPA and KA responses may suggest that the same receptor is activated by both agonists. Furthermore, the desensitization observed with both agonists would seem to indicate that KA receptor activation is taking place, since KA is not believed to activate AMPA receptors in a desensitizing fashion (Hollman et al., 1989, Nakanishi et al., 1990; Sakimura et al., 1990; Patneau et al., 1993) but does give desensitizing responses at KA receptors (Huettner, 1990; Sommer et al., 1992; Partin et al., 1993). The long duration of the desensitization also suggests mediation by KA receptors, since AMPA receptor desensitization is associated with recovery on the order of one second, rather than of several minutes (Kiskin et al., 1986). A further indication that the non-NMDA responses are mediated through KA receptors is the observation that cyclothiazide was unable to block the desensitization of AMPA responses. In other studies, this drug has been demonstrated

to effectively block desensitization of native and recombinant AMPA receptors but not of KA receptors (Partin et al., 1993; Wong and Mayer, 1993).

The apparent cross-desensitization between NMDA and non-NMDA responses in MeV neurons was somewhat unexpected, though not unprecedented. Similar phenomena have been reported in dorsal horn neurons (Kyrozis et al., 1995) and hippocampal neurons (Medina et al., 1994). There is little doubt that NMDA and the non-NMDA agonists are mediating their effects through separate receptors on MeV neurons, since the agonists and antagonists used are selective and the NMDA responses displayed Mg^{2+}- sensitivity that was not observed for the non-NMDA responses.

EAA responsiveness involving NMDA and non-NMDA Glu receptors has also been observed in peripherally located DRG primary afferent neurons or fibres (Lovinger and Weight, 1988; Agrawal and Evans, 1986; Huettner, 1990), and was in some cases presumed to be restricted to the small C-fiber subpopulation of neurons involved primarily in nociception (Agrawal and Evans, 1986). Similarly, in cultured DRG neurons, KA/domoate responses were observed in the smaller, but not the larger cells (Huettner, 1990). In contrast, no such population of primary afferents exists within the MeV and responses did not appear to be restricted to any particular subpopulation of MeV neurons, since they were elicited in randomly impaled cells throughout the nucleus. Where non-NMDA responses of DRG neurons have been observed, they are generally believed to be acting through KA receptors (Partin et al., 1993).

The failure of previous investigations to demonstrate EAA responsiveness of MeV neurons was most likely due to technical limitations. De Montigny and Lund (1980)

observed no responses while recording extracellularly from the MeV during microiontophoretic application of a variety of candidate neurotransmitters, including KA and Glu. In the present investigation, responses to EAAs were found to be relatively small in magnitude and hence may not be easily observable with extracellular recordings. Henderson et al. (1982) reported that intracellularly recorded MeV cells did not respond to superfusion of Glu or responded with an almost imperceptible depolarization that did not last for the entire perfusion period. In this case, the response could be difficult to observe because of the small size of the Glu responses and the desensitization properties of the receptors mediating the response; hence, perhaps a more rapid drug application and clearing method such as that used in the present study is required. Finally, MeV cells have long been assumed to be unresponsive to EAAs since they are spared following injections of excitotoxic doses of KA into the reticular formation of cats (Colonnier et al., 1979). The lack of an excitotoxic effect of KA on these neurons may be explained by the prolonged desensitization of the EAA receptors observed in this investigation, and also by the relatively small magnitude of the responses which may indicate that the MeV cells possess relatively few EAA receptors through which KA could mediate its toxic effects.

DRG neurons are also reported to be resistant to the excitotoxic effects of KA in the rat (Wolf and Keilhoff, 1983) in spite of the fact that these cells have been found to possess KA receptors and respond electrophysiologically to the EAA agonist (Huettner, 1990; Wong and Mayer, 1993; Partin et al., 1993; Agrawal and Evans, 1986; Sato et al., 1993; Wisden and Seeburg, 1993; Bahn et al., 1994; Tachibana et al., 1994). Thus, it is

possible that primary afferents of the MeV and DRG possess similar mechanisms of protection against KA induced excitotoxicity. The observations of desensitizing KA responses in freshly dissociated and cultured DRG neurons (Wong and Mayer, 1993; Huettner, 1990) may suggest that this property is primarily responsible for the resistance to excitotoxicity.

MeV neuron responses to EAAs displayed a large degree of variability in amplitude and duration from cell to cell. This may be a result of variations in the concentration of drug actually reaching the recorded neurons using pressure application in the slice preparation. In some instances, recorded cells were superficially located, whereas in other cases they were deeper within the slice. Because the application of the drug was always onto the surface of the slice, the effective concentration at the recorded neurons is unknown and variable. Glu responses were consistently smaller in magnitude than responses to the Glu receptor agonists. This could be a result of the uptake of Glu by glial cells within the slice so that only a limited amount of the drug reaches recorded cells, especially since MeV neuronal somata are reported to be almost entirely ensheathed by astrocytic processes in the rat (Copray et al., 1990c). However, attempts to enhance Glu responses with the Glu uptake inhibitor, trans-PDC, were unsuccessful. Consistent with our findings, Glu responses have also been found to be smaller in magnitude than those of Glu receptor agonists in DRG primary afferents (Agrawal and Evans, 1986; Huettner, 1990).

4.2.2 Serotonin Responses of Mesencephalic Trigeminal Neurons

Applications of 5-HT to MeV neurons elicited small depolarizations accompanied by decreases in neuronal R_{input} due to direct effects upon recorded neurons. The 5-HT response was not observed in all neurons tested, (41 of 53 neurons tested) perhaps indicating that the responsiveness is selective for one of the subpopulations of MeV neurons. However, response segregation between muscle-spindle afferents and periodontal mechanoreceptors seems unlikely as serotonergic inputs have been observed to contact both subtypes of neurons (Tashiro et al., 1989; Kolta et al., 1993). It is noteworthy to mention that all 12 non-responding neurons were recorded from within a two-week period near the end of the study, during which time no responsive neurons were encountered suggesting that the lack of responsiveness may have arisen from technical difficulties.

Characterization of the 5-HT responses revealed that the effects of 5-HT resulted from activation of I_h. The intracellular second-messenger system mediating this response most likely involves stimulation of AC and a resulting increase in intracellular cAMP levels, as indicated by the mimicking effects of the membrane-permeable analogue of cAMP, 8-bromo-cAMP, a stimulant of AC activity, forskolin, and a phosphodiesterase inhibitor, IBMX. Similar effects of 5-HT have been reported in various neuronal populations including mammalian and crustacean motoneurons (Takahashi and Berger, 1990; Kiehn and Harris-Warrick, 1992; Larkman and Kelly, 1992; Garrat et al., 1993), thalamo-cortical neurons (Pape and McCormick, 1989; McCormick and Pape, 1990b), substantia nigra pars compacta neurons (Nedergaard et al., 1991), CA1 hippocampal

neurons (Storm et al., 1996), and neurons of the brain stem nucleus prepositus hypoglossi (Bobker and Williams, 1989). In these studies, activation of I_h channel activity was also suggested to be mediated by increases in cAMP levels and was demonstrated to rely on positive shifts in the voltage-dependence of the I_h activation curve by up to +10mV, with no change in the steepness of the curve or in the fully activated I/V-relation. This suggests that receptor stimulation modulates I_h through an alteration in the voltage-dependence of the underlying channels rather than simply leading to a direct opening of channels. Further experiments employing voltage-clamp techniques will be required in order to determine if the 5-HT-induced activation of I_h in MeV neurons results from a change in the voltage-sensitivity of the channels or a direct opening of channels.

Other neurotransmitters, such as noradrenaline and histamine, functioning through receptors positively coupled to AC (β-adrenergic and H_2 receptors respectively), have also been reported to activate I_h (Pape and McCormick, 1989; McCormick and Pape, 1990b; McCormick and Williamson, 1991; Banks et al., 1993; Maccaferri et al., 1993; Storm et al., 1996). Conversely, neurotransmitters such as adenosine and opioids, acting on receptors negatively coupled to AC (A_1 and μ-opioid receptors respectively) have been demonstrated to inhibit noradrenaline or forskolin-stimulated I_h channel activation (Pape, 1992; Ingram and Williams, 1994; Rainnie et al., 1994), presumably through a negative shift in the voltage-dependence of the I_h activation curve. These findings indicate that I_h conductances are under the fine control of intracellular cAMP concentrations and that stimulation of receptors that are negatively and positively coupled to AC activity

reciprocally shift the I_h activation curve along the voltage axis, thereby controlling the availability of I_h channels over a wide range of membrane potentials. With this in mind, it is interesting to note that some of the projections onto MeV neurons are believed to contain various neurotransmitters other than 5-HT, such as noradrenaline, adenosine, dopamine and histamine. These could potentially alter intracellular cAMP levels leading to changes in I_h channel activity. While these substances were not tested in the present investigation, it seems likely that MeV neuronal I_h channels, and perhaps excitability, are under tight regulation by the integrative effects of 5-HT and some of these other neurotransmitter substances. Such a situation occurs in cardiac sino-atrial node cells where the integrative actions of cholinergic and adrenergic receptor stimulation on cAMP levels controls the voltage-dependence of I_f, and hence heart rate, since I_f is a component of the pacemaker apparatus in these cells (reviewed by DiFrancesco, 1993).

Typically, transmitters acting via cAMP modulate target proteins by activation of PKA leading to phosphorylation of the target protein. As noted in the Results section, PKA involvement in the cAMP-dependent modulation of I_h channel activity has to date been a matter of some controversy. In dissociated bullfrog sympathetic neurons the protein kinase inhibitor, H-8, was observed to reduce the amplitude of I_h, and to completely reverse the enhancement of I_h by both forskolin and IBMX (Tokimasa and Akasu, 1990); however, the same protein kinase inhibitor was reported not to affect I_h in thalamo-cortical neurons in a slice preparation (Pape, 1992). The discrepancy may have resulted from the different modes of application of the kinase inhibitor, as bath application for 20 to 30 minutes was used by the first group while the second group

relied on local pressure application to the recorded neurons. A more thorough investigation of protein kinase involvement in I_h channel modulation has been performed on CA1 hippocampal neurons. Intracellular application of a specific PKA inhibitor (Walsh peptide) to these cells was found not to supress the activation of I_h by β-adrenergic agonists (Pedarzani and Storm, 1995). The same group recently reported that application of the cAMP analog, Rp-cAMPS, which is a PKA inhibitor, enhances I_h in a manner similar to that of 5-HT, isoproterenol, dopamine, and histamine (Storm et al., 1996). Together these findings strongly suggest a direct cAMP effect on I_h channels independent of PKA involvement.

Conflicting results concerning PKA involvement in I_f channel regulation have also been reported in cardiac tissue. Chang et al. (1991) reported that the protein kinase inhibitor H-7 induced a negative shift along the voltage axes of the I_f activation curve in Purkinje fibers, and that both H-7 and H-8 reversed or prevented cAMP-induced increases in I_h of these cells. Conversely, inside-out macro-patches from sino-atrial node myocytes performed by DiFrancesco and Tortora (1991) suggest a direct cAMP-dependent regulation of I_h channels independent of protein kinase activity, since cAMP-induced shifts in the activation curve occurred regardless of the presence of PKA inhibitors, and applications of PKA or the active catalytic subunit did not activate I_f.

Responses of MeV neurons to 5-HT, presumably mediated by increases in cAMP, were found to be partially antagonized by protein kinase inhibitors in the present investigation. This observation suggests cAMP-dependent regulation of I_h channels occurs, at least in part, through PKA activation in these neurons. However, further

experiments are required to conclusively determine the role of PKA in I_h regulation in MeV neurons. Specifically, voltage-clamp experiments should be performed to monitor direct effects of protein kinase inhibitors on the I_h activation curve as well as on cAMP-induced changes in I_h channel function.

Complicating matters, are reports that second-messenger systems other than those involving cAMP are able to modulate I_h. Increases in cyclic guanosine monophosphate (cGMP) levels via nitric oxide stimulation of guanylyl cyclase activity have been observed to induce a positive shift in I_h activation in thalamic neurons (Pape and Mager, 1992) and blood-brain barrier endothelial cells (Janigro et al., 1994). In whole-cell voltage-clamp studies of sino-atrial node cells, increases in intracellular Ca^{2+} levels have been observed to positively shift I_f activation in a manner independent of protein kinase or calmodulin activity, suggesting a direct effect of Ca^{2+} on the channels (Hagiwara and Irisawa, 1989). However, recording of I_f activity in inside-out macro-patches from sino-atrial node cells failed to demonstrate a modulating action of Ca^{2+} on I_f (Zaza et al., 1991). Finally, a direct action of G-proteins on I_f has been proposed since preactivated G_s and its preactivated catalytic subunit α_s are capable of activating I_f channels under substrate-free conditions in inside-out membrane patches excised from sino-atrial node cells; furthermore, preactivated G_0 and G_i and their respective preactivated catalytic subunits inhibited I_f channels (Yatani et al., 1990). Based on these observations it is possible that I_h channels in MeV neurons are under the control of a variety of intracellular signals other than cAMP, and additional experimentation to delineate these signals would be appropriate.

Identification of the subtype of receptor(s) mediating 5-HT increases in I_h has remained elusive. All data concerning the subtype of receptor(s) are based on less than extensive pharmacological profiles that have generated conflicting results. For example, while the 5-HT_{1A} receptor agonist 8-OH-DPAT activates I_h in spinal motoneurons (Takahashi and Berger, 1990), this agonist has been found to be without effect on I_h in prepositus hypoglossi neurons (Bobker and Williams, 1989), thalamic neurons (McCormick and Pape, 1990b), substantia nigra pars compacta neurons (Nedergaard et al., 1991) and facial motoneurons (Larkman and Kelly, 1992). Similarly conflicting results have been reported for the 5-$HT_{1A/2}$ antagonist spiperone, the 5-$HT_{1C/2}$ antagonist ritanserin, and the 5-HT_2 antagonist ketanserin (Bobker and Williams, 1989; McCormick and Pape, 1990b; Takahashi and Berger, 1990; Nedergaard et al., 1991; Larkman and Kelly, 1992; Garratt et al., 1993) which have been demonstrated to antagonize 5-HT activation of I_h in some systems but not in others. The case of spiperone is especially confusing as it is reported to antagonize LSD-mediated activation of I_h (Garratt et al., 1993) but not 5-HT-mediated activation of I_h (Larkman and Kelly, 1992) in facial motoneurons.

We attempted to provide a preliminary pharmacological profile of the 5-HT receptor mediating responses in the MeV. Agonists and antagonists tested were chosen based on the assumption that the receptor is positively coupled to AC, suggesting a 5-HT_4, 5-HT_6, or 5-HT_7 (or perhaps 5-HT_{1A}) subtype of receptor involvement (see Introduction: 1.3.2 Serotonin Receptors) and on the observed binding of radiolabeled ketanserin in the MeV, suggesting 5-HT_2 receptor involvement (Kolta et al., 1993).

Consistent with the observations of Kolta et al.'s binding data, ketanserin antagonized MeV neuronal responses to 5-HT, however 5-HT-induced activation of I_h through 5-HT_2 receptors seems unlikely, as preliminary tests indicated that the potent 5-HT_2 receptor agonists, DOI and α-methyl-5-HT, were incapable of generating the response. Ketanserin has also been demonstrated to have micromolar affinity at 5-HT_6 and 5-HT_7 receptors and to be inactive at 5-HT_4 receptors (Boess and Martin, 1994), thus, we focussed our attention on the two aforementioned subtypes of receptors. Both 5-HT_6 and 5-HT_7 are potently activated by the agonist 5-CT (Boess and Martin, 1994) which has been observed to be selective for the enhancement of I_h in prepositus hypoglossi neurons (Bobker and Williams, 1989) and facial motoneurons (Larkman and Kelly, 1992). Likewise 5-CT was found to mimic 5-HT responses in MeV neurons, potentially implicating 5-HT_6 or 5-HT_7 receptor involvement. Unfortunately, attempts to antagonize the 5-HT response with methiothepin, a general 5-HT antagonist with very high affinity for both 5-HT_6 and 5-HT_7 receptors, were unsuccessful (although tests were not extensive). Furthermore, consistent with most of the data available from other systems, the 5-HT_{1A} receptor agonist 8-OH-DPAT, which has high affinity for 5-HT_7 receptors, was incapable of generating responses in our recordings, further discrediting the 5-HT_7 receptor as a mediator of 5-HT responses in MeV neurons and also ruling out possible 5-HT_{1A} receptor involvement. Based on these findings, we are at present unable to identify the subtype of receptor involved in the 5-HT-induced enhancement of I_h in MeV neurons. Positive identification will require the availability of more specific pharmacological agents as well as the application of molecular biological techniques, such as in situ hybridization and

receptor cloning.

4.3 Functional Considerations of Neurotransmitter Responses of Mesencephalic Trigeminal Neurons

In spite of the relatively small amplitude of MeV neuronal voltage responses to EAAs, recorded neurons frequently reached threshold for AP generation. It is possible that in these cells, the generation of voltage-dependent oscillations permits AP activation by relatively small depolarizations. Thus, while the responses to EAAs may appear inconsequential because of their small amplitude, when combined with the voltage-dependent oscillatory behaviour of MeV neurons they could result in significant changes in these cells' activity. In view of the oscillatory activity following evoked spikes, it would appear possible that a single AP invading a somewhat depolarized MeV soma could generate a burst of potentials that would be conducted into the axon, resulting in an amplification of the original signal. This type of modulation related to oscillatory activity has been suggested for TG neurons by Puil et al. (1989), and would seem also applicable to MeV neurons. Such a facilitatory modulation of the output of MeV muscle-spindle neurons could be of importance during periods of aggression and attack behaviour, when increased drive onto the motor neurons could be desired for increased biting force. Interestingly, stimulation of the amygdala and the hypothalamus, two neuronal centres intimately involved with mood and behaviour which have been demonstrated to project to the MeV (see afferent projections to the MeV), have been shown to facilitate jaw closure (Kawamura and Tsukamoto, 1960; Nakamura and Kubo,

1978; MacDonnell and Fessock, 1972). Alternatively, facilitation of the output of periodontal mechanoreceptor neurons would tend to inhibit jaw closure and promote jaw opening. This type of modulation could be desirable as a protective mechanism against damage to dental structures and could also play an important role during any period when the jaw must be held open for considerably long periods of time, such as during emesis, and in laboured respiration.

While it is clear that the effects of EAAs upon MeV neurons are excitatory in nature, as evidenced from the frequently observed generation of bursting activity upon their application, the effects of 5-HT are not so easily discerned. Presumably the final effects of 5-HT will depend on those of I_h at MeV neurons, effects which require further experimentation to elucidate. Work performed in the present investigation demonstrated that I_h blockade was inhibitory to MeV neuronal firing, and hence, intuitively I_h activation should be excitatory, suggesting that the 5-HT effect is excitatory in nature. Most likely, the I_h activation induced by 5-HT allows for the neuron to faithfully follow high frequency repetitive burst activity by limiting the degree of hyperpolarization during prolonged activation, as discussed above concerning the role of I_h in MeV neurons. As the source of the serotonergic input to the MeV has been suggested to originate from the dorsal raphe nucleus (Copray et al., 1990b) it is interesting to note that neurons of this nucleus increase their firing frequency before or during mastication (Jacobs, 1991), a period during which MeV neurons are very active.

As mentioned in the introduction, modulation of MeV electrical activity has been observed in response to activation of other CNS areas and these effects were

hypothesized to be the result of the activation of synaptic pathways impinging upon MeV neuronal somata. In this study I have provided possible candidates as the substrates mediating these effects. However, while both the EAAs and 5-HT provide a possible basis for facilitatory modulation of MeV neurons I am unable to suggest a mechanism for the predominantly inhibitory modulation suggested to occur in MeV neurons by Pettorossi (1983) and Kolta et al. (1990). The present work suggests that the excitability level of MeV neurons is dependent upon the activation state of I_h, hence, it is possible that a neurotransmitter substance that negatively modulates I_h may serve as a possible substrate for the inhibitory modulation of MeV neuronal activity observed in previous investigations.

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