SEROTONIN AND NORADRENALINE MODULATE EXCITATORY AMINO ACID-EVOKED CURRENTS IN ACUTELY ISOLATED INTERMEDIATE LAMINAE NEURONS FROM THE NEONATAL RAT SPINAL CORD

A Thesis

Presented to the

University of Manitoba

In Partial Fulfillment of the Requirements

for the Degree

MASTER OF SCIENCE

IN

PHYSIOLOGY

by

Stephen C. MacDonald

1996

ISBN 0-612-13329-X

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BY

STEPHEN C. MACDONALD

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Acknowledgments

"There are no such things as mistakes. They don't exist. There are only lessons." -C.R.S.

I would like to extend my heartfelt thanks to anyone and everyone who has contributed to helping me with my degree or by supporting me throughout it. I will be brief in these acknowledgments because it is difficult to put what I feel into words. The last few years have been full of lessons that I'll never forget.

I would like to start with my supervisor Larry Jordan. Thanks for giving me the opportunity to become a contributing member of the scientific community and getting me out of retail. I appreciate all the generosity and kindness that you have extended to me since I first walked into your office looking for a summer job in May 1992. I am looking forward to working with you over the next few years.

Shawn, my mentor and friend. Well we've grown a lot together you and I. Since you came to Winnipeg as a research associate, you've had quite an influence on my life. You've taught me many things that I'll keep with me my entire life.Your sense of humor is one of your strongest points. Don't ever lose it. I'm looking forward to what the next few years have in store for us.

To my other committee members, Brian Schmidt, Susan Shefchyk and David Magnusson. Thanks for the support, the comments and the encouragement. It was greatly appreciated.

Jason, you definitely have made my life interesting since you came from Windsor. In working with you so closely in the lab and travelling together as much as we did, we both grew a lot. Thanks for making it a memorable experience.

To my fellow graduate students who were there to help out or simply to talk and pass some time. Kris, Pierre, Bodo, Uta, Sherry, Aihua, Denise, Karen, Farah, Lorraine and Mike I just wanted to say thanks.

I would like to thank Mary-Jane Espey, my godsend from Halifax, for her lengthy discussions about pharmacology. I would also like to thank Brian Noga for the discussions we had on monoamines. Thanks to you both, your knowledge was very beneficial and I appreciated it immensely.

I am indebted to Xiaohong Dai, Lei Song, Matt Ellis, Jin and Carolyn Gibbs for the remarkable technical assistance. You all made my life so much easier and I want you all to know how much you help was appreciated.

I would like to thank the secretarial staff, Nancy, Gail, Judy and Maureen. It was really nice to be greeted with a smile and a sincere desire to help out every time I walked through your doors. All of the reminders and help making appointments was totally appreciated. My life would have been pretty chaotic without your help.

Angela, more than a part-time office assistant you were a friend right from the start. Your smile helped me get familiarized and settled when I first started as a summer student and didn't know my way around. Who knew in Grade 7 home room that we would wind up here together. The Gods must have been looking out for me when you got hired on here. I hope you get all the best in life.

And lastly, I would like to thank my family. To my Mom and Dad, this is for you. You were with me all the way through this and supported me through good and bad. I would have had a rough time without you. I love you both. To my brother David and sister-in-law Teri, thanks for listening to me and taking an interest in whatever came out of my mouth. You guys are great.

ABSTRACT

Descending serotonergic and noradrenergic systems modulate spinal cord activity. Neurons in the intermediate zone of the spinal cord has been previously implicated in locomotion yet it is unclear how the monoamines, serotonin and noradrenaline, affect neurotransmission in this region. In the present study, the effects of serotonin and noradrenaline on excitatory amino acid-evoked currents were examined using whole cell patch clamp recording in neurons acutely isolated from the intermediate zone of the neonatal rat spinal cord. Local perfusion of the excitatory amino acid agonists N-methyl-D-aspartate or kainate evoked an inward current. The current was either potentiated or attenuated by coapplication of serotonin or noradrenaline while application of either monoamine alone did not evoke any membrane currents. Modulation mediated by serotonin and noradrenaline displayed a voltage dependency in 11% of cells with the greatest modulation occurring around resting membrane potential. The effects of serotonin on excitatory amino acid-evoked currents was found to display a concentration dependence. Kainate-evoked currents were attenuated at serotonin concentrations above 70 μM while serotonin concentrations between 10 μM and 70 μM resulted in a potentiation. N-methyl-D-aspartate-evoked currents were on average attenuated at all serotonin concentrations tested although greatest attenuation occured at serotonin concentrations above 10 μM. Pharmacological examination of this modulation using specific receptor agonists and antagonists revealed that attenuation of excitatory amino acid-evoked currents is mediated by α_2-adrenergic and 5-HT_{1b} receptor subtypes. The potentiation of kainate-evoked currents by noradrenaline was found to be mediated by α_1- or β-adrenergic receptor subtypes. The receptor subtypes governing serotonin mediated potentiation were not determined. The results support the hypothesis that serotonin and noradrenaline may modify motor activity by modulating excitatory neurotransmission to select neurons in spinal networks specific to a given motor task.

List of Abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HTP	5-hydroxytryptophan
8-Br-cAMP	8-bromo-3',5' cyclic-adenosine monophosphate
ACh	Acetylcholine
ACSF	artificial cerebrospinal fluid
AHP	after hyperpolarization
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ATP	adenosine triphosphate
cAMP	3',5' cyclic-adenosine monophosphate
cAMP-PKA	cyclic-adenosine monophosphate dependent protein kinase A
CGS-12066B	CGS-12066B maleate (5-HT_{1b} agonist)
CNS	central nervous system
CHP	cyproheptadine (5-HT_2 antagonist)
DA	dopamine
DAG	diacyl glycerol
DL-HCA	D-L- homocysteic acid
EAA	excitatory amino acid
EDRO	edrophonium
EMG	electromyogram
ENG	electroneurogram
FRA	flexor reflex afferent
GTP	guanosine triphosphate
$I_{K(Ca)}$	calcium dependent potassium current
IML	intermediolateral nucleus
IP_3	inositol triphosphate
IPT	isoproterenol (β-adrenergic agonist)
L-DOPA	L-3,4-dihydroxyphenylalanine
Methox	methoxamine hydrochloride (α_1-adrenergic agonist)
MSR	monosynaptic reflex
NA	noradrenaline
NMA	N-methyl-D,L-aspartate
NMDA	N-methyl-D-aspartate
PKC	protein kinase C
Prop	propranolol (β-adrenergic antagonist, 5-HT_{1a} antagonist)
PSR	polysynaptic reflex
TTX	tetrodotoxin
Yoh	yohimbine (α_2-adrenergic antagonist)

INTRODUCTION

Different motor behaviours are generated by activation of neural networks located in the spinal cord. It is possible that functionally and anatomically separate networks exist which govern each behaviour. In a 1991 review on modulation, Harris-Warrick and Marder make reference to the hypothesis of a single "polymorphic" network which is reconfigured in many ways to produce different motor patterns. One can imagine this large polymorphic network as being made up of smaller neuronal circuits called central pattern generators which generate simple rhythmic behaviours. Reconfiguring these circuits can be a result of actions of agents such as peptides and monoamines or activation of pathways which release these substances. These agents may affect neurons in a network by modulating intrinsic properties of the cell such as rhythmic bursting, and generating plateau potentials or by modulating synaptic transmission between cells in a network. With respect to motor control, two important modulator substances are the monoamines serotonin (5-HT) and noradrenaline (NA). The actions of these two substances on motor activity will be addressed later in the introduction.

The spinal cord intermediate zone has been implicated in locomotion by the use of activity dependent labeling (Kjaerulff et al. 1994; Viala et al. 1988; Dai et al. 1990) and electrophysiological recording techniques (Grillner 1981; MacLean et al. 1995; Hochman et al. 1994). Neurons in Rexed's lamina VII have been shown to be rhythmically active during locomotion (MacLean et al. 1995) while some lamina X neurons display conditional bursting capabilities in the presence of NMDA (Hochman et al. 1994). Notably, neurons within this region have been demonstrated to possess action consistent with a locomotor 'half-centre' function (Jankowska et al. 1967). There has also been a population of neurons from the intermediate zone in the cat which has previously been described by Jankowska and colleagues

(Edgley and Jankowska 1987). These neurons possess strong group II afferent input, project to motoneurons in lower lumbar segments, and are rhythmically active during fictive locomotion (Shefchyk et al. 1990). Transmission to this population of cells has also been shown to be attenuated by 5-HT and NA (Bras et al. 1989). These observations all implicate the spinal intermediate zone in locomotion and direct the study of the actions of 5-HT and NA to neurons from this region.

In order to introduce how 5-HT and NA modulate motor behaviours such as locomotion, we will begin with a review of serotonergic and noradrenergic projections to the spinal cord and the distribution of specific receptor subtypes for these two monoamines in the lumbar spinal cord. A subsequent overview of excitatory amino acid (EAA) neurotransmission will follow afterwhich this information will all be applied to a more expansive review of the actions of 5-HT and NA on motor activity and EAA neurotransmission. The introduction will conclude with a discussion of the choice of preparation, and goals of the study.

Origins of serotonergic and noradrenergic projections to the spinal cord

The lumbar spinal cord receives projections from serotonergic nuclei in the brain stem. These nuclei were first characterized by Dahlstrom and Fuxe in 1964 who used histofluorescence methods to observe monoamine containing neurons in the rat brainstem. Serotonergic innervation of the intermediate zone and the ventral horn comes primarily from the raphe pallidus and raphe obscurus (areas B1 and B2 respectively) (Dahlstrom and Fuxe 1964) and a small number of projections from the more rostral area (B5) in the pons (Bowker et al. 1982). There also exists a dorsal pathway arising from the raphe magnus (area B3) which travels in the dorsolateral funiculus and terminates in the dorsal horn at all segments of the spinal cord (Bowker et al. 1982). This pathway will not be discussed further because of this study's focus on the intermediate zone and motor control. The serotonergic pathway innervating the intermediate gray

matter arises from the ipsilateral arcuate cell group, raphe pallidus and raphe obscurus and terminates in both ipsilateral and contralateral spinal intermediate zones. The ventral pathway projects from the raphe pallidus, raphe obscurus and nucleus reticularis ventralis via the ventral funiculus and ventrolateral funiculus to a given segment where it projects to both ipsilateral and contralateral ventral horns (for review see Skagerberg and Bjorklund 1985). Both intermediate and ventral pathways are largely serotonergic although non-serotonergic components have been reported (Skagerberg and Bjorklund 1985).

Noradrenergic projections to the spinal cord have been traced by staining for dopamine-β-hydroxylase (Westlund et al. 1982,1983). Noradrenergic projections to the spinal cord arise from the locus coeruleus and nucleus subcoeruleus in the pons (areas A6 and A7 respectively) (Dahlstrom and Fuxe 1964). These projections travel in the ipsilateral ventrolateral funiculus to the intermediate zone and the lumbar ventral horn. It was discovered that locus coeruleus axons terminated primarily in the superficial laminae of the dorsal spinal cord with relatively few fibres in the intermediolateral nucleus (IML) or ventral horn (Fritschy et al. 1987). These results were contradictory to a previous study which found the locus coeruleus projected heavily to the ventral horn (Nygren and Olsen 1977). It was subsequently reported that projections from the locus coeruleus terminated mainly in the dorsal horn and IML whereas sub-coeruleus axons terminated in the motor nuclei while both cell groups project to lamina X around the central canal (Grzanna and Fritschy 1991). These findings suggest that noradrenergic neurons are divided into anatomical groups with different functions.

Immunohistochemical study of the development of sertonergic projections to the spinal cord has revealed that serotonergic fibres in the spinal cord develop from a ventral to a dorsal direction (Rajaofetra et al. 1989; Tanaka et al. 1992; Ziskind-Conhaim et al. 1993). The first 5-HT immunoreactive fibres appear in the lumbar spinal cord at embryonic day 15 (E15) but do not

actually penetrate the grey matter until E18. At E18, both the IML region and the ventral horn are innervated and 5-HT synapses are present. By E19, 5-HT fibres appear in the region around the central canal. By postnatal day 1 (P1), fibres exist throughout the intermediate zone and begin to innervate the marginal zone of the dorsal horn. It is of interest to note that even at P30, 5-HT positive fibres are localized predominantly in the central and ventral grey matter, perhaps indicative of their role in motor control (Tanaka et al.1992). The developmental changes in 5-HT responses on spinal motoneurons during development were studied *in vitro* using isolated spinal cords from embryonic and neonatal rats (Ziskind-Conhaim et al. 1993). These authors reported that at E16-E17, 5-HT evoked slow rising depolarizations which disappeared by E18. At E18, 5-HT generated long lasting tetrodotoxin (TTX) and Mg^{2+} insensitive depolarizations which resulted in an average increase in motoneuron input resistance of about 11%. These authors suggested that the increase in input resistance is likely due to a decrease in K^{+} conductance. Fast-rising high frequency potentials were also evoked by 5-HT and could be blocked by glutamate, GABA, and glycine antagonists. These responses persisted until birth upon which motoneuron responses to 5-HT increased with average depolarizations of about 19 mV and repetitive firing of action potentials also occurring. Pharmacological analysis revealed that motoneuron depolarization and induction of synaptic potentials were mediated by multiple 5-HT receptor subtypes.

Noradrenergic development has been studied using antisera to dopamine-β-hydroxylase and noradrenaline. Development of the noradrenergic innervation of the lumbar spinal cord has been shown to begin on embryonic day 18 (E18) with individual fibres invading the ventral grey matter from the ventrolateral funiculus (Aramant et al. 1986; Rajaofetra et al. 1992). By postnatal day 0 (P0), noradrenergic fibres are found throughout the intermediate zone and ventral horn. In the dorsal horn, noradrenergic fibres do not appear until (P3) when they begin to enter the

marginal zone. By P3 the densest population of noradrenergic fibres exists in the motor nuclei of the ventral horn and the ventrolateral funiculus white matter. At this time, there exists a dense fine network of noradrenergic fibres throughout the entire length of the spinal cord. 5-HT and NA positive fibres exist well before specific functional roles begin and it has been suggested that there may be a trophic role on the part of these fibres in the development of the spinal cord (Rajaofetra et al. 1992).

Receptors to 5-HT and NA exist within the spinal intermediate zone.

There are several different classes of 5-HT receptors which have been classified based on operational, transductional, and structural characteristic (for review see Martin and Humphrey 1994). Receptor classes found in the intermediate zone of the rat spinal cord are the 5-HT_{1a}, 5-HT_{1b}, 5-HT_{2a} and 5-HT_{2c} receptor subtypes (Marlier et al. 1991; Thor et al. 1993). More specifically, 5-HT_{1a} receptors are primarily located around the central canal (lamina X). 5-HT_{1b} receptors are more widely distributed and exist throughout the cord with the exception of the motor nuclei (lamina IX). 5-HT_2 receptors are found in the ventral half of the spinal cord (laminae VII- IX) and sparsely throughout intermediate laminae V- VI. It is interesting to note that in such a heterogeneous population of cells there exists a differential distribution of 5-HT receptor subtypes.

Currently adrenergic receptors are divided in three broad categories, α_1-, α_2-, and β-adrenergic receptors each coupled to a particular intracellular signal transduction system (Bylund et al. 1994). Each category contains several receptor subtypes based on different binding affinities and pharmacological profiles. In situ hybridization studies in the rat spinal cord have shown that β_2-receptor mRNA is not notably expressed in the spinal cord while β_1-adrenoceptor mRNA is expressed mainly in neurons of lamina VII dorsal to the central canal (Nicholas et al.

1993). The α_2-adrenergic and α_1-adrenergic receptors are diffusely distributed throughout the intermediate zone although α_2-adrenergic receptors are more dense around the central canal (lamina X) (Roudet et al. 1993, 1994)

Different cellular effector systems have been found to be associated with different 5-HT and NA receptor subtypes. The 5-HT_1 , and α_2-adrenergic receptor class has been found to be negatively coupled to the adenylate cyclase enzyme. That is to say that activation of the α_2-adrenergic or 5-HT_1 receptors will lead to decreased levels of the second messenger 3',5' cyclic-adenosine monophosphate (cAMP) inside the cell. Conversely, the β-adrenergic receptor is coupled positively to adenylate cyclase and upon activation, results in an increase of cAMP levels inside the cell. The α_1-adrenergic and 5-HT_2 receptor class is coupled to the enzyme phospholipase C. With binding at these receptors, secondary messengers inositol triphosphate (IP_3) and diacyl glycerol (DAG) are produced. The production of intracellular messengers is an important event by which a receptor may mediate cellular processes and it may be the mechanism by which 5-HT and NA modulate EAA-evoked currents (for review see Fillenze 1990; Bylund et al. 1994; Martin and Humphrey 1994).

Excitatory amino acid neurotransmission

L-glutamate is the major excitatory neurotransmitter in the CNS. Glutamate receptors are divided into two classes. The metabotropic receptors are coupled to a G-proteins and receptor binding of the ligand activates intracellular second messenger systems. Ionotropic glutamate receptors make up the second class. These receptors are coupled to ion channels and are divided into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and kainate subtypes. L-glutamate is currently considered a mixed agonist having

actions at metabotropic and both NMDA and non-NMDA (AMPA/kainate) ionotropic receptor subtypes.

Ionotropic glutamate channels have been described as non-specific cation channels and are permeable to monovalent cations such as Na^+ and K^+. Although Na^+ and K^+ are the major current carriers through NMDA channels, the NMDA channel is also considerably permeable to Ca^{2+} (for review see Ascher and Johnson 1994). Calcium influx via NMDA channels has been implicated in cellular events such as synaptic integration and plasticity.

In 1987, Johnson and Ascher discovered that the presence of glycine in sub-micromolar concentrations potentiates the current evoked by NMDA. This glycine dependency is strychnine insensitive and is a result of glycine binding to a specific site on the NMDA channel itself. Further study of the effects of glycine on NMDA channels expressed in *Xenopus* oocytes revealed that glycine is essential for channel function and that glycine binding manifests itself as an increase in the open frequency of NMDA channels (Kleckner and Dingledine 1988). In the absence of extracellular glycine, no noticeable NMDA evoked current were observed yet addition of 3 μM glycine (EC_{50} = 670 nM), resulted in large NMDA-evoked currents (10-40 nA). The NMDA receptor appears to be the only ligand gated channel that requires two different agonists for activation. The AMPA and kainate channels do not possess such a site and are unaffected by coapplication of glycine.

The depressant actions of Mg^{2+} on the NMDA receptor were first described in 1980 by Ault and colleagues. In 1980, MacDonald and Wojtowicz described a region of negative slope conductance on the I-V plot for current evoked by NMDA, D-L-homocysteic acid (DL-HCA), and L-aspartate. They found that as the membrane potential increased (i.e. hyperpolarized) below -40 mV, the corresponding conductance decreased which is contradictory to Ohm's law. This phenomenon has previously been termed a negative slope conductance and has been implicated

in rhythmic oscillations of bursting neurons (Wilson and Wachtel 1974). This negative slope occurred only when Mg^{2+} was present in the extracellular environment and was dependent on membrane potential (Nowak et al. 1984). At membrane potential greater than (i.e. hyperpolarized) about -30 mV, Mg^{2+} molecules appear to become trapped in the channel thus preventing ion flow. Mg^{2+} blockade does not occur in the absence of extracellular Mg^{2+} or at potentials less than (i.e. depolarized) -30 mV. The conductance which is sensitive to the Mg^{2+} block has a value of approximately 50 pS. It has been suggested that the Mg^{2+} block phenomenon gives the NMDA channel a more important role in modulating synaptic pathways while the non-NMDA receptors function as the primary fast neurotransmitter. The conductance evoked by non-NMDA receptor activation does not exhibit this phenomenon and has a lower conductance level of approximately 10-15 pS. Extracellular Mg^{2+} ions have also recently been reported to increase glycine binding affinity in rat hippocampal neurons (Wang and MacDonald 1995). These authors reported that Mg^{2+} potentiates NMDA-evoked currents at positive membrane potentials and reduces desensitization of the receptor by allosterically interacting with the glycine binding site. This demonstrates another possible mechanism that Mg^{2+} may modulate the NMDA receptor.

The Mg^{2+} block and the Ca^{2+} permeability of the NMDA receptor are properties which make it an important component in rhythm generation. An attractive mechanism by which NMDA receptor activation functions to generate rhythmic bursting has previously been presented (Wallen and Grillner 1987; Hu and Bourque 1992). At resting membrane potential, activation of the NMDA receptors cause very little depolarization. Perhaps non-NMDA receptors may contribute to the depolarization. At membrane potentials around -40 mV, the voltage dependent Mg^{2+} block is removed and Na^{+} and to a lesser extent Ca^{2+}, rush into the cell via NMDA channels as well as voltage dependent Na^{+} and Ca^{2+} channels. Although not as much Ca^{2+} and Na^{+} enters the cell, the rise in intracellular Ca^{2+} activates a calcium dependent potassium current ($I_{K(Ca)}$) which functions to repolarize the membrane. As the membrane repolarizes past -40 mV,

the Mg^{2+} block of the NMDA channel returns further contributing to the repolarization of the membrane. The repolarization of the membrane to resting membrane potential stops the Ca^{2+} entry into the cell. As the intracellular calcium is sequestered into intracellular stores, the calcium dependent potassium conductance inactivates and the regenerative cycle is ready to begin again.

NMDA and non-NMDA receptors have been reported to be affected by second messenger systems. It was observed by MacDonald and colleagues that when using whole cell patch clamp recording in cultured hippocampal neurons, NMDA-evoked current decreased upon breakthrough into the cell (MacDonald et al. 1989). The current decreased to a plateau level of about 50% of the initial value within the first 15 minutes. It was later concluded that this behaviour was not due to an increase in intracellular calcium but rather to the intracellular dialysis occurring between the cell and the micropipette (Wang 1993). Due to the large size of the pipette tip, there was extensive diffusion between the cell and the volume of the micropipette which was infinitely larger. As a result, high energy compounds such as adenosine triphosphate (ATP) and guanosine triphosphate (GTP) were diffusing out of the cell causing the apparent rundown of NMDA-evoked currents but not the currents evoked by non-NMDA agonists. This suggested that protein phosphorylation modulated the NMDA receptor but not the non-NMDA receptor (MacDonald et al , 1989). It was later discovered by the same group that AMPA/kainate-evoked currents did exhibit rundown (Wang et al 1991). Application of the catalytic subunit of protein kinase A (PKA) potentiated responses to AMPA and kainate while inhibiting PKA caused an attenuation of AMPA/kainate-evoked currents. It was revealed that in the first study, (MacDonald et al. 1989) the electrode tip size was much smaller. By using electrodes with a larger tip diameter, non-NMDA-evoked currents also exhibited rundown similar to currents evoked by NMDA. This would indicate that although non-NMDA-evoked currents do rundown, there is a longer time course. Similarly, Randic and colleagues have also

reported that EAA-evoked currents could be modulated by the PKA/cAMP cascade in an acutely isolated spinal dorsal horn neuron preparation (Cerne et al. 1992, 1993). These authors found that NMDA-evoked currents and synaptic responses were potentiated by perfusing with 8-bromo-cAMP or by including cAMP or the catalytic component of PKA in the recording electrode. Studies of the effects of activating adenylate cyclase on responses to glutamate and kainate have shown an increase in the opening frequency and mean open time of non-NMDA glutamate channels (Greengard, 1991). These studies all suggest that EAA channels can be modulated by cAMP-dependent phosphorylation.

The protein kinase C (PKC) second messenger system has also been reported to potentiate currents evoked by NMDA by decreasing the voltage dependent Mg^{2+} block of the NMDA channel (Chen and Huang 1992). Other studies have shown that AMPA/kainate receptors are potentiated by application of the catalytic subunit of PKC and inhibited by application of the inhibitory subunit in a concentration dependent manner (Wang et al 1994). That is, at low concentrations of kainate the catalytic subunit of PKC attenuated the kainate-evoked current while at high concentrations of kainate the subunit potentiated the current. This study demonstrated that application of okadaic acid, a phosphatase inhibitor, also caused a response similar to that of PKC. This implicated protein phosphatases in the regulation of EAA receptors by second messenger systems. These results all suggest that EAA receptors can be modulated by second messenger systems. This may be a mechanism by which 5-HT and NA modulate excitatory transmission to reconfigure neural networks.

5-HT and NA can modulate motor activity

In 1963, Lundberg and colleagues reported that intravenous administration of the NA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) to an acute spinal cat depressed transmission in the short latency flexor reflex afferent (FRA) pathway while increasing the flexor reflex to a

skin pinch. These effects were antagonized by the α-adrenergic antagonist phenoxybenzamine. L-DOPA was also found to reduce excitatory and inhibitory transmission from the FRA to both ascending tracts and motoneurons (Anden et al. 1963). In a subsequent study, the inhibition of the short latency FRA component by L-DOPA revealed a longer latency component which was released from tonic inhibition (Anden et al 1966a). The increased flexor reflex was attributed to the longer latency component. A pharmacological analysis of the L-DOPA effects revealed that blocking the first enzyme in the synthesis of dopamine (DA) from L-DOPA, the actions of L-DOPA were blocked (Anden et al. 1966b). Similar results were found when blocking the formation of NA from DA. These authors concluded that the L-DOPA was converted into NA by terminals in the spinal cord and modulated the FRA pathway upon release from noradrenergic terminals.

Similarily, intravenous administration of the 5-HT precursor, 5-hydroxytryptophan (5-HTP) also inhibited transmission from the FRA pathway to primary afferents, motoneurons and ascending pathways, and increased motoneuron excitability as seen by an increased VR discharge (Anden et al. 1964). Adrenergic antagonists had no effect on blocking these effects which implied that 5-HT was working on a different receptor than the L-DOPA. Similar work with 5-HTP revealed that 5-HTP markedly increased the size of the monosynaptic reflex (MSR) while depressing polysynaptic and dorsal root reflexes (Anderson and Shibuya 1966). They also reported an increased ventral root discharge rate which correlated with the increase in magnitude of the MSR. The actions of L-tryptophan were also examined and it was revealed that L-tryptophan caused an increase in both monosynaptic and polysynaptic responses. They proposed that the difference in actions of 5-HTP may be because the 5-HT was being taken up by noradrenergic neurons and converted into 5-HT wheras L-tryptophan could only be converted into 5-HT by serotonergic neurons. Thus the effects of L-tryptophan were said to more closely

mimick the effect of 5-HT. These studies of L-DOPA and 5-HTP on the FRA pathway were landmark studies in that they deomstrated how precursors of monoamines from descending terminals could modulate spinal pathways.

Locomotion is a complex behaviour in which the coordination between multiple oscillators is essential for coordinated movement (for review see Grillner et al 1991; Grillner and Matsushima 1991). Modulatory substances with effects in the spinal cord could alter coordination between oscillators of different limbs. NMDA receptor activation has been implicated in inducing locomotion in preparations such as Xenopus embryos (Dale and Roberts 1985), the lamprey (Grillner 1981), the neonatal rat (Smith and Feldman 1987), and cat (Douglas et al. 1993), but both 5-HT and NA have been shown to be important agents in initiating and modulating these motor patterns. Recently, by using of chronically implanted microdialysis probes there has been direct evidence that 5-HT and DA are released in the spinal cord during locomotion (Gerin et al. 1995). Barbeau and Rossignol (1991) used the spinal cat preparation to study the effects of 5-HT, NA and DA on locomotion. This model was used because no monoaminergic terminals could be found past the lesion site yet the postsynaptic receptors still persisted after the lesion. Intrathecal application of monoaminergic agonists or antagonists allowed an assessment of the drug effects without the effect of other transmitters contaminating the results. They found that intrathecal administration of clonidine (an α_2-agonist) or L-DOPA induced treadmill locomotion in acutely spinal cats. This was specific to the noradrenergic system as 5-HT and DA agonists could not induce locomotion. However, a chronic spinal cat is capable of some locomotion several weeks post lesion and locomotor rhythms can be modified by administration of noradrenergic, serotonergic and dopaminergic agonists. These all have fairly specific actions on locomotion. Clonidine administration increased step cycle duration, and prolonged flexor and extensor electromyogram (EMG) burst duration. Administration of a

serotonergic agonist quipazine resulted in an increased step cycle duration and also increased the duration and amplitude of EMG activity in extensors, flexors, and axial muscles. Dopaminergic agonist injection resulted in increased flexor EMG activity which often resulted in sustained flexion which blocked locomotion. Co-application of clonidine and quipazine resulted in combined effects of both agents in promoting the locomotor pattern. These results support the influence of 5-HT and NA in promoting locomotion in the cat.

The *in vitro* neonatal rat spinal cord preparation has revealed that pattern generators underlying locomotion can be modulated by monoamines to yield different motor outputs. It has been reported that slow frequency discharges from ventral roots occur in the presence of NMDA and 5-HT (Cazalets et al. 1990). Addition of NA to the bath evoked a high frequency motor rhythm which was superimposed over the slow rhythm. In preparations with the hindlimbs attached, alternating limb movement only occurred during the slow frequency pattern generated by 5-HT while the high frequency pattern only resulted in fast muscle contractions without any hindlimb movement. The effects of 5-HT and NA had relatively long washout times and it was suggested that they may exert their actions by way of intracellular transductory processes. Subsequent study of the slow rhythmic pattern using pharmacological antagonists revealed that the slow pattern was slowed down further by 5-HT_2 antagonists and blocked by non-specific 5-HT antagonists. This would imply that a mix of 5-HT receptor subtypes is necessary to generate locomotor like activity (Cazalets et al 1992). In the same model, it has been shown that different putative locomotion-inducing substances evoked different motor patterns (Cowley and Schmidt 1994). A combination of acetylcholine (ACh) and edrophonium (EDRO) typically resulted in rhythmic alternation of left and right hindlimb electroneurograms (ENGs) although interlimb flexor and extensor bursts were in phase. N-methyl-D,L-aspartate (NMA) sometimes produced the same pattern as well as other non-locomotor patterns. Conversely 5-HT was usually effective in generating locomotor-like patterns in the hindlimbs with interlimb flexor and extensor ENGs

out of phase. The ENG recordings of NMA and ACh/EDRO rarely resulted in patterns consistent with locomotion in intact rats. These authors concluded that 5-HT was the best single agent of those tested for inducing locomotor-like patterns in the neonatal rat.

Aminergic modulation has also been extensively studied in the somatogastric ganglion of the crustacean (Flamm and Harris-Warrick 1986; for review see Harris-Warrick and Flamm 1986). The somatogastric ganglion contains only about 30 neurons which generate two rhythmic stomach movements called the gastric and pyloric rhythms. The central pattern generator responsible for the pyloric rhythm contains only about 14 neurons in 6 different classes yet studies of the effects of DA, 5-HT and octopamine reveal different motor patterns resulting from modulation of the pyloric cicuit. All three of these agents induce bursting pacemaker potentials in one class of neuron (the AB neuron). All three amines also evoke oscillations with different ionic mechanisms which result in different characteristic shapes. The oscillations induced by dopamine are TTX resistant while the serotonin and octopamine-induced oscillations are blocked. Conversely the oscillations induced by dopamine are abolished in a low Ca^{2+} solution. Of interest to note is that another class of neuron (the PD neuron) is not a direct target of serotonin yet shows increased burst activity with 5-HT due to the fact that the PD neuron is electrotonically coupled with the AB neuron which is activated by 5-HT. These authors remind us that the actions of a neuromodulator must be viewed in context with all connections intact to view the final result of the modulation.

The development of the effects of 5-HT have also been examined in two amphibians, *Xenopus* and *Rana temporaria.* In *Xenopus* embryos, it was reported that the actions of 5-HT on motor rhythms develop in a rostrocaudal fashion. Bath application of 5-HT dramatically increased the ventral root discharge in rostral segment but not caudal segments of a hatchling *Xenopus* larvae. Twenty-four hours after hatching 5-HT increased the duration of ventral root discharge in caudal segments as well. These effects were subsequently found to exhibit a $5\text{-}HT_{1a}$-

like receptor pharmacology (Scrymgeour-Wedderburn and Sillar 1993). In *Rana*, 5-HT receptor activation results in increased burst duration and intensity of ventral root discharge (Woolston et al 1994). In neurons which are rhythmically active during locomotion, 5-HT is required for NMDA induced intrinsic membrane oscillations to occur (Sillar and Simmers 1993). These oscillations are dependent on Mg^{2+} and are abolished by NMDA receptor antagonists. It is hypothesized that the mechanism of 5-HT action in *Rana* is through second messenger systems which modulate the NMDA receptor itself at the agonist docking site or possible the Mg^{2+} binding site. These authors suggest that an interaction between 5-HT and NMDA receptors occurs which results in the expression of membrane oscillations which may contribute to locomotor behaviour in amphibians.

Locomotion in the lamprey evoked by NMDA or D-glutamate has also been modulated by 5-HT (Christenson 1989; Harris-Warrick and Cohen 1985). 5-HT has been observed to reduce the frequency of ventral root discharge, enhance the intensity of the burst discharge and prolong the intersegmental phase lag. Grillner and colleagues have suggested that the mechanism by which 5-HT modulates this network is by reducing the late AHP component of action potentials in spinal neurons (Van Dongen et al 1986). This component of the AHP is a result of the current evoked by $I_{K(Ca)}$ and has subsequently shown to be depressed by 5-HT (Wallen et al. 1989). This 5-HT-mediated reduction of $I_{K(Ca)}$ results in an increased spike frequency evoked by an excitatory stimulus and has recently displayed a pharmacology consistent with a 5-HT_{1a}-like receptor (Wikstrom et al 1995). Thus 5-HT functions to increase the gain of the neuron. Administration of 5-HT_{1a} receptor agonists mimic the effect of 5-HT yet decreasing intracellular levels of cAMP (as would happen with a mammalian 5-HT_{1a} receptor) has no effect in reproducing the effects of 5-HT. These author concluded that the 5-HT receptor responsible for the actions of 5-HT in the lamprey shares some common features to the mammalian 5-HT_{1a} receptor but not the same

intracellular mechanism. According to this hypothesis, 5-HT can potentiate activity in any neuron where the AHP is affected.

NA has also been shown to block the action of $I_{K(Ca)}$ mediated AHPs in rat pyramidal cells (Madison and Nicholl 1986a). These authors reported that this behaviour is mediated by β-adrenergic receptor activation and also results from an increase in intracellular cAMP levels as a mammalian β-adrenergic receptor should (Madison and Nicholl, 1986b). Study of 5-HT revealed a decrease in the slow AHP in pyramidal neurons by decreasing $I_{K(Ca)}$, although the authors could not attribute this effect to a particular receptor subtype. The 5-HT response was described as being biphasic with a hyperpolarization followed by a depolarization. The hyperpolarizing component was mediated by receptor of the $5\text{-}HT_{1a}$ subtype while the depolarizing component was not due to a reduced $I_{K(Ca)}$ and could not be attributed to a specific receptor subtype (Andrade and Nicholl 1987). These studies demonstrate that the mechanism that 5-HT and NA may affect the slow AHP may be related to second messenger systems.

Another phenomenon which should be addressed is the generation of plateau potentials by 5-HT in turtle (Hounsgaard and Kiehn 1985), crustacean (Kiehn and Harris-Warrick 1992), and cat (Hounsgaard and Kiehn 1989) spinal motoneurons. These plateau potentials may contribute to burst-like behaviour seen in spinal motoneurons during motor behaviours. Motoneurons exposed to a depolarizing stimulus in the presence of 5-HT will express bistable membrane characteristics and move to a higher stable membrane potential which can be turned off by a brief hyperpolarizing stimulus. Plateau potentials can not be induced in the absence of 5-HT and are Ca^{2+} dependent, being abolished when Ca^{2+} is replaced with Co^{2+} or Mn^{2+}. They are also sensitive to nifedipine, an L-type Ca^{2+} channel blocker, implicating Ca^{2+} channels in their maintenance. 5-HT has also been shown to attenuate $I_{K(Ca)}$. It was suggested that 5-HT induces plateau potentials by decreasing $I_{K(Ca)}$ and thus decreasing the slow AHP. Apamin is a $I_{K(Ca)}$

channel blocker which promotes Ca^{2+} mediated plateaus in the turtle (Hounsgaard and Mintz 1988). In the crustacean stomatogastric ganglion, 5-HT has been shown to induce plateau potentials in cells involved in foregut movement (Kiehn and Harris-Warrick 1992). In this study, stimulation of the gastropyloric receptor cell (a serotonergic/ cholinergic sensory neuron which projects to the dorsal gastric motoneuron.) resulted in a nicotinic EPSP which induced a plateau potential in the dorsal gastric motoneuron. Local application of 5-HT to the dorsal gastric motoneuron caused a TTX resistant slow depolarization which mimicked the non-nicotinic plateau induction of gastropyloric cell stimulation. These authors concluded that the 5-HT application conferred the ability to evoke a plateau potentials by modulation of multiple conductances while ACh provides a rapid depolarization to trigger the plateau potential. This demonstrated that synergistic actions of two neuromodulators can result in the generation of plateau potentials. The generation of plateau potentials in spinal motoneurons demonstrates another locale in the spinal cord where 5-HT functions to promote motor activity.

Studies in the cat and rat have implicated 5-HT and NA in mediating reflex responses. Jankowska and colleagues demonstrated in the cat model that NA and 5-HT can depress transmission in group II but not group I afferent fibre pathways in the intermediate and ventral horn and occasionally in the dorsal horn (Bras et al 1989). Jankowska and colleagues have described a population of interneurons involved in locomotion which are located in the spinal intermediate zone and receive strong group II afferent input (Edgley and Jankowska 1987; Edgley et al. 1988). Some of these neurons have also been shown to be rhythmically active during locomotion (Shefchyk et al 1990). Pharmacological studies have revealed that noradrenergic attenuation of group II field potentials in the intermediate zone and ventral horn was mediated by α_2-adrenergic receptors while 5-HT mediated attenuation of group II afferent responses in the dorsal horn was due to multiple 5-HT receptor activation (Bras et al 1990). In a

subsequent study, attempts to mimic these effects by stimulating the serotonergic and noradrenergic brainstem nuclei which project to the spinal cord also resulted in depression of group II field potentials in mid-lumbar spinal segments (Noga et al. 1992; Skoog and Noga 1991). Thus in a population of neurons active during locomotion, 5-HT and NA depress transmission from sensory afferents to interneurons in the spinal cord. This provides another example of how 5-HT and NA function to attenuate sensory information processing. This will be addressed later in the discussion.

The role of 5-HT and NA on descending inhibition has been studied using the *in vitro* neonatal rat spinal cord. In the neonatal rat, descending inhibitory pathways develop after birth (Gilbert and Stelzner 1979). From P1 to P0, stimulation of thoracic cord results in an inhibition of the MSR (Miyata et al 1987). This effect declines over the first few months of life and has been attributed in part to actions of 5-HT (Yomono et al 1992). It seems that the role of 5-HT in spinal reflexes is variable. Few have succeeded in attributing a specific receptor subtype to reflex modulation by 5-HT. It has been reported that inhibition of reflex responses is via 5-HT acting a 5-HT_{1a} receptors although they also suggested that the inhibition of the MSR could be due to 5-HT acting at a mixture of 5-HT receptor subtypes (Crick and Wallis 1991). Another study found that attenuation of the MSR was mediated by 5-HT but not NA or DA (Yomono et al 1992). They were unable to assign a particular 5-HT receptor subtype to this effect. It was subsequently concluded that 5-HT acting at 5-HT_2 receptors was the principal mediator of inhibition of the MSR and important in inhibiting the polysynaptic reflex (PSR) (Wallis et al 1993). They concluded that NA was not involved in descending inhibition of the neonatal rat, yet they found that clonidine application (an α_2 -adrenergic agonist) resulted in a potent inhibition of slow reflex responses (Wallis et al. 1993). It is interesting to note that of all of these studies, Wallis et al. (1993) used the youngest rats (P1 to P2). Crick and Wallis used three to eight day old rats and

Yomono et al. used one to five day old rats. As we know descending inhibition develops after birth. Perhaps Wallis et al. were successful in finding a 5-HT receptor subtype because of the young age of the animal and after time, possibly more complex interactions develop which complicate pharmacological studies. It would not be surprising to see age-dependent factors when studying animals at such an active developmental stage.

Application of 5-HT has been shown to directly depolarize rat spinal motoneurons (Takahashi and Berger 1990). Under voltage clamp conditions, 5-HT evokes a membrane current called $I_{5\text{-}HT}$. This current has been attributed to a K^+ conductance which exhibits characteristics similar to the inward rectifier current. An unpublished observation from this study was that 5-HT also enhanced low voltage activated Ca^{2+} currents. This is consistent with the studies of 5-HT in generating plateau potentials which implicated a Ca^{2+} current in the maintenance of plateaus. Another study found that 5-HT generally results in a depolarization of spinal motoneurons (81%) but occasionally 5-HT application results in a hyperpolarization (9%)(Wang and Dun 1990). Efforts to determine the pharmacology of the effects mediated by 5-HT have been quite variable. It has been suggested that the variability is from so many different preparations and recording techniques used (Wallis et al. 1991). It was suggested by Wang and Dun that 5-HT decreases a K^+ conductance by a $5\text{-}HT_2$ receptor to evoked the depolarization and increases a K^+ conductance by a $5\text{-}HT_{1a}$ receptor to evoke the hyperpolarization. Some groups have simply attributed the effects to a 5-HT receptor unlike any that has been characterized (Connell and Wallis 1989). The common aspect of these studies is that they agree that the mechanism that 5-HT depolarizes motoneurons is by decreasing a K^+ conductance (Wang and Dun 1990; Wallis et al 1991). Both 5-HT and NA have been shown to induce depolarizations in spinal neurons of the hemisected rat spinal cord preparation (Elliot and Wallis 1992). These depolarizations evoked by NA displayed a pharmacology consistent with α_1-adrenoceptors and those evoked by 5-HT have been

demonstrated to be due to a 5-HT_2 receptor. These results would implicate receptors coupled to IP_3/DAG second messenger system in the increased neuronal excitability evoked by 5-HT and NA.

Another way that 5-HT and NA may modulate neural networks is by altering the efficacy of EAA neurotransmission. Many studies have reported actions of 5-HT and NA on EAA evoked responses throughout the nervous system. In 1966, Engberg and Ryall reported that NA and 5-HT had inhibitory actions on neurons in the lumbar cat spinal cord manifesting as a decrease in spontaneous firing, synaptic responses or of firing induced by application of DL-HCA. A study of the inhibitory effects of NA revealed that NA depressed DL-HCA-induced firing in 5/9 cells and had a negligible effect on Renshaw cells. 5-HT also displayed a similar inhibition of spinal cord neurons although it was not as extensively studied due to problems at the time of getting 5-HT into solution. 5-HT did reduce the rate of firing evoked by DL-HCA in 13/48 neurons. They reported no actions of 5-HT on Renshaw cells or motoneurons. One interesting point to note is that not all cells tested were sensitive to NA yet there was a correlation between densities of NA containing terminals and the location of NA sensitive cells. In the intermediate zone they found both cells which were depressed by NA and cells which were not possibly representing the heterogeneity of the population. One limitation of this study was that in using an intact spinal cord, one could not discern whether observed effects were due to a primary action of NA on the neuron in question or a secondary action on another neuron connected to it. These authors also stated that the proportion of neurons affected by NA was not accurate because in using microelectrode recordings, larger cells are preferentially impaled. This unfortunately excluded smaller cells from their study. In the ventral horn of the cat spinal cord, noradrenergic fibres closely oppose small interneurons situated in the motor nuclei (Jordan et al. 1977). Responses induced by L-glutamate were inhibited in 68% of these neurons by coapplication of NA. The

remaining 32% were unaffected. These authors proposed that NA in the ventral horn functions to inhibit input to the motoneurons by actions on neighboring interneurons.

It is now accepted that excitatory transmission and neuronal excitability may be subject to modulation by other neurotransmitters. This may occur by one neurotransmitter, such as 5-HT or NA activating a second messenger system which in turn affects the membrane channels controlled by a different neurotransmitter such as L-glutamate. 5-HT and NA have been shown to affect EAA responses in different parts of the CNS. In the spinal cord dorsal horn, it has been demonstrated that 5-HT selectively attenuates currents evoked by NMDA in acutely isolated cells (Murase et al. 1990). They noticed that 5-HT depressed NMDA responses via a 5-HT_{1a}-like receptor in 52% of cells, potentiated NMDA responses in 8% and had no effect on the remaining 40%. The mixed responses to 5-HT reflect the heterogeneity of their cell population. In the cerebellum, 5-HT consistently supressed glutamate, NMDA and kainate-evoked currents in Purkinje cells implicating a strong role in modifying cerebellar output (Hicks et al. 1989). Oddly enough these authors reported that the currents evoked by NMDA were the least affected by 5-HT. Perhaps this may reflect an emphasis on fast excitatory transmission in the cerebellum. The depressant effects of 5-HT were subsequently studied on all EAA-evoked currents in the cerebellum (Netzeband et al. 1993). These authors reported similar results as (Hicks et al. 1989) for responses evoked by glutamate and kainate as well as minimal modulation of AMPA receptors. Metabotropic glutamate receptors were entirely unaffected by 5-HT. This group attributed the attenuation to the 5-HT_{1a} receptor subtype which is the dominant 5-HT receptor subtype in the cerebellum. Whereas it appears that 5-HT functions only as an inhibitory neuromodulator in the cerebellum, NA has both inhibitory and excitatory effects (Mori-Okamoto 1991). The excitatory actions of NA have been attributed to β-adrenoceptors while the inhibitory effects are mediated by α_2-receptors. In cat neocortical slices, 5-HT has been reported to

potentiate responses evoked by L-glutamate, NMDA and quisqualate (Nedergaard et al 1986, 1987). This group also reported that responses evoked by NMDA could also be potentiated by NA. A similar study in the rat revealed that 5-HT potentiated NMDA-evoked responses (possibly by a 5-HT_2 receptor), while quisqualate responses were unaffected (Reynolds et al. 1988). This evidence suggests that modulatory actions of 5-HT may not affect all EAA receptors between species.

Spinal motoneurons have also been a target of modulation by 5-HT. Activity evoked by glutamate has been potentiated in rat spinal motoneurons by 5-HT_{1b}, and 5-$HT_{2a/2c}$ receptor subtypes while 5-HT_{1a} receptor activation resulted in an attenuation of the activity (White and Neuman 1983; Jackson and White 1990). Recently, Smith and colleagues reported a potentiation of kainate evoked currents by DA in cultured chick motoneurons (Smith et al. 1995). Application of dopamine caused a potentiation of kainate-evoked currents by a D1 receptor which is positively coupled to adenylate cyclase causes. It was suggested that phosphorylation of the kainate ionophore by DA binding results in an increase channel opening probability and larger kainate-evoked currents. These studies all suggest that the function of EAA receptors can be modulated by the actions of monoamines thus resulting in a finer control of ionophores and the currents generated by them.

As an overview, it is evident that 5-HT and NA have diverse effects on motor activity. Difficulty in discovering which specific receptor subtypes mediate aspects of motor control reflects the complex interactions that these two monoamines have in the spinal cord. One mechanism that 5-HT and NA action may affect motor control is by modulating membrane currents evoked by EAAs. It would be beneficial to study the interactions of 5-HT and NA in the spinal cord to elucidate possible mechanisms by which they may affect motor behaviours such as locomotion

The preparation

An acutely isolated cell preparation has been chosen to study the modulation of EAA channels by 5-HT and NA. There was need for a preparation that would allow for many agents to be tested and washed out rapidly as well as being synaptically isolated from other cells. There was no need to examine circuitry involved so a dissociated cell preparation seemed the ideal choice to use in this study.

Dissociated cells are free from any synaptic interference from neighboring cells. One can be positive that the responses and changes are due to the drug applications and not to extrinsic input from other cells. This preparation also lacks diffusion barriers from neighbouring cells and extracellular matrix that would be present in a slice or whole cord preparation. This allows for rapid application and washout of many different agents. Likely occurrences when using the voltage clamp technique are 'space clamp' problems. Large cells or cells with extensive dendritic arborizations might be difficult to 'clamp' at a given holding potential. This problem manifests itself as delayed responses to voltage steps or as currents activating at membrane potentials where they should not be activated. In using a dissociated cell preparation, most of the processes are removed during the isolation procedures thus eliminating much of the cell surface area and the space clamp problems associated with it.

Whole cell voltage clamp recording was employed in this study (Hamill et al. 1981). The whole cell patch clamp technique on isolated cells offers the advantage of increased access to the interior of the cell. The diameter of the electrode tip (approx. 1-2 μm) offers a low access resistance to the interior of the cell relative to a conventional sharp electrode, thus making it possible to manipulate the intracellular components of the cell. By diffusion of the electrode

solution into the cell, the investigator can be aware of exactly what the intracellular constituents of the cell are and include agents in the electrode solution to block ionic conductances. In this study intracellular K^+ was replaced with Cs^+ to block most K^+ conductances. This was done to be certain that the attenuation of EAA-evoked currents was not due to rundown of K^+ conductances which rundown rapidly upon achieving the whole-cell configuration. Manipulating the intracellular solution may seem powerful but there are also disadvantages to this application. Cellular components such as high energy phosphates (ATP, GTP) will leak out of the cell into the micropipette causing "rundown" of some membrane currents in a matter of minutes (Wang et al. 1993). To avoid difficulties in interpreting the data due to rundown, the test drug responses were "bracketed" by control drug responses. This will be discussed in more detail in the methods section.

Spinal cords from neonatal rats (P1-P4) were used because they were young enough that the extracellular matrix and connections of the spinal cord was still quite immature. This facilitated dissociation and reduced the time needed to incubate in digestive enzymes. This age was also used because descending serotonergic and noradrenergic projections have reached the lumbar cord by this point (Rajaofetra 1989, 1992). The immaturity of the extracellular matrix also permitted more diffusion of O_2 through the tissue than in an adult. As there was typically about four hours between slicing and dissociation, this was a factor to consider when trying to keep the tissue healthy. Lower temperatures of the *in vitro* preparation (around 25°C) decrease the O_2 requirement of the cell while increasing the amount of O_2 dissolved in solution (Forsythe and Coates 1988). As advantageous as this may seem, a limitation to this is that cellular components such as enzymes, certain ion channels and second messenger systems will not function at those levels seen around normal body temperature (37 °C).

The actual dissociation procedure used to isolate the cells also has some drawbacks. During the enzymatic stage of dissociation, proteolytic enzymes must be rigorously controlled lest they digest some cell surface proteins such as receptors and ion channels. Also, regardless of how gentle, the mechanical stage of dissociation is still very traumatic on the tissue and results in the death of many cells. Perhaps mechanical dissociation also selectively spares certain sizes of cells over others. This might complicate results in that one might be unconsciously selecting for a particular population of neurons. Another possibility is that receptor distribution may change upon dissociation. As connections are broken between cells, the receptors concentrated at a particular location may spread over the cell surface. For the purposes of this study, this does not seem to be a complicating factor as we are studying modulation of ion channels, not whole network properties.

It has also been suggested that the relevance of a cell in isolation with most processes removed it is difficult to see in relation to an intact spinal cord. In a neural network where so many interactions are occuring within and between cells it is difficult to imagine that monoaminergic modulation of glutamate receptors on an isolated sphere of cytoplasm may contribute substantially to reconfiguring a particular pattern generator. In defence of this, there are many cellular processes going on within an individual cell which may contribute substantially to its function in a network. It is impossible to explore the integral properties of a neuron while it is part of an intact spinal cord. The reduced preparation permits the exploration of integral cell properties and relies on the imagination of the researcher the apply them back to a more intact system. Although not as physiological as an *in vivo* network, this study is being undertaken in a system that is more physiological than ion channels expressed in *Xenopus* oocytes. For studying properties of neural networks, this is obviously not the preparation to use but for elucidating the behaviour of individual neurons within a network the isolated cell preparation was the ideal choice.

Goals of the study

It has previously been demonstrated that one manner by which monoamines may modulate motor activity is by potentiating or attenuating excitatory glutamatergic transmission. It would be appealing to hypothesize that monoaminergic reticulospinal neurons affect the behaviour of individual neurons in the spinal intermediate zone thus promoting locomotor activity. Since glutamate receptor activity is essential for locomotion, and the spinal intermediate zone has been implicated in locomotion, the present study attempts to explore the actions of 5-HT and NA on currents evoked by EAA agonists in neurons from this region. The hypothesis in this study is that one manner by which 5-HT and NA exert their actions on motor activity is by modulating excitatory glutamatergic transmission. Thus the first goal was to see if 5-HT and NA had an influence on post-synaptic glutamate receptors in neurons from the intermediate zone. As previously stated, an acutely isolated cell preparation was chosen to explore this question in order to prevent synaptic contamination from other cells. Tissue from the intermediate zone was microdissected from a spinal slice and dissociated into individual neurons. This prevented complications in determining whether the effect of 5-HT or NA was primary, on the neuron in question or secondary via actions on a cell connected to it. Application of EAA agonists and monoamines by a local perfusion system would also show definitively that the actions were post-synaptic while whole cell recording would monitor the evoked currents. There would also exist fewer diffusional barriers of more intact preparations and allow for more rapid drug application.

Although cells active during locomotion have been localized to the intermediate zone, indeed not all neurons in this region are active. The intermediate zone is comprised of a heterogeneous population of cells and it would not be at all surprising to see variable effects of 5-HT and NA between cells. As an addition to the hypothesis then, one could expect monoamine

application to result in an increased activity in some cells while resulting in a diminished activity in others. Thus the second goal of the study was to determine whether 5-HT and NA have consistent actions on EAA-evoked currents in all cells or whether EAA evoked currents are potentiated in some cells and attenuated in others.

Upon determining the effects of 5-HT and NA on EAA-evoked currents, the next course of action would be to characterize the behaviour more fully by examining the effect of monoamine dose and studying the pharmacology of any effects. The last goal of the study therefore is to vary the concentration of 5-HT or NA to explore any concentration dependence of the effects and to use specific receptor agonists and antagonists to determine which 5-HT and NA receptor subtypes mediate actions on glutamate receptors.

In the future, it would be interesting to observe the effects of 5-HT and NA on cells which have been defined as being active in a motor task. Perhaps as a long term goal, the modulatory actions of 5-HT and NA may be examined in intact spinal circuits. An attractive study would be to examine the role of neuromodulators implicated in the initiation and control of locomotion on functionally identified components of the locomotor network. Currently activity dependent labeling techniques are being developed to specifically label cells active during a locomotion. Once this technique is perfected, it will be possible to examine neurons active during locomotion and compare them to those which are not.

Modulation of excitatory neurotransmission has been shown to be modulated by 5-HT and NA in other systems and it would not be surprising to see a similar action in this region of the CNS. However by achieving these first goals and characterizing the actions of 5-HT and NA more fully in this region, one can hope to elucidate a possible mechanism by which 5-HT and NA function to reconfigure pattern generators for locomotion in the rat.

METHODS

Dissection and Slicing

Sprague-Dawley rats (postnatal day 1-5) were decapitated, eviscerated and chilled to 4°C in artificial cerebrospinal fluid (ACSF) containing: NaCl 125 mM, KCl 2.5 mM, glucose 25 mM, MgCl 1 mM, $CaCl_2$ 2 mM, $NaHCO_3$ 26 mM, $NaH_2PO_4 \cdot H_2O$ 1.25 mM and oxygenated with 95% O_2, 5% CO_2. The spinal cord was then exposed ventrally and carefully removed from the vertebral column. The dura mater was removed and the lumbar enlargement was subsequently isolated. The tissue was then embedded in 2.5% agar type E (Sigma) cooled to about 40°C. The block of agar containing the tissue was subsequently sliced in chilled ACSF on a Pelco® vibratome at thicknesses of 400 μm. The slices were kept in a plastic mesh suspended in a bath of ACSF at 32°C until dissociation.

A combination of enzymatic and mechanical dissociation was used to obtain isolated cell suspensions. It has been discovered that enzymatic dissociation gives a better yield but can sometimes have detrimental effects on the cells. Mechanical dissociation (trituration) results in a relatively low yield. A combination of the two is widely used to yield cell suspensions (Leibovic 1986). Slices were removed from the agar and incubated at room temperature (20-22°C) in a 4 mL chamber containing 3 mL of ACSF and 0.5 mg pronase E (Sigma protease type XIV) for 7-10 minutes.. The slices were then incubated at room temperature for the same duration in a petri dish containing 3 mL of ACSF and 0.5 mg of thermolysin (Sigma protease type X) to inhibit the pronase activity. Regarding the choice of enzyme, an enzyme was needed which would digest extracellular matrix proteins while having minimal effect on cell surface receptor proteins. It has been previously shown that some proteases such as trypsin have deleterious effects on NMDA

receptor channels. Incubation of tissue in trypsin decreased the size of NMDA evoked currents in hippocampal neurons (Akaike 1988). It has been suggested that the protease, pronase is more gentle on cellular membrane proteins while also yielding one of the best dissociations (Mody et al 1989; Leibovic 1986; Murase 1990). Incubation time in both enzymes was dependent on the age of the animal. P1 rats were incubated for 7 minutes, P2 rats for 8 minutes, P3-P4 rats for 9 minutes and 10 minute incubations were reserved for P5 rats. This was determined by trial and error during my development of this preparation. After incubation in both enzymes, the slices were then washed once in a Ca^{2+} free HEPES buffered solution containing: NaCl 150 mM, KCl 5 mM, $MgCl_2$ 1 mM, HEPES 10 mM, glucose 10 mM, pH 7.3 for 5-10 minutes. During this wash the intermediate zone was microdissected from the slice under a dissecting microscope using a fine scalpel blade. The purpose of the microdissection was to increase the selectivity of the area in question and decrease the heterogeneity of the cell suspensions. The resulting tissue pieces were placed in 35 mm culture dishes containing 1.5 mL of HEPES buffered ACSF solution containing: NaCl 150 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, HEPES 10 mM, glucose 10 mM, pH 7.3 and triturated through a series of fire polished pasteur pipettes of increasingly smaller diameters. During trituration care was taken to ensure that the tissue passed smoothly through the tip and was not pressed or blown away. Care was also taken to avoid blowing bubbles in the cell suspension as the surface tension of the bubbles would tear the cells apart. The cell suspension is allowed to settle for approximately 30 minutes before recording.

Recording

Whole-cell voltage clamp recordings were made (Hamill et al. 1981) with an Axopatch 1D amplifier (Axon Instuments). Microelectrodes were pulled on an upright electrode puller (Narishige PP-83) and filled with an electrolyte solution which approximated the intracellular milieu. The electrode solution contained CsF 140 mM, EGTA 11mM, HEPES 10 mM, KOH 35

mM, $CaCl_2$ 1 mM, pH 7.3. Occasionally, 2mM Mg-ATP was added and CsF was replaced by KF, K-gluconate, or Cs-methanesulfonate. Resulting tip resistances were between 2 and 5 MΩ. The low resistance of the electrode tip permits exchange of solution between the cell and the electrode. This intracellular dialysis can be applied as a means of manipulating the intracellular contents of the cell.

Upon achieving whole-cell configuration, the cell was perfused with HEPES buffered solution fed via a 12 barrel perfusion system. The perfusion system consisted of 12 barrels gravity fed into a common output and controlled by a set of solenoid switches. The common output was mounted with a square glass capillary tube with an internal diameter of 500 μm and was placed approximately 100 μm from the cell.

Data was acquired at 666 Hz using pCLAMP software (Axon Instruments), and stored on computer. Continuous records were captured on Gould chart recorder and VCR tape. Data analysis was accomplished using the pCLAMP analysis and SigmaPlot software. After seal formation on the cell, stray capacitance was minimized using the cancellation circuitry of the amplifier. Upon achieving whole-cell configuration, the membrane was voltage clamped at -80 mV. The cell was then taken through a series of 200 msec voltage steps. Neurons were identified based on the occurrence of a fast inactivating sodium current evoked by supratheshold depolarizing voltage steps.

The drug application paradigm consisted of a control application of 5-HT or NA, application of EAA agonist, application of EAA-agonist and monoamine, and lastly application of the same EAA agonist. This repeat application of EAA agonist allowed a bracketing of the "test" response with "control" responses. This bracketing precludes complications that may result in interpretation due to rundown, receptor desensitization and reversibility of modulatory actions. All drugs were dissolved in distilled water as stock solutions and frozen in aliquots until

use. On each experiment day, drugs were diluted in HEPES buffered solution for recording and adjusted to pH 7.3.

For NMDA application (50 μM- 100 μM), glycine (1 μM) was co-applied in order to satisfy the glycine requirement of the NMDA channel. Glycine was only added concomitantly with NMDA and not to the dishes during incubation. For about half of the NMDA applications, NMDA was diluted in Mg^{2+}-free HEPES buffered recording solution. The absence of extracellular Mg^{2+} resulted in an NMDA response at the holding potential of -80 mV. Another point to consider is the light sensitivity of 5-HT. 5-HT oxidizes in the presence of light and thus precautions were taken to minimize degradation. Experiments with 5-HT were conducted in the dark and the perfusion barrels containing 5-HT were covered.

During drug application, the cell was taken through a series of 600 msec long voltage steps from a holding potential of -80 mV. This slightly hyperpolarized holding potential was chosen because in a membrane which contains a mixed population of channels, studying modulation of a particular ion channel may be complicated by voltage-dependent ion channels which are activated at more depolarized potentials. The lengthy duration of drug application may seem unphysiological. This would be a complicating factor if channel kinetics and desensitization were the focus of this study. Since the focus was modulation of glutamate channels, the EAA-agonist response was allowed to plateau before the step protocol was executed. Current-voltage relationships of steady-state responses were constructed by subtracting the agonist-evoked responses from those obtained in the absence of any drug application.

Modulation of EAA-evoked currents by 5-HT and NA was normalized and given as a percent change relative to control values. Modulatory actions of 5-HT and NA on EAA-evoked currents were quite variable. A minimum 15% change in control current was set to define neuromodulation by 5-HT or NA based on the size of the control current. At this minimum value,

the differences were readily noticable and there was little doubt that the change was associated with the application of 5-HT or NA. It was also observed that NMDA-evoked currents were usually small thus, if the EAA agonist could not evoke an inward current larger than the inherent noise of the respective channel (approximately 10 pA for the kainate channel, and approximately 25 pA for the NMDA channel), the cell was not included in the analysis. All values are given as mean ± standard deviation.

RESULTS

In cells taken from 49 neonatal rats, perfusion of either kainate or NMDA evoked an inward current. It was observed that subsequent coapplication of 5-HT or NA either potentiated or attenuated the EAA-evoked current. Raw current traces showing modulation of currents evoked by kainate and NMDA can be seen in Figure 1. Application of 5-HT alone (n=112 cells) and NA alone (n=52 cells) had no effect on cell membrane conductance (Figure 2). The occurrence of occlusion or facilitation between the two substances was not examined in this study. Occurrences of what may have seemed like either facilitation or occlusion were infrequent at best and thus were not examined more fully. It should also be noted that perfusion of HEPES buffered solution alone did not evoke any membrane currents in any of the cells examined.

The excitatory amino acid agonist kainate (5 μM to 1 mM) was applied to 104 cells and evoked an inward current in all cells. 5-HT (100 nM to 1 mM) was coapplied in 76 of the 104 cells. Currents evoked by kainate were potentiated by 5-HT in 30 cells, and attenuated in 24 cells. The remaining 22 cells were not modulated. In 42 cells, NA (10 μM to 50 μM) was coapplied with kainate. Currents evoked by kainate were potentiated by NA in 13 cells, and attenuated in 11 cells. The remaining 18 cells were not modulated. The occurrence and percentage change in EAA-evoked current is summarized in Figure 3 and Table 1.

The excitatory amino acid agonist NMDA (50 μM to 100 μM) was applied to 57 cells and evoked an inward current in 45 cells. 5-HT (100 nM to 1 mM) was coapplied in 40 cells of which 7 were potentiated and 25 were attenuated. 8 cells showed no modulation. NA (10 μM to 50 μM) was coapplied in 22 of the 45 cells. Of these, 7 were potentiated, 7 were attenuated and the remaining 8 showed no modulation. These results are summarized in Figure 4 and Table 1.

The modulatory actions of 5-HT and NA were also examined on the putative endogenous neurotransmitter, L-glutamate. Out of 8 cells tested, L-glutamate (50 μM) evoked an inward current in 7 cells. 5-HT was co-applied in 5 cells in which 4 were attenuated and 1 was not modulated. NA was co-applied in 2 cells in which both were attenuated. Occurrence and amount of modulation are summarized in Table 1.

Generally when 5-HT and NA both had modulatory effects on the same cell, the effects were the same. That is in a given cell where 5-HT and NA both modulated the EAA-evoked current they both either potentiated or attenuated the current. Out of 39 cells in which both 5-HT and NA were tested, 30 cells showed a commonality of action. In 7 cells 5-HT modulated the kainate-evoked current while NA had no effect and in one cell, NA modulated the current evoked by kainate while 5-HT had no effect. In 6 cells, 5-HT had a modulatory effect on the NMDA-evoked current while NA had no effect. Conversely, in 3 other cells, NA had modulatory actions on the NMDA-evoked current while 5-HT did not. Only one cell was found in which 5-HT and NA had different effects on the current evoked by NMDA.

The frequency of differential modulation of EAA currents was also noted to see if one EAA agonist-evoked current was predominantly potentiated while the other was attenuated in the sample population. Out of 15 cells in which both kainate and NMDA were examined, both currents were potentiated in 3 cells, and attenuated in five cells. Currents evoked by NMDA were potentiated while currents evoked by kainate were attenuated in 3 cells. The remaining 4 cells displayed potentiation of kainate-evoked currents while NMDA-evoked currents were attenuated. This is not to suggest that modulation of currents evoked by NMDA and kainate are under separate control but only that there does not appear to be any significant occurrence of one particular combination.

The dependence of the modulatory actions of 5-HT and NA on membrane voltage was examined in 41 cells for kainate and 40 cells for NMDA. In 15% of the cells tested with kainate and 33% of the cells tested with NMDA, the greatest amount of modulation occurred at more hyperpolarized potentials (less than -30 mV) with very little to no modulation occurring at depolarized potentials. Modulation of currents evoked by NMDA was most prominent between -60 and -30 mV when the extracellular recording solution contained magnesium. This was due to the voltage-dependent magnesium block of the NMDA receptor at potentials below -50 mV. When the extracellular recording medium contained no magnesium, the currents evoked by NMDA exhibited a similar voltage-dependency as the kainate-evoked currents. Unfortunately there was substantial rundown of potassium currents during recording, and this could possibly affect the occurrence of a voltage dependent effect. Figure 5 demonstrates the voltage dependency of kainate (A), and NMDA (B) -evoked current modulation by serotonin. Also illustrated are results from a cell which did not exhibit this behaviour (C). For the cell in Figure 4C, the I-V relationship follows Ohm's law, as current levels decrease as the membrane potential decreases. Figure 6 demonstrates a voltage dependency for two cells in which the current evoked by L-glutamate was attenuated by 5-HT and NA.

The concentration dependency of the modulatory effects of 5-HT and NA was also examined. In 11 cells the kainate concentration was kept constant while the 5-HT concentration was varied between 100 nM and 1 mM. At 5-HT concentrations less than 10 μM there was very little change in the size of the current evoked by kainate. However from concentrations of 10 μM to 70 μM 5-HT, the kainate current was potentiated with maximal potentiation occurring at 50 μM 5-HT. At concentrations above 70 μM the kainate-evoked current was attenuated. This is shown in Figure 7. The concentration dependency of 5-HT on currents evoked by NMDA were examined in 11 cells. The NMDA concentration was held constant at 100 μM and the 5-HT

concentration was stepped between 100 nM and 1 mM. There was mean attenuation of the NMDA-evoked current at all 5-HT concentrations tested with a sharp increase in attenuation occurring at concentrations larger than 10 μM 5-HT. These data are summarized in Figure 8.

It has been suggested that one must exercise caution when using the fluoride ion in any solutions. Fluoride has the property in that it stabilizes membrane structure during patch recording and aids in seal formation. Unfortunately fluoride has also been shown to have actions on certain second messenger systems (Rall and Sutherland 1958, Howlett et al. 1979, Bigay et al. 1987, Godfrey and Watson 1988, Tiger et al. 1990). In four cells, the gluconate$^-$ or methanesulfonate$^-$ anion was used instead of F^- as the predominant anion in the intracellular recording solution. During application of 10 μM 5-HT, currents evoked by kainate (50 μM) were attenuated by 15% (n=1), and NMDA-evoked currents (100 μM NMDA) were attenuated by 34 ± 7.5% (n=3). In one cell, application of 10 μM NA attenuated the by kainate-evoked current (50 μM kainate) by 30%. In this small sample no potentiation of EAA-evoked currents was seen.

A pharmacological approach was taken to see if the modulation was mediated by specific receptor subtypes of 5-HT and NA. The results of receptor pharmacology for attenuation of each EAA receptor are summarized in Table 2. The attenuation mediated by 5-HT was not sensitive to the $5\text{-HT}_{2a/2c}$ receptor antagonist cyproheptadine (CHP) (10 μM), nor the 5-HT_{1a} receptor antagonist propranolol (Prop) (10 μM). 5-HT mediated attenuation was mimicked by the selective 5-HT_{1b} agonist CGS-12066B maleate (10 μM). This would imply that 5-HT attenuation of EAA-evoked currents is 5-HT_{1b} receptor mediated. Current traces showing the pharmacology of 5-HT-mediated attenuation of currents evoked by kainate are shown in Figure 9. Attenuation by NA was not affected by the β-adrenergic receptor antagonist Prop. (10 μM) but it was blocked completely by the α_2-adrenergic antagonist yohimbine (Yoh) (1 μM). This would suggest that

NA attenuation of EAA-evoked currents was α_2-noradrenergic receptor mediated. Kainate-evoked current traces demonstrating this finding are shown in Figure 10.

The potentiation of kainate evoked currents was examined using isoproterenol (IPT), a β-adrenergic agonist, and methoxamine (Methox) (10 μM), an α_1 -adrenergic agonist. In 3 cells, application of 50 μM kainate with methoxamine caused no significant change in the control current. Application of 50 μM kainate with 1 μM IPT, caused a substantial potentiation of the control current. In contrast application of 50 μM kainate with 10 μM or 50 μM IPT caused no significant change in the control current. This could perhaps be due to the fact that at a concentration of 1 μM, IPT is relatively selective for the β-adrenergic receptor. At higher concentrations, IPT begins to have some actions of α_2-adrenergic receptors (Ahlquist 1948, Tsukahara et al. 1983). α_2- and β-adrenergic receptors act by inhibiting and potentiating adenylate cyclase respectively so when both receptors are activated it is understandable that there would be very little or no modulation due to competition between the actions of the two receptors. In another 6 cells, activation of either β- or α_1-adrenergic receptors caused a potentiation at all concentrations of agonist tested (Methox 10μM- 50μM, IPT 1μM- 50μM), except in one cell, where IPT had no effect and only methoxamine potentiated the kainate evoked current. It is possible that in the intermediate zone, which is a heterogeneous group of cells, this second group represents another population of cells without a full complement of receptor subtypes and lacking α_2-adrenergic receptors. This would account for seeing consistent potentiation at all concentrations. These current traces are shown in Figure 11.

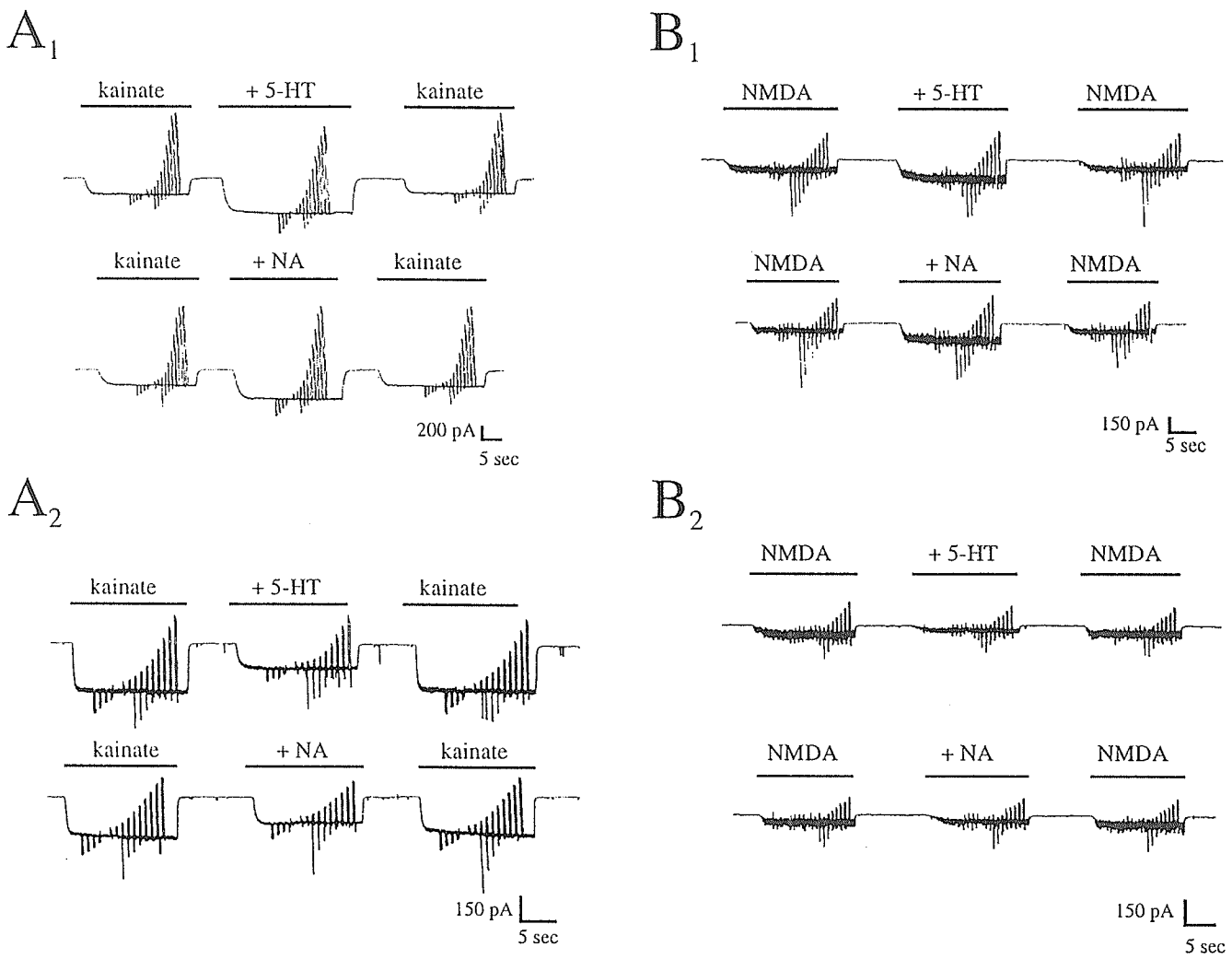
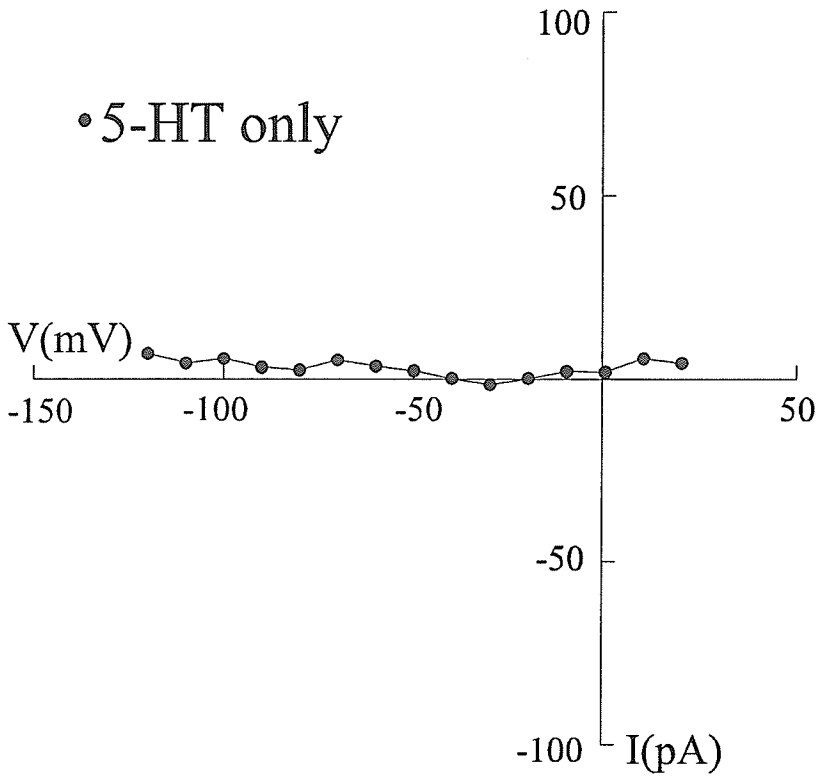

A1
kainate
+ 5-HT
kainate
kainate
+ NA
kainate
200 pA
5 sec
A2
kainate
+ 5-HT
kainate
kainate
+ NA
kainate
150 pA
5 sec
B1
NMDA
+ 5-HT
NMDA
NMDA
+ NA
NMDA
150 pA
5 sec
B2
NMDA
+ 5-HT
NMDA
NMDA
+ NA
NMDA
150 pA
5 sec

Figure 1

Raw current traces showing inward current evoked by 50 μM kainate and poteniated (**A1**) or attenuated (**A2**) by co-application of 50 μM NA or 50 μM 5-HT. Vertical bars on the current traces represent the current levels in response evoked as the neuron is taken through the voltage step protocol. Long horizontal bars denote period of drug application. Potentiation (**B1**) and attenuation (**B2**) of currents evoked by 100 μM NMDA are also shown. Horizontal bars denote period of drug application. Each "EAA-monoamine" response was "bracketed" by control "EAA-only" trials before and after the test trial. Holding potential is -80mV.

A.


• 5-HT only
100
50
V(mV)
-150
-100
-50
50
-50
-100
I(pA)

B.

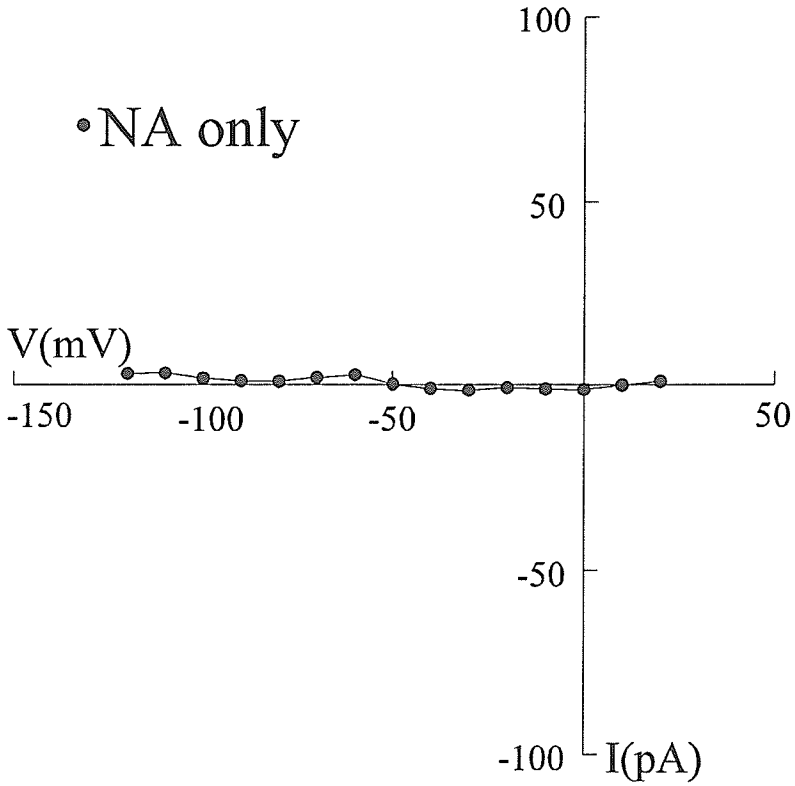

• NA only
100
50
V(mV)
-150
-100
-50
50
-50
-100
I(pA)

Figure 2

Representative examples of I-V relationships demonstrating that 5-HT **(A)** and NA **(B)** did not evoke any membrane currents between -120 mV and +20 mV.

Kainate-evoked current modulation

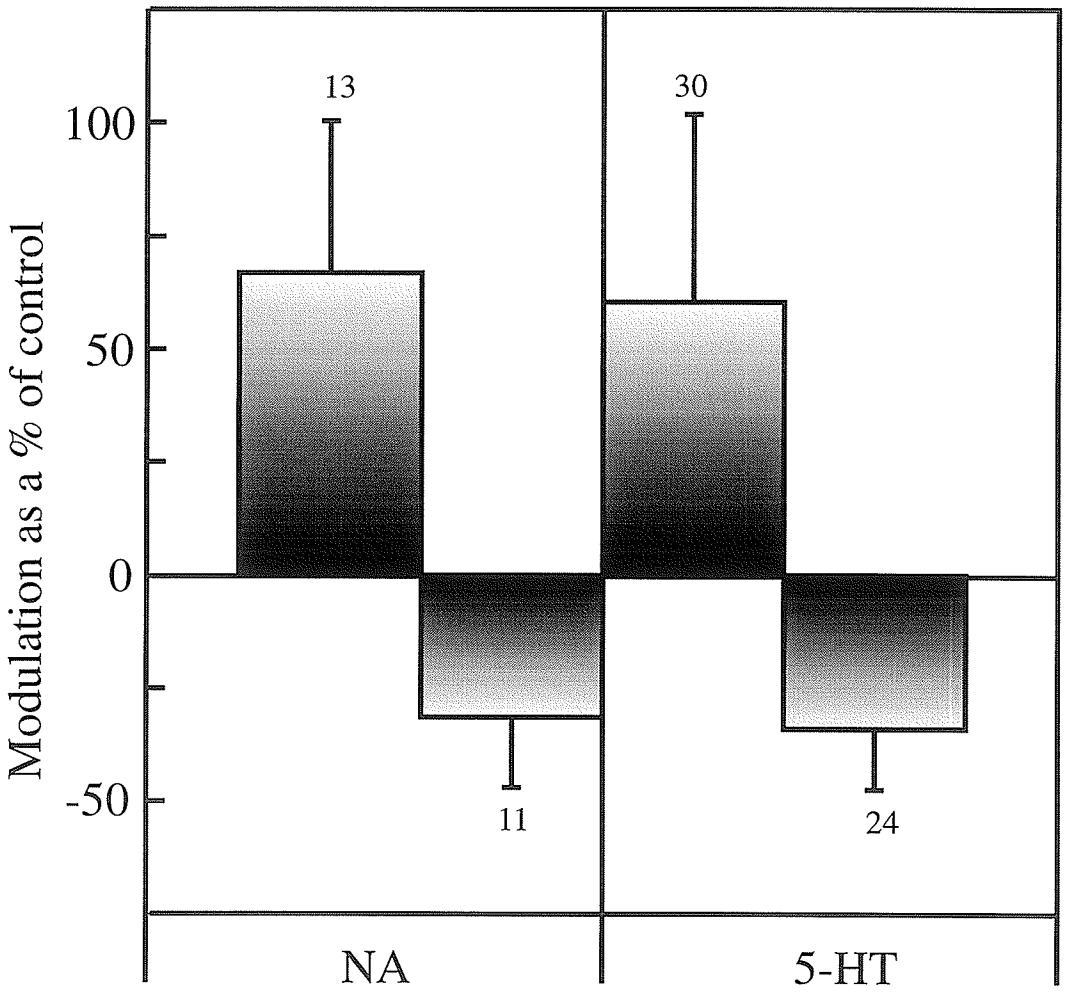


Figure 3

Histogram summarizing the modulation of current evoked by kainate (50 μM) application. Modulation by NA (50 μM) and 5-HT (50 μM) is shown on the left and right respectively. Modulation given as a percentage deviation from control. Bars represent mean amount potentiated and attenuated for each monoamine with standard deviation bars. Sample size in denoted at the end of the standard deviation bars. The number of cells with unaffected kainate-evoked currents is placed at the bottom of each column.

NMDA-evoked current modulation

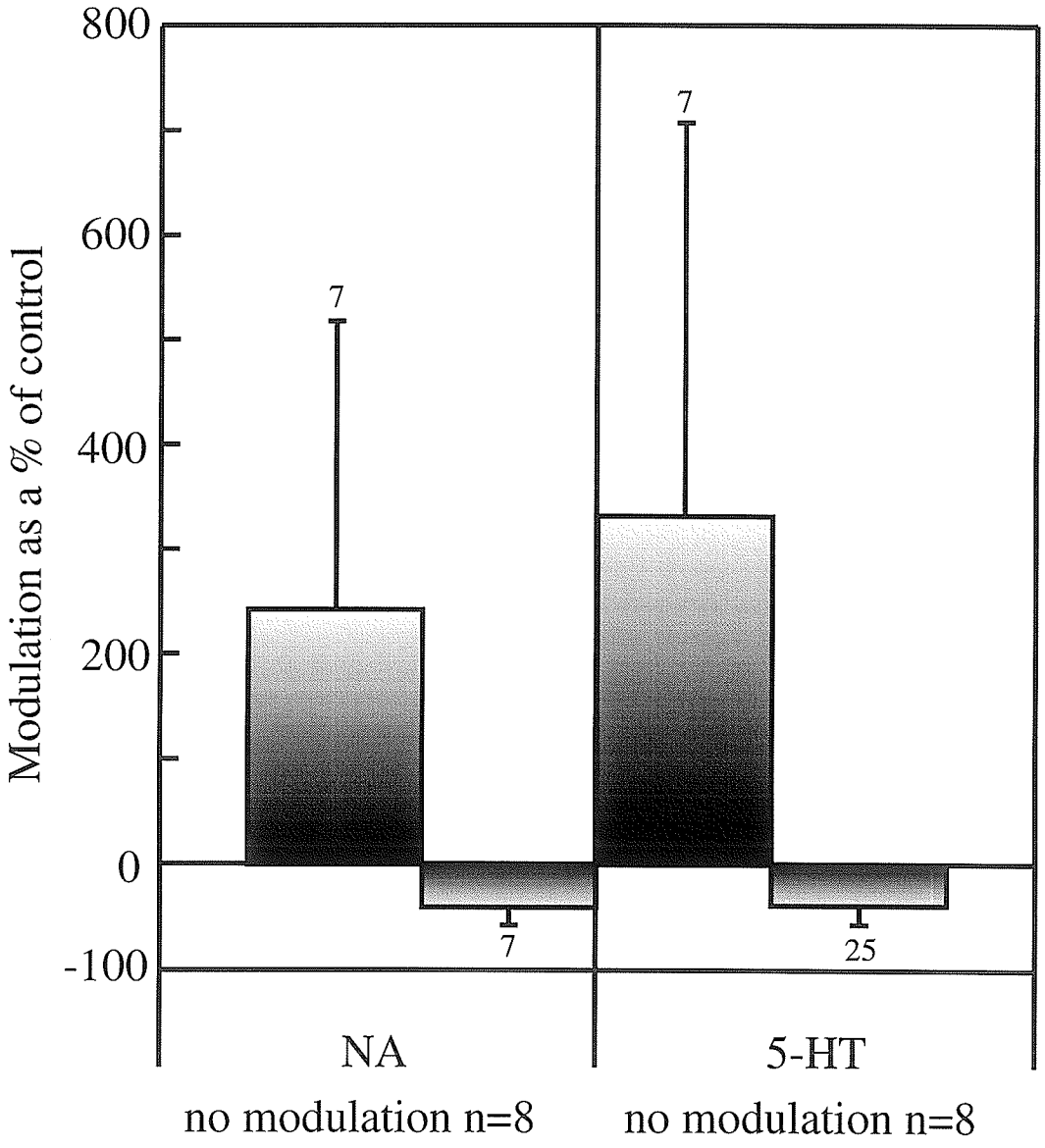


Figure 4

Histogram summarizing the modulation of current evoked by NMDA (100 μM) application. Modulation by NA (50 μM) and 5-HT (50 μM) is shown on the left and right respectively. Modulation given as a percentage deviation from control. Bars represent mean amount potentiated or attenuated for each monoamine. Standard deviation bars and sample size are included for each. The number of cells with unaffected NMDA-evoked currents is placed at the bottom of each column.

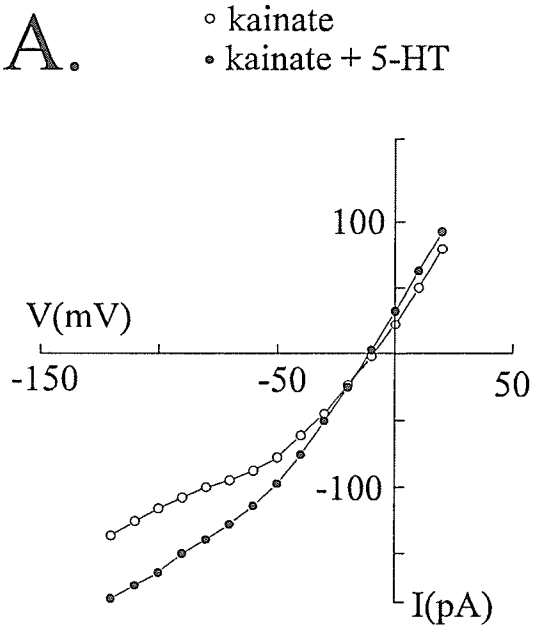
Table 1: Modulation Summary: Percentage change from control ± standard deviation including sample size (n) for each.

	NA potentiation	NA attenuation	NA no modulation	5-HT potentiation	5-HT attenuation	5-HT no modulation
kainate	66.97±33.3 n=13	-31.3±15.5 n=11	n=18	60.55±41.2 n=30	-34±13.4 n=24	n=22
NMDA	242.2±275 n=7	-41.0±16.7 n=7	n=8	331.9±375 n=7	-38.7±18.4 n=25	n=8
L-glutamate		-41±20.5 n=2			-33.3±14.2 n=4	n=1

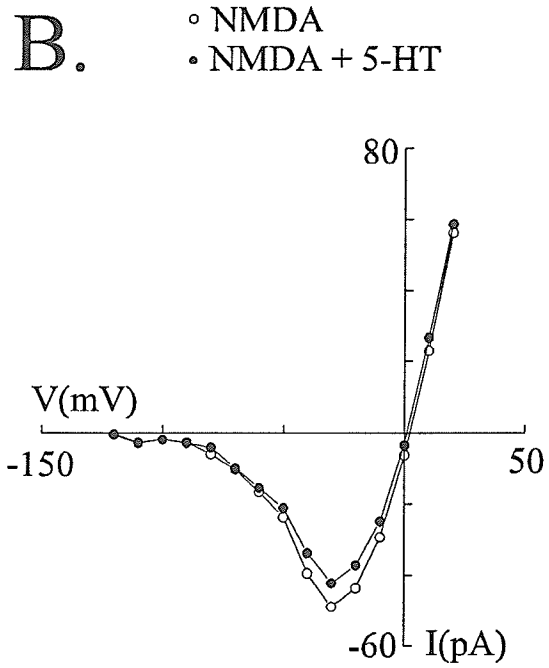
Table 1

Quantitative summary of modulatory effects of 5-HT (50 μM) and NA (50μM) on EAA-evoked currents. Concentrations of EAA are: kainate (50 μM), NMDA (100 μM), and L-glutamate (50 μM). All current values recorded at holding potential of -80 mV. Values represent mean percent modulation ± standard deviation. Sample size is included for each.

A.


◦ kainate
• kainate + 5-HT
100
V(mV)
-150
-50
50
-100
I(pA)

B.


◦ NMDA
• NMDA + 5-HT
80
V(mV)
-150
50
-60
I(pA)

C.

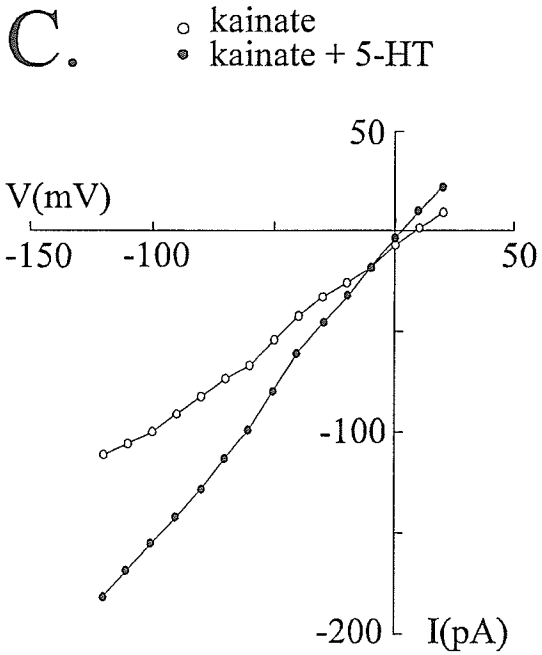

◦ kainate
• kainate + 5-HT
50
V(mV)
-150
-100
50
-100
-200
I(pA)

Figure 5

Representative examples of I-V relationships showing the possible voltage dependency of 5-HT mediated modulation of EAA-evoked currents. **A:** 5-HT potentiates kainate-evoked currents at membrane potentials less than -40 mV. Above -40 mV, there is negligible modulation. **B:** 5-HT attenuates NMDA-evoked current with modulation occuring between -10 and -40 mV. Notice the characteristic shape of the NMDA I-V relationship due to the voltage dependent Mg^{2+} block of the NMDA channel. **C:** A cell in which kainate-evoked currents were potentiated by 5-HT but the modulation was not dependent on membrane potential. Both control and test responses were linear in accordance with Ohm's law.

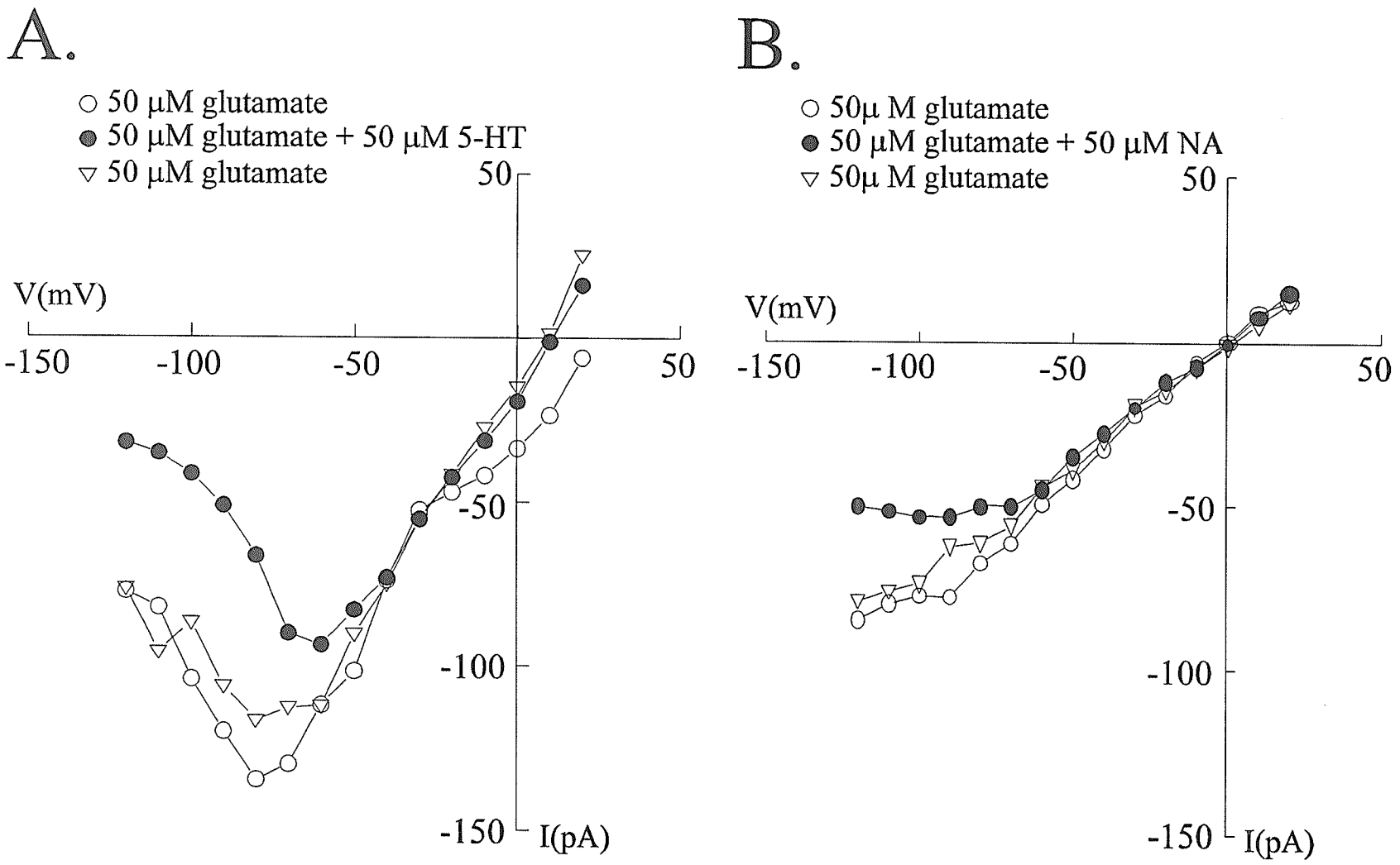

A.
○ 50 μM glutamate
● 50 μM glutamate + 50 μM 5-HT
▽ 50 μM glutamate
V(mV)
-150
-100
-50
50
50
-50
-100
-150
I(pA)
B.
○ 50μ M glutamate
● 50 μM glutamate + 50 μM NA
▽ 50μ M glutamate
V(mV)
-150
-100
-50
50
50
-50
-100
-150
I(pA)

Figure 6

Representative examples if I-V relationships for modulation of L-glutamate-evoked currents by 5-HT and NA showing voltage dependency. **A:** L-glutamate-evoked currents exhibit attenuation by 5-HT at membrane potentials less than -40 mV. **B:** L-glutamate-evoked currents exhibit attenuation by NA at membrane potentials less than -60 mV. Unfilled circles are pretest L-glutamate application. Unfilled triangles are post test L-glutamate application to "bracket" the test response.

Modulation of kainate-evoked current by
a range of 5-HT concentrations

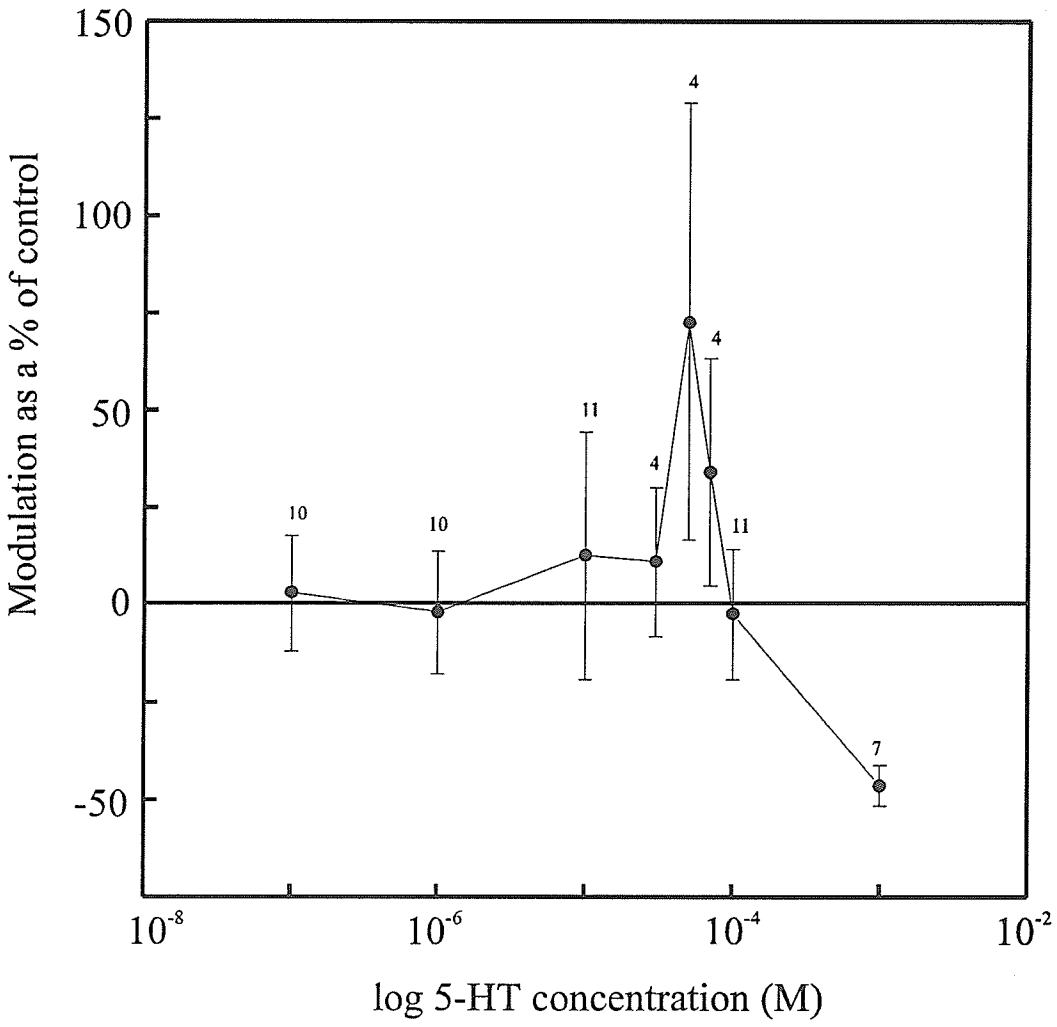


Figure 7

Concentration dependency of kainate-evoked current modulation by 5-HT. In 7 cells, the kainate concentration was kept constant while the 5-HT concentration was tested at 100 nM, 1 μM, 10 μM, 100 μM and 1 mM. Due to premature cell death during recording, not all concentrations of 5-HT were tested in these cells. In 4 cells, 30 μM, 50 μM, and 70 μM concentrations of 5-HT were also tested to elucidate the range in which potentiation was observed. These data are pooled and normalized modulation is plotted versus logarithmic concentration of 5-HT. Potentiation of kainate-evoked currents is observed between 1 μM 5-HT and 70 μM 5-HT. At 5-HT concentration above 100 μM, there is an attenuation of kainate-evoked currents. These data show that both potentiation and attenuation can occur in the same cell depending on the concentration of 5-HT used. Points represent mean modulation ± standard deviation bars for the sample size denoted above each point. Values are measured at a holding potential of -80 mV.

Modulation of NMDA-evoked current by
a range of 5-HT concentrations

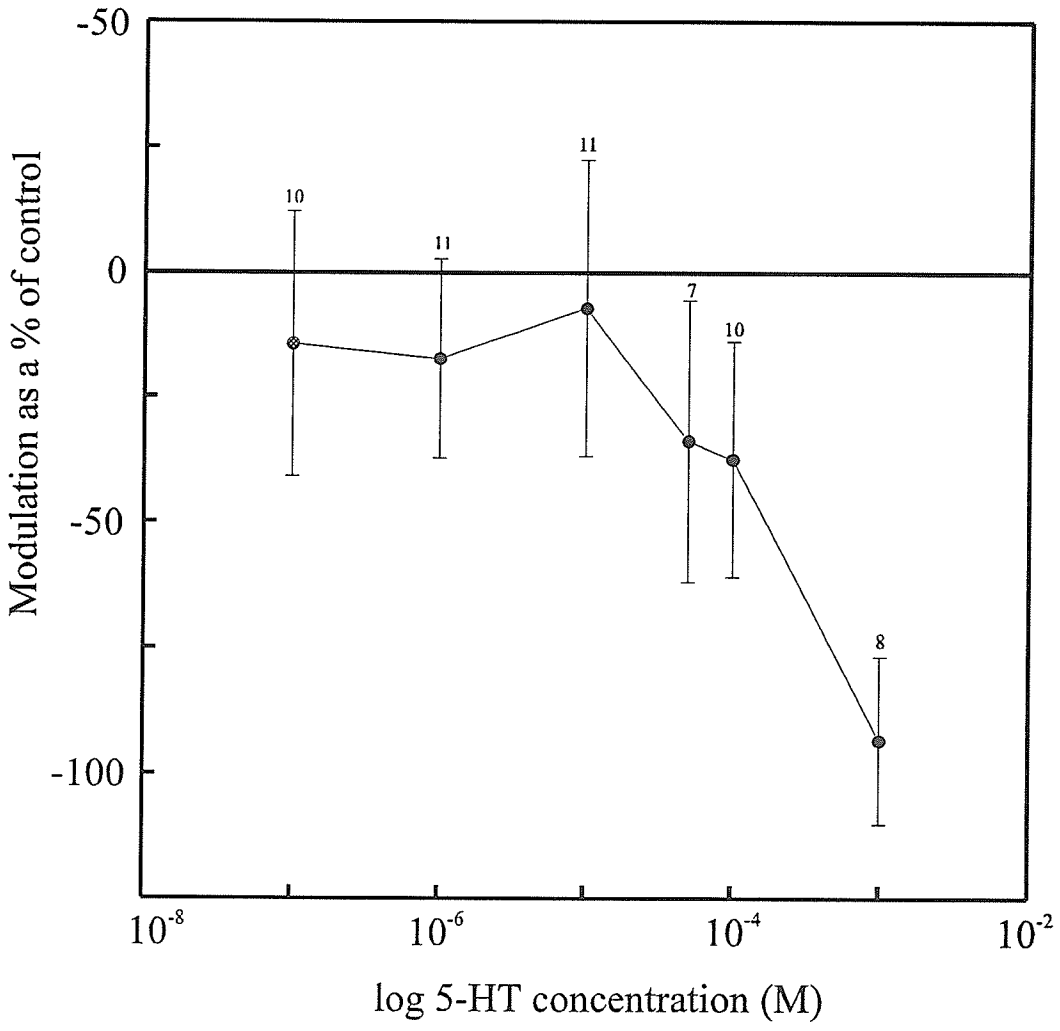


Figure 8

Concentration dependency of NMDA-evoked current modulation by 5-HT. In 11 cells, the NMDA concentration was kept constant while the 5-HT concentration was varied between 100 nM and 1 mM. Due to premature cell death during recording, not all concentrations of 5-HT were tested in all 11 cells. Normalized modulation is plotted versus logarithmic concentration of 5-HT. Generally there is attenuation at all concentrations of 5-HT with a sharp attenuation occuring at concentrations of 5-HT above 10 μM. Points represent mean modulation ± standard deviation bars for the sample size denoted above each point. Values are measured at a holding potential of -80 mV.

Table 2: Summary of the actions of monoaminergic antagonists and agonists on monoaminergic attenuation of kainate and NMDA-evoked currents.
(↓ = attenuation of current, ↑ = potentiation of current, — = no modulation)

5-HT receptors:

Agonist	*5-HT*	*propanolol* ($5\text{-}HT_{1a}$ *antagonist)* + *5-HT*	*cyproheptadine* ($5\text{-}HT_{2a/2c}$ *antagonist)* + *5-HT*	*CGS-12066B* ($5\text{-}HT_{1b}$ *agonist)*
NMDA	↓ (n=2)	— (n=2)	— (n=2)	↓ (n=1)
kainate	↓ (n=5)	— (n=5)	— (n=5)	↓ (n=3)

NA receptors:

Agonist	*NA*	*yohimbine* (α_2 *antagonist)* + *NA*	*propanolol* (β *antagonist)* + *NA*
kainate	↓ (n=3)	— (n=3)	— (n=3)
NMDA	↓ (n=1)	— (n=1)	↓ (n=1)
NMDA	— (n=1)	↑ (n=1)	— (n=1)

Table 2

Summary of pharmacology of EAA-evoked current attenuation by 5-HT and NA. **Top:** Effects of 5-HT receptor antagonists and agonists on EAA-evoked current modulation by 5-HT with sample size in parenthesis. **Bottom:** Effects of NA receptor antagonists on EAA-evoked current modulation by NA with sample size included in parenthesis. Observe the last example of a cell which was not affected by NA yet α_2-adrenergic receptor activation resulted in a potentiation of the NMDA-evoked response. Symbols represent change in current evoked by EAA (↑ = potentiation of current, ↓ = attenuation of cuurent, — = no modulation of current).

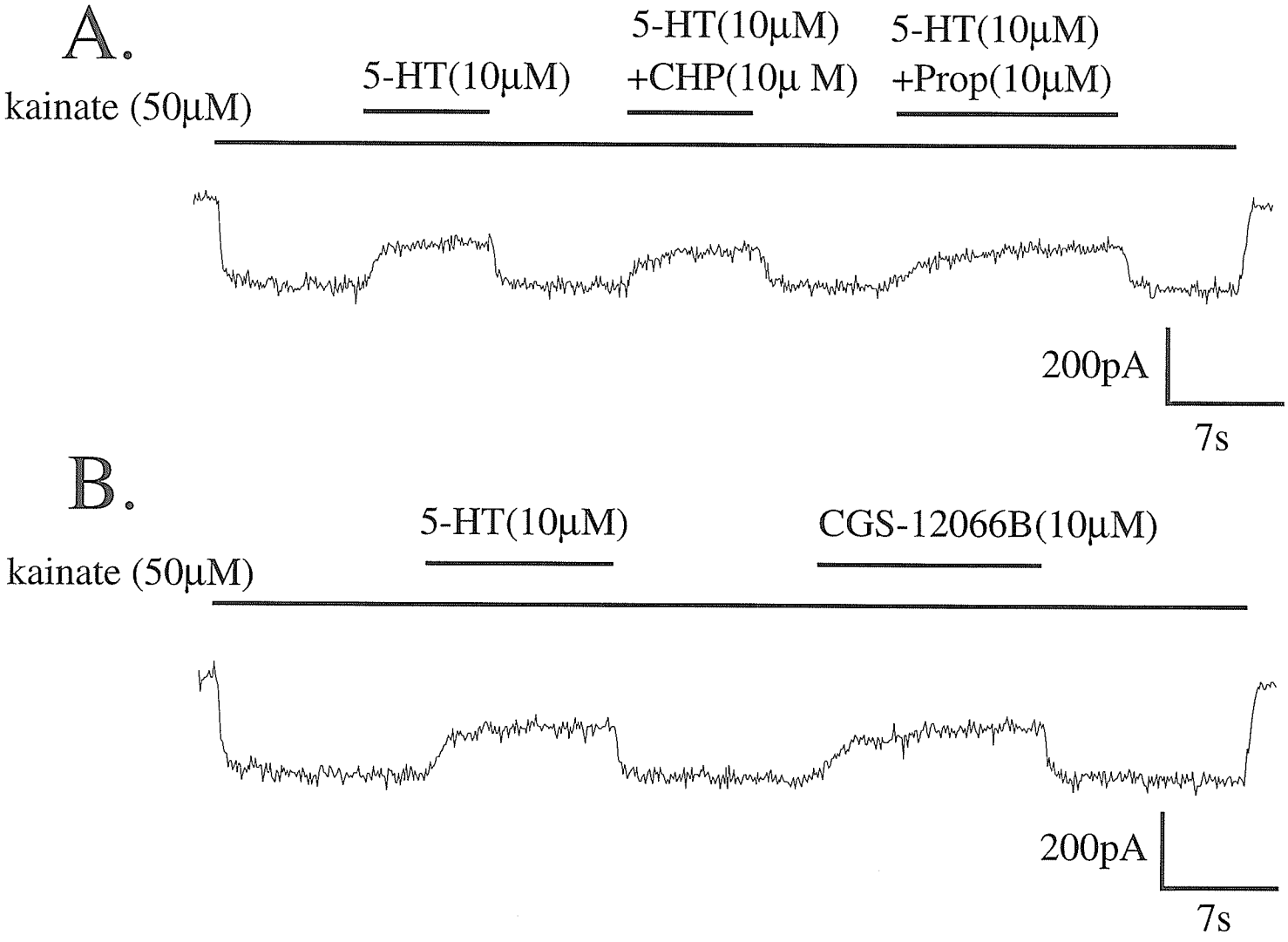

A.
kainate (50μM)
5-HT(10μM)
5-HT(10μM)
+CHP(10μ M)
5-HT(10μM)
+Prop(10μM)
200pA
7s
B.
kainate (50μM)
5-HT(10μM)
CGS-12066B(10μM)
200pA
7s

Figure 9

Pharmacology of attenuation of kainate-evoked currents by 5-HT.Raw current traces with drug administration denoted above by horizontal bars. **A:** Application of kainate evoked and inward current which was attenuated by 5-HT. Addition of Cyproheptadine (5-HT 2a/2c antagonist) or propranolol (5-HT 1a antagonist) did not block the attenuation. **B:** Application of kainate evoked and inward current which was attenuated by 5-HT. Substitution of 5-HT with CGS-12066B maleate (5-HT 1b agonist) mimicked the effect of 5-HT. Holding potential is -80 mV.

A.

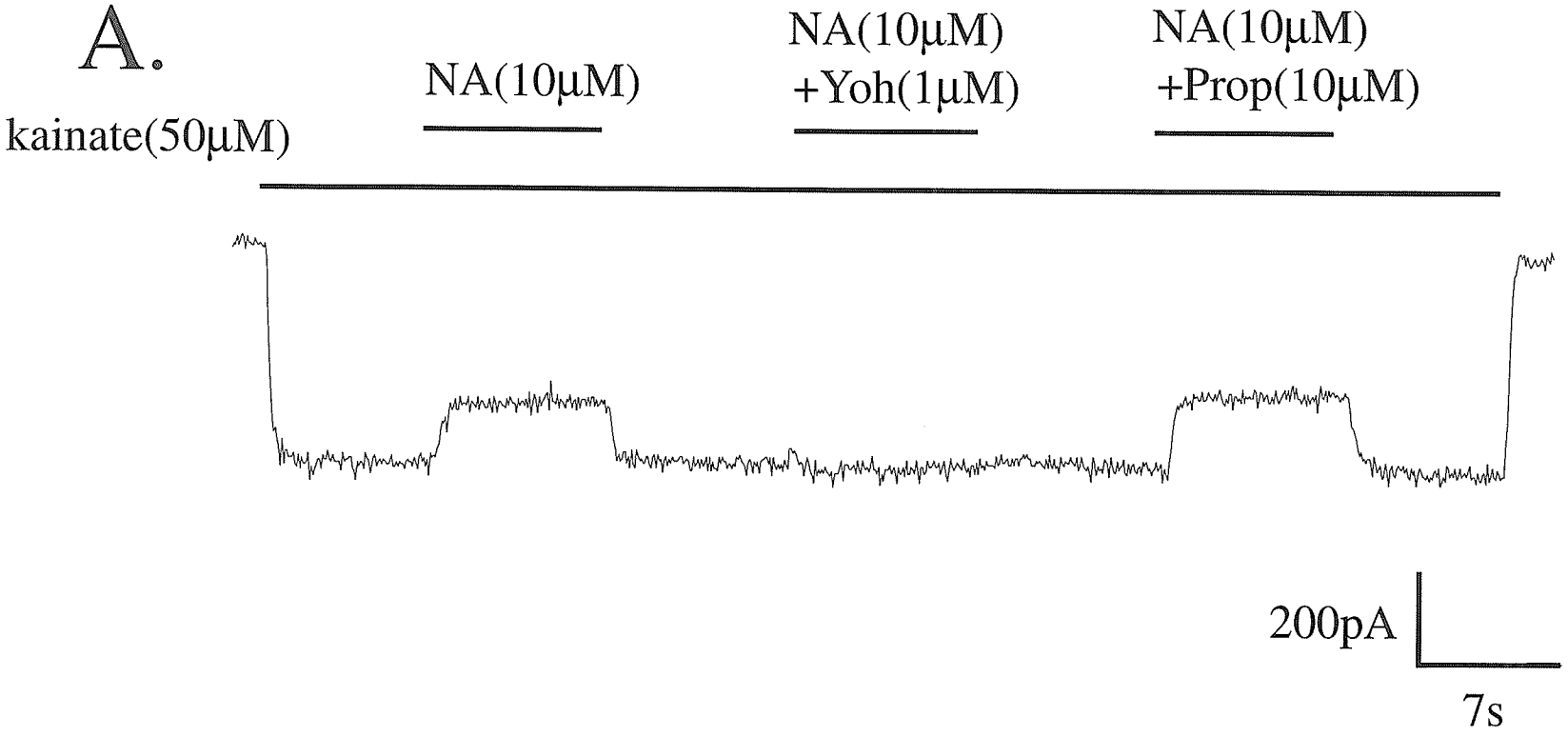

kainate(50μM)
NA(10μM)
NA(10μM)
+Yoh(1μM)
NA(10μM)
+Prop(10μM)
200pA
7s

Figure 10

Pharmacology of attenuation of kainate-evoked currents by NA. Raw current trace with drug administration denoted above by horizontal bars. **A:** Application of kainate evoked an inward current which was attenuated by addition of NA. Addition of yohimbine (α_2-adrenergic antagonist) blocked the NA mediated attenuation. Addition of propranolol (β-adrenergic antagonist) did not block the NA mediated attenuation. Holding potential is -80 mV.

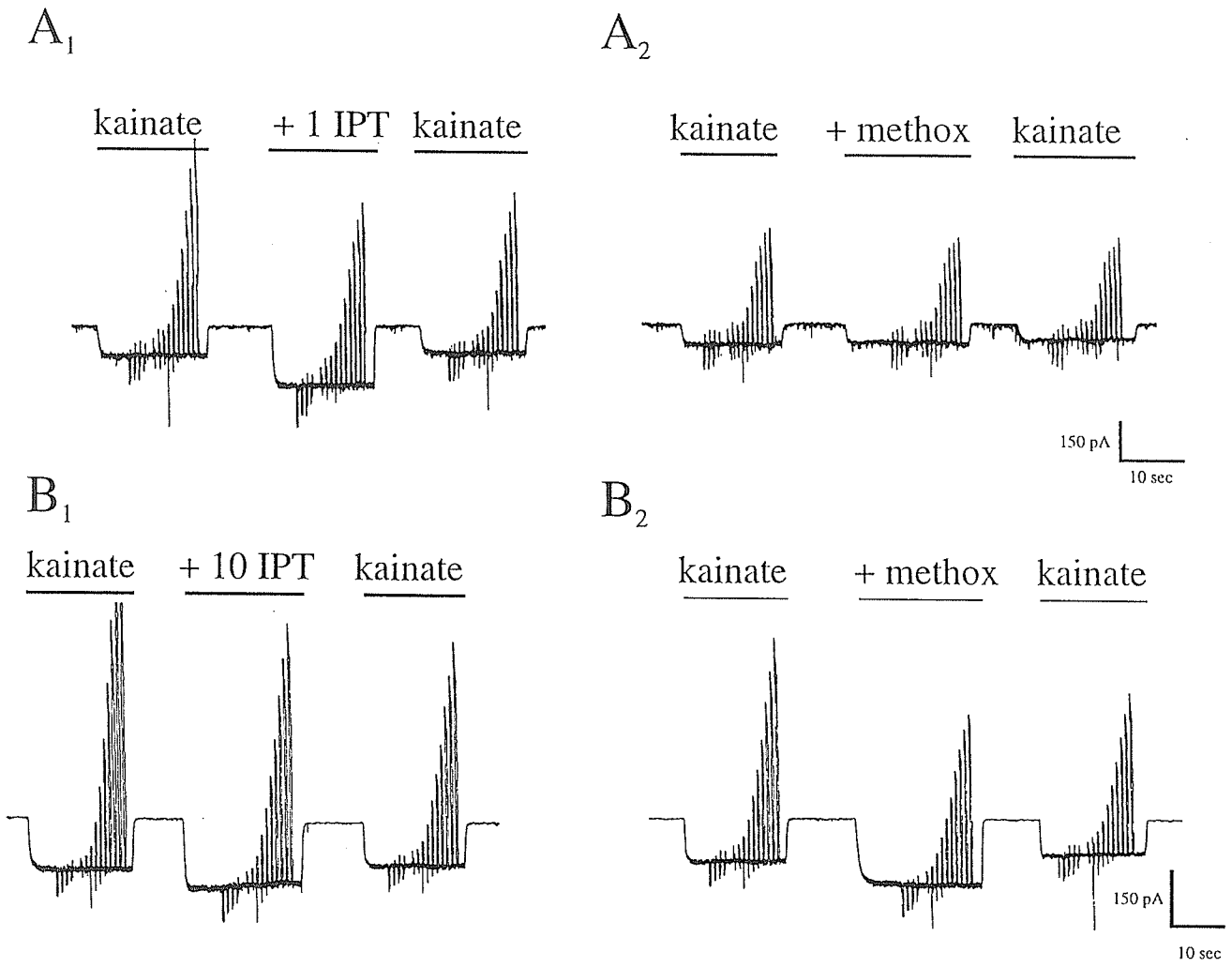

A1
kainate
+ 1 IPT
kainate
A2
kainate
+ methox
kainate
150 pA
10 sec
B1
kainate
+ 10 IPT
kainate
B2
kainate
+ methox
kainate
150 pA
10 sec

Figure 11

Pharmacology of potentiation of kainate-evoked currents by adrenergic agonists. Representative examples of both groups of cells affected by adrenergic agonists. **(A)** One group of cells has kainate-evoked currents potentiated by the β-adrenergic agonist isoproterenol while the α_1-adrenergic agonist methoxamine-HCl had not effect. Conversely in the second group of cells **(B)** both α_1- and β-adrenergic agonists potentiated the kainate-evoked current. Holding potential is -80 mV.

DISCUSSION

These results indicated that 5-HT and NA can potentiate or attenuate EAA-evoked currents in cells isolated from the intermediate zone of the spinal cord. This is also the first time that kainate-evoked currents in the spinal cord have been demonstrated to be modulated by 5-HT and NA. One interesting feature of this study is that for all cells except one, 5-HT and NA shared a commonality of effect on the EAA current. That is, in a given cell, both monoamines either potentiated or attenuated a particular EAA-evoked current. As shown, application of 5-HT and NA alone did not evoke membrane currents. This satisfies the definition of a neuromodulator used by Sizer et al. (1992) who state that to act as a neuromodulator, a substance should "influence the response to excitatory amino acids, without having any detectable effect itself on the neurone in question".

In trying to identify which cells are involved in this study, this would therefore exclude cells on which 5-HT and NA exert direct actions. One problem in dissociating cells from a heterogeneous tissue such as the spinal cord is that there are many different cell types present. The region dissociated after microdissection could in fact possess motoneurons as well as some sympathetic preganglionic neurons. These neurons may be excluded from our sample population for two reasons. Firstly, motoneurons have been shown to be larger than 15 μm in diameter at this stage of development (Takahashi 1990), while sympathetic preganglionic neurons are also large possessing mean soma diameters larger than 22 μm (Krupp and Feltz 1993). Cells that were recorded from in this study had a mean soma diameter of 11± 2.4 μM. Secondly, as has been previously stated, 5-HT and NA were found to have no direct actions on evoking membrane currents in the sample of this study. However, 5-HT (10 μM) has been shown to induce an inward current which directly depolarizes spinal motoneurons (Takahasi and Berger 1993). Both 5-HT and NA have also been reported to exert direct actions on sympathetic preganglionic

neurons. Ma and Dun (1986) reported direct actions of 5-HT on sypathetic preganglionic neurons in the neonatal rat spinal cord. These authors reported that application of 5-HT (1 μM to 100 μM) resulted in a depolarization accompanied by an increase in membrane resistance. Their results suggest that 5-HT acts by decreasing a voltage sensitive K^+ current. These results were similar to those found by Pickering et al. (1994) who found that 5-HT application (500 nM to 30 μM) strongly depolarized neurons and was accompanied by an increase in input resistance. 5-HT also evoked rhythmic membrane potential oscillations in neurons which were normally quiescent. Pharmacological studies have implicated 5-HT_2 receptor subtypes functioning by inhibiting an outwardly rectifying K^+ current.

Noradrenaline (10 μM to 50 μM) has been shown to depolarize sympathetic preganglionic neurons by activation of α_1-adrenergic receptors in the *in vitro* cat spinal slice preparation (Yoshimura et al 1987). These authors believe that NA mediates a slow EPSP of several seconds in duration which is evoked by focal electrical stimulation in the lateral horn. All of these results suggest that 5-HT and NA used at similar concentrations as in this study have direct actions on motoneurons and sympathetic preganglionic neurons. By this token, it is unlikely that cells in this study are motoneurons or sympathetic preganglionic neurons.

There appeared to be no consistency between cells as to whether the EAA-evoked currents were potentiated or inhibited. This is understandable because, as previously stated, the intermediate zone of the spinal cord is composed of a heterogeneous cell population. One would in fact expect to have some currents potentiated and others attenuated. It has been suggested that 5-HT functions to potentiate motor rhythm generation while suppressing sensory pathway function (Jacobs and Fornal 1993). The results of this study would support this hypothesis if one could identify the functional significance of a particular neuron.

It was also observed that there was a wide variability in the size of EAA-evoked currents among the cell group using a constant concentration of agonist. Concentrations of 50 μM kainate and 100 μM NMDA evoked currents which were quite variable in size but still large enough to see changes upon 5-HT and NA administration. Mean current amplitude for kainate evoked currents was -313± 260 pA. Mean NMDA evoked current was -183± 150 pA. It was because of the variability in current size that we chose to normalize the EAA-evoked currents and describe the 5-HT and NA mediated modulation as a percent change from control. Another point which should be noted is that currents evoked by NMDA were generally very small thus any potentiation of a small current will appear very large when normalized. Thus, the extremely large and variable actions of 5-HT and NA on currents evoked by NMDA as summarized in Figure 3 and Table 1 should be interpreted with caution.

Study of the pharmacology of EAA receptor modulation mediated by 5-HT and NA, implicated the 5-HT_{1b} and the α_2-adrenergic receptors in attenuating EAA-evoked responses and α_1- and β-adrenergic receptors in potentiating currents evoked by kainate. It must be strongly emphasized that the samples sizes are small and these findings only implicate these receptors and do not definitively identify them as being involved in modulation of glutamate receptors. One could hypothesize that 5-HT and NA modulate EAA-evoked currents via second messenger mediated cellular effector systems. Phosphorylation of membrane proteins is now believed to be one of the primary modes of regulating cellular processes and as previously mentioned may also affect EAA receptors. Two second messenger cascades which relate to 5-HT and NA are the cAMP dependent protein kinase A cascade (cAMP-PKA) and the inositol triphosphate/ diacyl-glycerol cascade (IP_3/ DAG) (for review see Krebs 1989, Nishizuka 1989). It has been shown that 5-HT_1 receptor subtypes as well as α_2-adrenergic receptor subtypes are negatively coupled to adenylate cyclase. Thus ligand binding to a receptor results in a decrease of intracellular cAMP

levels. Conversely the β-adrenergic receptor subtypes are positively coupled to adenylate cyclase and result in an increase in cAMP while 5-$HT_{2a/2c}$ receptors and α_1-adrenergic receptors are coupled to the IP_3/DAG cascade (for review see Fillenze 1990, Martin and Humphrey 1994). As previously mentioned the 5-HT and NA receptor subtypes which have been implicated in EAA-evoked current modulation do exist in the intermediate zone of the spinal cord. Both α_2-adrenergic and 5-HT_{1b} receptor subtypes exist in the intermediate zone and are both negatively coupled to adenylate cyclase. Previous studies in the spinal cord have shown that attenuation of NMDA-evoked currents in the dorsal horn are 5-HT_{1a} mediated (Cerne et al 1993). In the mouse NMDA-evoked behaviours have been attenuated by 5-HT_{1a} receptor activation while quisqualate-evoked behaviours are enhanced by activation of 5-HT_2 receptors (Mjellem et al. 1993). Also consistent with this finding is that 5-HT and NA have been reported to depress group II afferent transmission in the cat spinal cord (Bras et al. 1990). These authors reported that NA attenuated group II transmission by way of an α_2-adrenergic receptor in the intermediate zone while in the dorsal horn group II depression by 5-HT was mediated by a 5-HT_{1a} receptor (Bras et al. 1990). Both of these studies in the dorsal horn implicate the 5-HT_{1a} receptor subtype in attenuating EAA responses (Bras et al. 1990; Murase et al 1990). This is understandable as the 5-HT_{1a} receptor is localized primarily in the dorsal horn and around the central canal. The 5-HT_{1b} receptor is also negatively coupled to adenylate cyclase and is more densly distributed around the intermediate zone (Thor et al.1993, Marlier et al. 1991). It is therefore not surprising that our results would reveal the 5-HT_{1b} receptor and not the 5-HT_{1a} receptor in mediating EAA-evoked current attenuation. If activating receptor subtypes negatively coupled to adenylate cyclase results in an attenuation of EAA-evoked currents then it would follow that β-adrenergic receptor activation which is positively coupled to adenylate cyclase should result in a potentiation of EAA-evoked currents. This has been reported to potentiate depolarizations induced by NMDA in the frog

spinal cord (Wohlberg et al. 1987) and is consistent with the finding in this study that application of β-adrenergic agonists can potentiate kainate-evoked current response. In other systems such as the rat neocortex, the PKC cascade has been implicated in mediating the excitatory effects of EAAs by activation of 5-HT_2 and α_1-adrenergic receptors which are coupled to the IP_3/DAG cascade. Both receptors exist in this region of the spinal cord and may mediate the potentiating effects of 5-HT and NA on EAA-evoked currents. It was not determined which 5-HT receptor subtype mediated EAA-evoked current potentiation, although in light of other studies, the 5-HT_2 receptor seems a likely candidate. Of course the involvement of second messenger systems is purely speculation but is an attractive hypothesis to account for some of these results. One discrepancy between cells in the present sample is that at a given concentration of 5-HT or NA, the EAA-evoked currents in a cell were either potentiated or attenuated. The modulation summary histograms and Table 1 were obtained by using 50 μM kainate and 100 μM NMDA with 50 μM 5-HT or NA. This discrepancy can be accounted for by the possibility that different complements of receptor subtypes may exist on each cell. Perhaps cells involved in a locomotor pattern generator possess more of a receptor subtype which would result in a net potentiation of the excitatory response whereas cells involved in sensory information processing would possess an ample amount of the receptor subtype which would result in a net attenuation of the synaptic response.

By varying the concentration of 5-HT against a fixed kainate concentration, it was found that this modulation was concentration dependent. At lower concentrations of 5-HT (100 nM - 10μM) the kainate evoked currents were slightly attenuated. Between 10 and 70 μM 5-HT the kainate evoked current was potentiated and at 5-HT concentrations of 100 μM and above, the kainate evoked current was attenuated again. This was exciting in that it demonstrated that currents evoked by kainate could be either potentiated or attenuated in a particular cell based on

the concentration of 5-HT applied. The 5-HT receptor subtypes possess different binding affinities to their respective endogenous ligands. It is possible that at low concentrations of 5-HT, the 5-HT_1 receptor subtypes are being activated causing an attenuation of the kainate-evoked current, while at higher concentrations, the 5-HT_2 receptor subtypes become activated causing a potentiation. This would account for the differential effects of 5-HT on modulating the kainate evoked currents. At the higher concentrations of 5-HT where substantial attenuation of the kainate-evoked current is seen, it is possible that the shear amount of 5-HT applied is resulting in a block of the kainate channel. Concentration studies on the currents evoked by NMDA revealed very little potentiation. 5-HT-mediated potentiation of currents evoked by NMDA occurred at lower 5-HT concentrations with an increasing attenuation as the 5-HT concentration increased. One could hypothesize that the window of potentiation for NMDA-evoked currents was between two of the points tested or perhaps NMDA evoked currents are more sensitive to 5-HT and are potentiated at concentrations less than 100 nM. If this were the case than it would imply a differential modulation of the EAA receptors. From a physiological perspective, at low 5-HT concentrations or synaptic release, NMDA receptors would be potentiated while kainate receptors attenuated. Conversely, at high 5-HT concentrations resulting from synaptic release kainate receptors would be potentiated and NMDA receptors attenuated. Thus by varying the actual release of 5-HT onto a given cell, the descending systems may present a way by which precise modulation of synaptic processes may occur. This could perhaps explain how cells involved in sensory information processing are attenuated and cells involved in a motor pathway are potentiated to evoke a given motor task.

Another observation is that 15% of kainate-evoked currents and 33% of NMDA-evoked currents displayed a voltage dependency of modulation. The greatest modulation existed at hyperpolarized potentials with very little or no modulation occurring at more depolarized potentials. It was initially thought that observing this behaviour on an I-V relationship was

simply due to a run down of K^+ and not actually a physiological effect. This effect did persist when most K^+ currents were blocked by Cs^+ in the intracellular recording solution. An explanation of this phenomenon may be that the monoamines may have actions on voltage-dependent conductances not affected by high internal Cs^+ (Anywyl 1990; Gray and Johnston 1987; Ishibashi and Akaike 1995). This was a very exciting observation which unfortunately did not occur consistently. It is understandable that the greatest modulation should occur around resting membrane potential such that a cell at rest would experience the greatest effects of the monoamines in recruiting that cell into a behaviour. Perhaps the inconsistency of this voltage dependence is due to rundown of some membrane currents which minimize the modulatory effect of 5-HT and NA at more depolarized potentials or that the voltage dependency is only present in a select population of neurons which should not be affected at depolarized potentials once a cell is recruited.

One point that was of interest and some confusion was that for most experiment days, the modulatory effects of 5-HT and NA were consistent throughout all of the cells recorded from that day. That is to say, if one cell was potentiated by 5-HT and NA, then all other cells recorded from that experiment day were also potentiated. There were, however, four days out of 49 in which both potentiation and attenuation of an EAA-evoked currents in different cells were observed. One possible cause might be changes in ambient temperature which were not controlled for. This is quite unlikely though as fluctuations in room temperature were never extreme. It has been suggested that letting the cells incubate in low glycine concentrations (1 μM) may be beneficial to the cells. This was not done in this study and may be a factor in the diversity of the results. Another point is that on average only 2 or 3 cells were recorded from on a given day. This was due to my ability to patch cells as well as the health of the preparation which

declined very rapidly after about 8 hours. Perhaps if more cells were recorded from in an experiment day then this occurrence would not be observed.

The fluoride ion, F^-, has been used as the principle electrode solution anion because of its ability to stabilize a cell for recording (Kay 1992). Unfortunately, it has also been reported that fluoride has many effects on second messenger systems. Extracellular application can interfere with PKA activity, activate G-proteins and inhibit receptor coupled phospholipase C (Rall and Sutherland 1958, Howlett et al. 1979, Bigay et al. 1987, Godfrey and Watson 1988,Tiger et al, 1990). Intracellular application has been reported to interfere with protein phosphatases. It is now evident that the F^- containing electrode solution should not be used to examine behaviours which may be mediated by second messenger systems. At this point it is not known how fluoride ions in the intracellular electrode solutions might be affecting 5-HT/NA mediated modulation of EAA-evoked currents. Most data obtained in this study were acquired before the actions of F^- were fully known to us. After discovering the actions of F^- on second messenger sytems, we substitued the F^- ion in the electrode solution with gluconate$^-$ or methane-sulfonate$^-$ as recommended by Kay (1992). It was reassuring to see that some modulatory effects of 5-HT and NA on EAA-evoked currents persisted however the fact that only attenuation was observed may indicate that the F^- ion may have an effect on the modulatory actions of 5-HT and NA.

This study presents data which suggest one mechanism by which 5-HT and NA may modulate neural networks and influence motor activity. 5-HT and NA have modulatory actions on EAA receptors and may redirect excitatory neurotransmission within a neural network to elicit a particular motor behaviour such as locomotion. Although unexpected at first, the sparse results of the pharmacological exploration in this study do coincide with the effects of 5-HT and NA observed in other motor behaviours. This study will be useful in understanding the actions resulting from serotonergic and noradrenergic release in the spinal cord. These results support

the involvement of 5-HT and NA as mediators of EAA receptor modulation by actions via their specific receptor subtypes. It is possible that the modulatory actions of 5-HT and NA on EAA receptors are mediated by second messenger systems.

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