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Mechanisms intrinsic to 5-HT_{2B} receptor-induced potentiation of NMDA receptor responses in frog motoneurones

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1 In the presence of NMDA receptor open-channel blockers [Mg^{2+}; (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate (MK-801); 1-amino-3,5-dimethyladamantane (memantine)] and TTX, high concentrations (30–100 μM) of either 5-hydroxytryptamine (5-HT) or α-methyl-5-hydroxytryptamine (α-Me-5-HT) significantly potentiated NMDA-induced depolarizations of frog spinal cord motoneurones.

2 Potentiation was blocked by LY-53,857 (10–30 μM), SB 206553 (10 μM), and SB 204741 (30 μM), but not by spiroxatrine (10 μM), WAY 100,635 (1–30 μM), ketanserin (10 μM), RS 102221 (10 μM), or RS 39604 (10–20 μM). Therefore, α-Me-5-HT's facilitatory effects appear to involve 5-HT_{2B} receptors.

3 These effects were G-protein dependent as they were prevented by prior treatment with guanylyl-5′-imidodiphosphate (GMP-PNP, 100 μM) and H-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2 (GP antagonist 2A, 3–6 μM), but not by pertussis toxin (PTX, 3–6 ng ml^{-1}, 48 h preincubation).

4 This potentiation was not reduced by protein kinase C inhibition with staurosporine (2.0 μM), U73122 (10 μM) or *N*-(2-aminoethyl)-5-isoquinolinesulfonamide HCl (H9) (77 μM) or by intracellular Ca^{2+} depletion with thapsigargin (0.1 μM) (which inhibits Ca^{2+}/ATPase). Exposure of the spinal cord to the L-type Ca^{2+} channel blockers nifedipine (10 μM), KN-62 (5 μM) or gallopamil (100 μM) eliminated α-Me-5-HT's effects.

5 The calmodulin antagonist *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide (W7) (100 μM) diminished the potentiation. However, the calcium/calmodulin-dependent protein kinase II (CaM Kinase II) blocker KN-93 (10 μM) did not block the 5-HT enhancement of the NMDA responses.

6 In summary, activation of 5-HT_{2B} receptors by α-Me-5-HT facilitates NMDA-depolarizations of frog motoneurones *via* a G-protein, a rise in $[Ca^{2+}]_i$ from the entry of extracellular Ca^{2+} through L-type Ca^{2+} channels, the binding of Ca^{2+} to calmodulin and a lessening of the Mg^{2+} -produced open-channel block of the NMDA receptor.
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Keywords: *N*-methyl-D-aspartate (NMDA) receptors; frog; spinal cord motoneurones; 5-HT_{2B} receptors; open-channel block; Mg^{2+} ions; G-proteins; Ca^{2+} ions; calmodulin; calcium/calmodulin-dependent protein kinase II

Abbreviations: α-Me-5-HT, α-methyl-5-hydroxytryptamine; ACPD, *trans*-(±)-(1S,3R)-amino-1,3-cyclopentanedicarboxylic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ω-CgTx, ω-conotoxin GVIA, Cys^1-Lys-Ser-Hyp-Gly-Ser-Ser-Cys^8-Ser-Hyp-Thr-Ser-Tyr-Asn-Cys^{15}-Cys^{16}-Arg-Ser-Cys^{26}-Tyr-NH_2; CaM kinase II, calcium/calmodulin-dependent protein kinase II; cAMP, 3′,5′-cyclic adenosine monophosphate; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; DR, dorsal root; $G\alpha_i$, G-protein α_i subunit; $G\alpha_o$, G-protein α_o subunit; $G\alpha_q$, G-protein α_q subunit; $G\alpha_t$, G-protein α_t subunit; GALL, gallopamil, 5-[(3,4-dimethoxyphenylethyl) methylamino]-2-isopropyl-2-(3,4,5-trimethoxyphenyl) valeronitrile hydrochloride, G-protein, guanosine triphosphate-binding protein; GMP-PNP, guanylyl-5′-imidodiphosphate; GP antagonist 2A, GP-2A,H-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2; GTP, guanosine triphosphate; H-9, *N*-(2-aminoethyl)-5-isoquinolinesulfonamide dihydrochloride; 5-HT, 5-hydroxytryptamine (serotonin); iGluR, ionotropic glutamate receptor; IP_3, inositol 1,4,5-triphosphate; KN-62, 1-[*N*,O-*bis*-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; KN-93, 2-[*N*-(4-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; LY-53,857, 6-methyl-1(1-methylethyl)-ergdine-8β-carboxylic acid; MEM, memantine; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate; NIFED, nifedipine; NMDA, *N*-methyl-D-aspartate; PI, phosphoinositol; PKC, protein kinase C; PMA, phorbol-12-myristate 13-acetate; PTX, pertussis toxin; SB 204741, *N*-(1-methyl-5-indoyl)-*N*-3-methyl-5-isothiazolyl)urea; SB 206553, *N*-3-pyrinyl-3,5-dihydro-5-methyl-benzo (1,2-b; 4,5-b') dipyrrole-1(2 H); RS 39604, 1-[4-amino-5-chloro-2-(3,5-dimethoxyphenyl)methyloxy]-3-[1-[2-methylsulfonylamino]ethyl]piperidin-l]propan-1-one; RS 102221, 8-[5-(5-amino-2,4-dimethoxyphenyl)-5-oxopentyl]-1,3,8-triazaspirol[4,5]decane-2,4-dione; THAP, thapsigargin; TTX, tetrodotoxin; U73122, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]-1H-pyrole-2,5-dione; VR, ventral root; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; WAY 100635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl-cyclohexanecarboxamide

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Introduction

The major task of spinal motoneurones is to translate a large, heterogeneous inflow of signals from afferent and descending fibers and interneurones into an output that will precisely control the firing of muscle fibers (Rekling *et al*., 2000). The current view is that these signals include not only fast synaptic information mediated by transmitters directly coupled to ion channels (ionotropic transmitters), but also a superimposed, extra tier of control mechanisms activated by neuromodulators such as serotonin (5-hydroxytryptamine, 5-HT) and other monoamines, amino acids, and peptides activating metabotropic receptors. In recent years, specific interactions between metabotropic transmitters and ionotropic transmitters mediating spinal reflexes and rhythmic motor pathways have been reported (e.g., Murase *et al*., 1990; Holohean *et al*., 1992a; 1999a; Blank *et al*., 1996; Beato & Nistri, 1998; Wallis *et al*., 1998; MacLean & Schmidt, 2001).

L-Glutamate is the dominant fast excitatory neurotransmitter in the CNS. Acting at directly coupled ionotropic glutamate receptors (iGluRs), it functions as the main transmitter at most excitatory synapses on motoneurones in the spinal cord of both mammals and amphibians (Davies *et al*., 1982). Three broad subtypes of iGluRs have been traditionally identified by their agonist specificity and pharmacological and functional properties: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-proprionate (AMPA), and kainate. NMDA receptors have been postulated to be particularly important in the control of motoneurone excitability (Rekling *et al*., 2000).

In mammals and amphibians, a descending serotonergic pathway from the raphe nuclei of the brainstem (Soller, 1977; Soller & Erulkar, 1979) synapses directly on the somatodendritic membrane of motoneurones (Alvarez *et al*., 1998). These motoneurones express a number of different serotonergic (5-HT) receptor subtypes (Mengod *et al*., 1990; Hellendall *et al*., 1993; Helton *et al*., 1994; Ridet *et al*., 1994). Although the descending serotonergic pathway is known to exert profound effects on motoneurone excitability, the precise consequences of its activation and of 5-HT's actions on motoneurones have been disputed (reviewed in Anderson, 1983; Rekling *et al*., 2000). Both depressive and facilitatory interactions between 5-HT and L-glutamate have been described; there is contention about the pharmacological identity of the 5-HT receptors responsible for them (cf. VanderMaelen & Aghajanian, 1982; White & Neuman, 1983; Jackson & White, 1990; Murase *et al*., 1990; Rasmussen & Aghajanian, 1990; Holohean *et al*., 1992a, b).

Our previous frog spinal cord studies have shown that selective activation of different 5-HT receptor subtypes has direct hyperpolarizing and depolarizing actions on the membrane potential of motoneurones, and has modulatory actions on motoneuronal excitability mediated by NMDA receptor activation (Holohean *et al*., 1990; 1992a, b). In order to understand the modulatory role of 5-HT it was necessary to take into account evidence that the NMDA channel *in situ* is blocked by low levels of Mg^{2+} in the extracellular space. In the frog cerebral spinal fluid, Mg^{2+} is found in a concentration of 0.92 mM (Davidoff *et al*., 1988). Previously, we have shown that the metabotropic glutaminergic (mGLU) agonist ACPD potentiates NMDA-induced motoneuronal depolarization but only when Mg^{2+} is included in the bathing solution (Holohean *et al*., 1999a). ACPD facilitation of NMDA induced responses in frog motoneurones was found to involve the Group I mGLUR, a G-protein, IP_3-mediated release of intracellular Ca^{2+}, calmodulin, and the release of the Mg^{2+} block of the NMDA receptor channel (Holohean *et al*., 1999a).

5-HT$_2$ receptors are similar to Group I mGLURs in that they are also linked *via* a G-protein to IP_3/DAG signal transduction mechanisms. In this paper, we show a unique 5-HT$_{2B}$-specific modulation of the NMDA receptor that is Mg^{2+}sensitive, requires the activation of a G-protein of type $G\alpha_q$, Ca^{2+}, calmodulin and L-type Ca^{2+}channels; however, IP_3-mediated release of Ca^{2+} stores was not involved. This indicates that although 5-HT$_{2B}$ receptors stimulate phosphatidylinositol hydrolysis in heterologous expression systems (Wainscott *et al*., 1993; Kursar *et al*., 1994; Schmuck *et al*., 1994; Barnes & Sharp, 1999), this is not the mechanism involved in 5-HT$_{2B}$-mediated enhancement of NMDA receptor activation in frog motoneurones. Some of these results have appeared in preliminary form (Holohean *et al*., 1999b).

Methods

Our experiments used a protocol that was approved by the Animal Care and Use Committee of the Veterans Affairs Medical Center and followed National Institutes of Health guidelines (outlined in *The Care and Use of Laboratory Animals*). Adult frogs (*Rana pipiens*, 30–55 g) were anesthetized by cooling on crushed ice to the point of unresponsiveness. After decapitation, the brain was destroyed by pithing, and a laminectomy performed to remove the spinal cord and spinal roots. The lumbar spinal cord was hemisected sagittally and a hemicord with attached IXth dorsal (DR) and ventral roots (VR) was transferred to a sucrose gap chamber as described previously (Davidoff & Hackman, 1980). The spinal cord was allowed to recover from surgery for at least 45 min prior to drug applications. The cord was superfused with Ringer's solution containing (in mM): NaCl 114, KCl 2.0, $CaCl_2$ 1.9, $NaHCO_3$ 10, and glucose 5.5. To record from motoneurones, TTX (0.783 μM) was added to all solutions to block the indirect effects of interneuronal and afferent activation. In some experiments, Mg^{2+} (1.0 mM) was added to the Ringer's. The pH was adjusted to 7.4 by bubbling with 95% O_2/5% CO_2. A Peltier thermoelectric cooling device maintained the temperature at 18°C.

Drugs and agonists were bath-applied by switching the superfusing solution to one containing known concentrations of drugs and agonists. Rapid (1–2 s) solution changes were achieved by the use of a solenoid valve assembly.

In order to minimize damage to the motoneurones and to keep their intra- and extracellular elements intact, DC recordings of electronically conducted changes in the membrane potential were made by placing the IXth VR across a 3 mm sucrose gap. Calomel electrodes, connected *via* agar-Ringer's bridges, measured the difference in potential between the spinal cord bath and the distal end of the VR maintained in a pool of Ringer's solution. The preparation was left ungrounded. After amplification, the signals were recorded using a Gould 2400 rectilinear pen writer. These methods

provide stable, reproducible recordings of motoneurone membrane potentials in an intact spinal cord preparation.

Peak amplitude of responses to NMDA and other agonists were measured in all experiments. All data are expressed as mean ± s.e.m. Statistical significance of differences was assessed using Student's *t*-test for correlated means.

Drugs

Unless otherwise indicated, drugs were dissolved in Ringer's solution shortly before use to minimize chemical degradation. Dimethyl sulfoxide (DMSO) (0.25%) was used to dissolve staurosporine, thapsigargin, gallopamil, RS 102221, KN-62 and U73122 before diluting with Ringer's solution. Control solutions containing DMSO alone were tested and produced no effects. The pH was adjusted when necessary.

The following compounds were used in these experiments: 1-amino-3,5-di-methyladamantane (memantine), ω-conotoxin GVIA (ω-CgTX), dimethyl sulfoxide (DMSO), 5-hydroxytryptamine (5-HT), ketanserin tartrate, 2-[*N*-(4-hydroxyethyl))-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN-93), 6-methyl-1(1-methylethyl)-ergdine-8β-carboxylic acid (LY-53,857), nifedipine, *N*-methyl-D-aspartate (NMDA), 8-[5-(5-amino-2,4-dimethoxyphenyl)-5-oxopentyl]-1,3,8-triazaspirol[4,5]decane-2,4-dione (RS 102221), *N*-3-pyrinyl-3,5-dihydro-5-methyl-benzo (1,2-b;4,5-b′) dipyrrole-1(2H) (SB 206553), spiroxatrine, thapsigargin, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl-cyclohexanecarboxamide (WAY 100,635), α-methyl-5-hydroxytryptamine (α-Me-5-HT), were purchased from Sigma/RBI; guanylyl-5′-imidodiphosphate, tetralithium salt (GMP-PNP), H-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-D-Trp-D-Trp-Met-NH_2 (GP antagonist 2A), *N*-(2-aminoethyl)-5-isoquinolinesulfonamide dihydrochloride (H-9), 1-[*N*,O-*bis*-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), pertussis toxin (PTX), staurosporine, tetrodotoxin (TTX), 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]-1H-pyrole-2,5-dione (U73122) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) from Calbiochem; 1-[4-Amino-5-chloro-*N*-(1-methyl-5-indoyl)-*N*-3-methyl-5-isothiazolyl)urea (SB 204741) and 2-(3,5-dimethoxyphenyl)-methyloxy]-3-[1-[2-methylsulfonylamino]ethyl]piperidin-4-yl]-propan-1-one (RS 39604) from Tocris Cookson; (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801) was donated by Merck; 5-[(3,4-dimethoxyphenylethyl) methylamino]-2-isopropyl-2-(3,4,5-trimethoxyphenyl) valeronitrile hydrochloride (GALL) was donated by Knoll Pharmaceuticals, Ludwigshafen, Germany.

Results

Our previously reported data showed that application of high concentrations of 5-HT or the 5-HT$_2$ agonist α-methyl-5-hydroxytryptamine (α-Me-5-HT) produces a depolarization of frog motoneurones bathed in Ringer's solution (5-HT, 30 μM, 2.1 ± 0.1 mV, $n=4$, data from Holohean *et al*., 1990) (α-Me-5-HT, 30 μM, 1.2 ± 0.2 mV, $n=6$; not shown). The α-Me-5-HT-induced depolarizations of motoneurone membranes were eliminated when the spinal cord was exposed to ketanserin (10 μM, 30 min) (not shown).

In spinal cords bathed in nominally Mg^{2+}-free medium, Figure 1a shows that activation of 5HT$_2$ receptors by 5-HT (in concentrations between 30 and 100 μM, 5 min) reduced depolarizations produced by applications of NMDA (100 μM, 10 s pulses) (5-HT: 100 μM, 81 ± 3%, $n=3$, $P<0.05$; α-Me-5-HT: 100 μM, 82 ± 6%, $n=4$, $P<0.05$). In contrast, when a physiological concentration of Mg^{2+} (1.0 mM, 15 min) (Davidoff *et al*., 1988) was added to the superfusate, application of 5-HT (30–100 μM, 5 min) or α-Me-5-HT (30–100 μM, 5 min) significantly increased motoneuronal depolarizations produced by NMDA (Figure 1b, Table 1). In concentrations of Mg^{2+} (10 mM, 15 min) sufficient to block transmitter release, α-Me-5-HT (30 μM, 5 min) enhanced NMDA-induced depolarizations (Table 1). The effects of both 5-HT and α-Me-5HT on NMDA-induced depolarizations were partially reversible immediately after their removal from the superfusate and completely reversible within 45–60 min. Mg^{2+} binds to a site within the open NMDA receptor channel. In the presence of other NMDA open-channel blockers – MK-801 (3–6 μM, 15 min) and memantine (MEM, 20 μM, 15 min) – α-Me-5-HT also significantly facilitated NMDA depolarizations (Figure 2, Table 1).

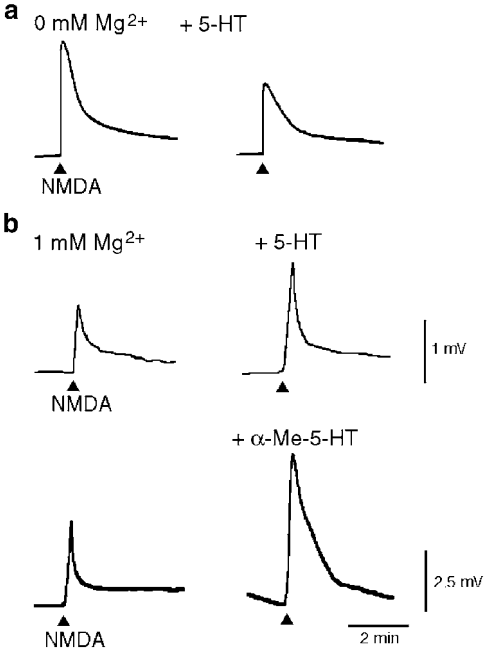

Figure 1 5-HT potentiates NMDA-evoked motoneurone depolarizations in Ringer's solution containing. Mg^{2+}. (a) Applications of NMDA (100 μM, 10 s) in nominally Mg^{2+} -free Ringer's solution before and after exposure to 5-HT (30 μM, 5 min). The traces show changes in frog motoneurone membrane potential (electronically conducted along the IXth VR). In these, and in all subsequent records, negativity is indicated by an upward pen deflection and signifies a depolarization of motoneurones whose axons exit from the cord in the IXth VR. (b) The same experiment carried out in another spinal cord in Ringer's solution containing 1.0 mM Mg^{2+} showing facilitation produced by 5-HT and by α-Me-5-HT (30 μM, 5 min). Both experiments were performed in Ringer's solution containing TTX (0.783 μM). Applications of NMDA (100 μM, 10 s) are indicated by arrows below the baseline. Note that in this and subsequent figures, the baselines in presence of 5-HT or α-Me-5-HT have been readjusted to the control baseline in order to facilitate the observation of changes in the NMDA responses.

Table 1 5-HT- and α-Me-5-HT-induced changes of NMDA-induced motoneurone depolarizations in the presence of NMDA open-channel blockers

	NMDA responses (% of control-NMDA in channel blocker)	
	In 5-HT	*In α-Me-5-HT*
Channel blocker		
Mg^{2+} (1.0 mM)	134±16% ($n=4$)*	162±12% ($n=11$)**
Mg^{2+} (10.0 mM)		144±3% ($n=3$)*
MK-801 (3–6 μM)		156±18% ($n=8$)*
Memantine (20 μM)		202±44% ($n=3$)*

Effects of 5-HT (30 μM, 5 min) and α-Me-5-HT (30 μM, 5 min) on NMDA responses expressed as a mean percentage±s.e.m. of control NMDA (100 μM, 10 s) responses in the same cords (Note – the NMDA concentration was 200 μM in 10 mM Mg^{2+} experiments.)
Mg^{2+}, MK-801 and memantine were applied for 15 min. Channel blockers and TTX (0.783 μM) were present in the Ringer's solution during the entire experiment. Mg^{2+} was not added to the Ringer's solution in experiments with MK-801 and memantine. *$P<0.05$; **$P<0.001$ (Student's paired t-test).

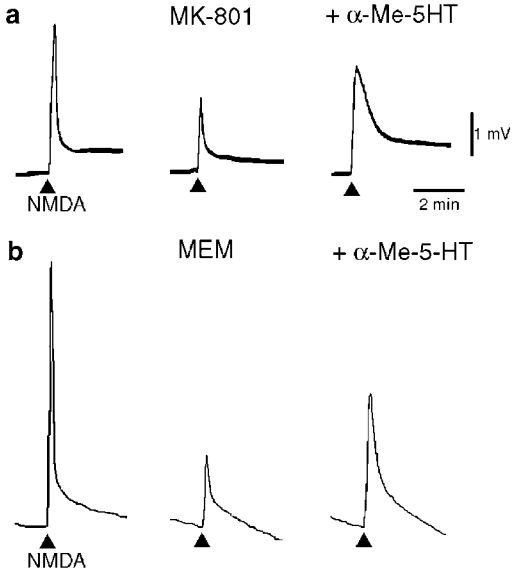

Figure 2 α-Me-5-HT-induced potentiation of NMDA responses in spinal cords exposed to NMDA receptor channel blockers. (a) Spinal cord exposed to MK-801 (6 μM, 15 min). (b) Spinal cord exposed to memantine (MEM, 10 μM, 15 min). Both experiments were performed in Ringer's solution containing TTX (0.783 μM). Applications of NMDA (100 μM, 10 s) are indicated by arrows below the baseline. Calibration bars apply to both (a and b).

Effects of 5-HT antagonists

To determine the receptor selectivity of the α-Me-5-HT-induced enhancement of NMDA depolarizations in Mg^{2+}-containing Ringer's solution, we tested the effects of compounds thought to function as selective 5-HT antagonists. As summarized in Table 2, the nonselective 5-HT$_{2A,2B,2C}$ antagonist LY-53,857 (10–30 μM, 30 min), the nonselective 5-HT$_{2B,2C}$ antagonist SB 206553 (10 μM, 30 min), and the selective 5-HT$_{2B}$ antagonist SB 204741 (30 μM, 30 min) completely blocked the potentiating effect of α-Me-5-HT on NMDA depolarizations. In contrast, the 5-HT$_{1A}$ antagonists spiroxatrine (10 μM, 30 min) or WAY 100,635 (10–30 μM, 30 min), the 5-HT$_{2A,2C}$ antagonist ketanserin (10 μM, 30 min), the 5-HT$_{2C}$ antagonist RS 102221 (10 μM, 30 min), or the 5-HT$_4$ antagonist RS 39604 (10–20 μM, 30 min) did not reduce the facilitating effect of 5-HT on responses to NMDA. None of the antagonists had direct effects on the motoneurone membrane potential; effects on control NMDA responses in Mg^{2+}-Ringer's were minimal (Table 2). It appears that activation of 5-HT$_{2B}$ receptors is responsible for the ability of α-Me-5-HT to facilitate NMDA depolarizations in medium containing Mg^{2+}.

Table 2 Effects of 5-HT antagonists on control NMDA responses and α-Me-5-HT-induced potentiation of NMDA responses

5-HT antagonists	*NMDA responses (% of control NMDA response)*	*NMDA responses in α-Me-5-HT (% of control-NMDA in antagonist)*
LY-53,857 (10–30 μM)	95±3 ($n=7$)	95±2 ($n=7$)
SB 206553 (10 μM)	96±3 ($n=7$)	98±9 ($n=7$)
SB 204741 (30 μM)	99±2 ($n=6$)	100±6 ($n=6$)
Spiroxatrine (10 μM)	91±7 ($n=3$)	124±7 ($n=3$)*
WAY 100,635 (10–30 μM)	95±6 ($n=7$)	148±22 ($n=7$)*
Ketanserin (10 μM)	103±2 ($n=4$)	173±24 ($n=6$)*
RS 1022221 (10 μM)	108±5 ($n=4$)	124±7 ($n=4$)*
RS 39604 (10–30 μM)	97±4 ($n=6$)	131±13 ($n=6$)*

Effects of 5-HT antagonists and α-Me-5-HT on NMDA (100 μM, 10 s) responses are expressed as a mean percentage±s.e.m. NMDA responses in the presence of antagonist alone for 30 min and with the addition of α-Me-5-HT for 5 min in the same cords. Antagonists, Mg^{2+} (1 μM), and TTX (0.783 μM) were present in the Ringer's solution during the entire experiment. *$P<0.05$ (Student's paired t-test).

G-proteins and 5-HT-induced effects on NMDA responses

Binding of an agonist to 5-HT$_2$ receptors is thought to involve intracellular signaling mechanisms activated *via* G-protein cleavage. We used compounds (pertussis toxin (PTX), guanylyl-5′-imidodiphosphate (GMP-PNP), H-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2 (GP antagonist 2A)) known to affect processes involving G-proteins. The premise that a G-protein is involved in α-Me-5-HT's actions on NMDA responses in Mg^{2+}-containing medium was studied by (1) incubating hemisected frog spinal cords in standard Ringer's solution either with or without the $G_{i/o/t}$ α-subunit antagonist PTX (3–6 ng ml^{-1}) for 48 h at 4°C, a protocol previously found to be effective in the frog spinal cord (Holohean *et al.*, 1999a), (2) adding GMP-PNP (100 μM, 30 min) to saturate GTP-binding sites to prevent further activation of G-proteins (Kopf & Woolkalis 1991; Paz *et al.*, 1994) or (3) adding the G_q α-subunit antagonist GP antagonist 2A (3–6 μM, 30 min) to the superfusate (Mukai *et al.*, 1992; Hunt *et al.*, 1999). At the concentrations used, neither GMP-PNP nor GP antagonist 2A had a significant effect on the control NMDA response (95.3±1.0%, $N=5$ and 99.3±1.6%, $N=3$ of control NMDA response, respectively). PTX did not block 5-HT's facilitating actions (Figure 3a), but GMP-PNP (not shown) and GP antagonist 2A (Figure 3b) eliminated 5-HT's effect on NMDA receptor activation (Table 3).

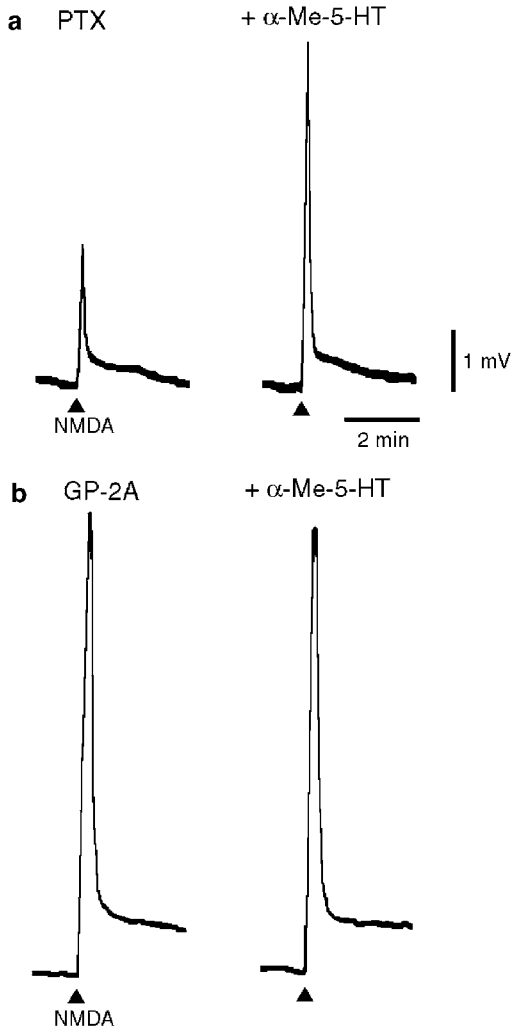

Figure 3 Potentiating effect of α-Me-5-HT on NMDA responses is unaffected by exposure to pertussis toxin, but is blocked by GP antagonist 2A. (a) Spinal cord incubated with PTX (6 ng ml^{-1}) for 48 h at 4°C. (b) Spinal cord exposed to GP antagonist 2A (GP-2A) (6 μM, 30 min). Applications of NMDA (100 μM, 10 s) are indicated by arrows below the baseline. Calibration bars apply to both (a and b).

Table 3 Effects of agents with actions on G-proteins on control NMDA responses and α-Me-5-HT-induced potentiation of NMDA responses

Treatment	*NMDA responses (% of control NMDA)*	*NMDA responses in α-Me-5-HT (% of control- NMDA in treatment)*
PTX (3–6 ng ml^{-1})		200±41 ($n=3$)*
GMP-PNP (100 μM)	95±3 ($n=3$)	100±12 ($n=3$)
GP antagonist 2A (3–6 μM)	99±2 ($n=4$)	97.2±8 ($n=4$)

PTX effects on α-Me-5-HT-induced facilitation of NMDA responses are expressed as a mean percentage±s.e.m. of NMDA (100 μM, 10 s) responses after incubation of spinal cords in PTX (48 h, 4°C) and are compared to cords incubated for the same period of time in medium without PTX. The effects of GMP-PNP (30 min) or GP antagonist 2A (30 min) on NMDA (100 μM, 10 s) control responses and α-Me-5-HT-induced (5 min) facilitation of NMDA responses are expressed as a mean percentage±s.e.m. Mg^{2+} (1 mM), and TTX (0.783 μM) were present in the Ringer's solution during all experiments. *$P<0.05$ (Student's paired t-test).

Table 4 Effects of various treatments on control NMDA responses and α-Me-5-HT-induced potentiation of NMDA responses

Treatment	*NMDA responses (% of control NMDA)*	*NMDA responses in α-Me-5-HT (% of control- NMDA in treatment)*
Staurosporine (2.0 μM)	90±4 ($n=5$)	134±8 ($n=5$)*
H-9 (77 μM)	101±1 ($n=5$)	141±14 ($n=5$)*
Thapsigargin (0.1 μM)	100±3 ($n=7$)	123±6 ($n=7$)*
Nifedipine (10 μM)	99±4 ($n=6$)	89±3 ($n=6$)
Gallopomil (100 μM)	99±3 ($n=5$)	100±4 ($n=5$)
ω-Conotoxin GVIA (0.14 μM)	104±5 ($n=3$)	129±18 ($n=3$)*
Amiloride (300 μM)	98±4 ($n=3$)	153±17 ($n=3$)*
W-7 (100 μM)	38±11 ($n=4$)	86±10 ($n=4$)
KN-62 (5 μM)	101±1 ($n=3$)	96±5 ($n=3$)
KN-93 (10 μM)	104±4 ($n=3$)	224±19 ($n=3$)*
U73122 (10 μM)	98±2 ($n=3$)	166±6 ($n=3$)*

Effects of 30 min treatments on NMDA (100 μM, 10 s) control responses and α-Me-5-HT-induced potentiations (5 min) of NMDA responses. Data expressed as mean percentage± s.e.m. of control NMDA responses. The control for NMDA responses in treatment solution was obtained in Ringer's solution and the control for NMDA in α-Me-5-HT was the response to NMDA in the treatment solution without α-Me-5-HT. Mg^{2+} (1.0 mM), and TTX (0.783 μM) were present in the Ringer's solution during each experiment. *$P<0.05$ (paired t-test).

Second messengers and Ca^{2+}

It has been hypothesized that when 5-HT$_2$ receptors in the spinal cord and other neural tissues are activated the primary cellular response is PI hydrolysis with subsequent activation of protein kinase C (PKC) and release of Ca^{2+} from intracellular stores. We examined the possible involvement of PKC in the potentiation of NMDA using two potent PKC inhibitors – staurosporine (2.0 μM, 30 min) (Tamaoki *et al*., 1986) and H-9 (77 μM, 30 min) (Hidaka *et al*., 1984; Hidaka & Kobayashi, 1992). In addition, we tested the effect of the phospholipase C antagonist U73122 (10 μM, 30 min) (Bleasdale *et al*., 1990). As Table 4 indicates, staurosporine, H-9 and U73122 were ineffective in reducing the facilitatory effects of α-Me-5-HT. To explore the possibility that the Ca^{2+} released from intracellular loci is responsible for the 5-HT receptor facilitation of NMDA responses, thapsigargin (THAP, 0.1 μM, 30 min) – a compound with the ability to deplete internal inositol 1,4,5-triphosphate (IP$_3$) activated Ca^{2+} stores (Treiman *et al*., 1998) – was applied to frog spinal cords at a dose previously found to be effective (Holohean *et al*., 1999a). Thapsigargin had no direct effect on NMDA responses and did not prevent the facilitation of NMDA responses (Figure 4a, Table 4).

In contrast, evidence that an extracellular Ca^{2+} source is necessary for 5-HT-induced facilitation of NMDA responses was provided by findings that L-type Ca^{2+} channel blockers nifedipine (10 μM, 30 min), gallopamil (100 μM, 30 min) or KN-62 (5 μM, 30 min) (Sihra & Pearson, 1995) eliminated

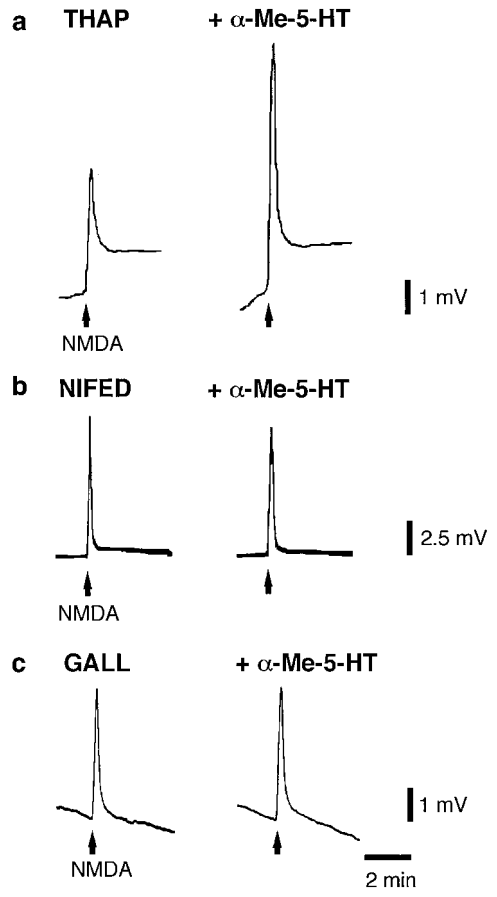

Figure 4 Nifedipine and gallopomil block the potentiating effect of α-Me-5-HT on NMDA responses but thapsigargin does not. (a) Application of thapsigargin (THAP, 0.1 μM, 30 min). (b) Application of nifedipine (NIFED, 10 μM, 30 min). (c) Application of gallopimal (GALL, 100 μM, 30 min). Applications of NMDA (100 μM, 10 s) are indicated by arrows below the baseline.

potentiation of NMDA-induced depolarizations (Figure 4b and c, Table 4). In contrast to the effects of L-type Ca^{2+} channel blockers, block of N-type Ca^{2+} channels with ω-conotoxin GVIA (ω-CgTX, 0.14 μM), or T-type Ca^{2+} channels with amiloride (300 μM) had no significant effect on the α-ME-5HT-induced enhancement of responses to NMDA (Table 4).

Calcium ions possess numerous intracellular targets including the Ca^{2+}-binding protein calmodulin. The calmodulin antagonist W7 (100 μM) significantly diminished NMDA and α-Me-5-HT-facilitated NMDA responses (Table 4) – an indication that one target of the increased $[Ca^{2+}]_i$ was the Ca^{2+}-binding protein calmodulin (Tanaka *et al*., 1982). Ca^{2+} and calmodulin are known to activate CaM Kinase II, an enzyme capable of phosphorylating NMDA receptors (McGlade-McCulloch *et al*., 1993; Gardoni *et al*., 1999). KN-93 (10 μM, 30 min), a potent and specific CaM Kinase II inhibitor does not eliminate α-Me-5-HT's facilitation of NMDA depolarizations (Table 4).

Discussion

There is little doubt that 5-HT functions as a neurotransmitter or neuromodulator in various vertebrate neurones; but opinions about how it functions are diverse. Both depressive and facilitatory interactions between various 5-HT receptors and iGluRs, the excitatory amino-acid receptors putatively mediating segmental spinal reflexes, have been described in spinal and brainstem motoneurones (cf. VanderMaelen & Aghajanian, 1982; White & Neuman, 1983; Jackson & White, 1990; Murase *et al*., 1990; Rasmussen & Aghajanian, 1990; Holohean *et al*., 1992a, b). In this study, we found that high concentrations of 5-HT and α-Me-5-HT potentiated frog motoneurone responses produced by applications of NMDA when the spinal cord was bathed in medium containing a physiological concentration of Mg^{2+} ions. Frog Ringer's solution does not customarily contain Mg^{2+} ions, but frog cerebrospinal fluid contains the cation in a concentration of 0.92 mM (Davidoff *et al*., 1988). It is presumed that the interstitial fluid surrounding frog neurones *in vivo* contains Mg^{2+} in approximately that concentration as well.

Of interest, in the presence of the NMDA channel blockers memantine and MK-801, α-Me-5-HT facilitated NMDA responses as well as-or better than-Mg^{2+}. Mg^{2+}, MK-801, and memantine bind to sites in the ion channel opened by NMDA receptor activation, thereby restricting NMDA receptor function (Huettner & Bean, 1988; Blanpied *et al*., 1997). Our data thus indicate that α-Me-5-HT potentiates NMDA responses in frog motoneurones as a result of a reduced channel block. Presumably the process involves a modified conformation of the NMDA receptor internal channel. This idea is in keeping with previous reports that 5-HT is able to regulate Mg^{2+} blockade of the NMDA channel (Chen & Huang, 1992; Blank *et al*., 1996; MacLean & Schmidt, 2001).

It is unlikely that the potentiation of NMDA responses is caused indirectly by a 5-HT or α-Me-5-HT-induced motoneurone membrane depolarization – a depolarization might lead to a reduction of the level of Mg^{2+} block. We have previously shown that depolarization of frog motoneurones to levels equivalent to the motoneurone depolarization produced by α-Me-5-HT did not enhance NMDA responses (Holohean *et al*., 1999a). In the present study, the α-Me-5-HT-facilitation of NMDA responses was seen when ketanserin eliminated the amine-generated depolarization.

The 5-HT$_{2B}$ receptor has the properties of what was previously classified as the 5-HT$_1$-like receptor in the stomach fundus (also designated as the 5-HT$_{2F}$ receptor). We previously demonstrated that high concentrations of 5-HT and α-Me-5-HT activated 5-HT$_2$ receptors on frog motoneurones (Holohean *et al*., 1990). In the present study, the facilitating effect on frog motoneurone NMDA depolarizations produced by the same concentrations of these two compounds appears to cause 5-HT$_{2B}$ receptor activation. The assumption that 5-HT$_{2B}$ is the receptor responsible for NMDA potentiation is based on the reported high affinity of α-Me-5-HT for 5-HT$_{2B}$ receptors and buttressed by our observations using a range of 5-HT antagonists (Richardson *et al*., 1985). We found that the selective 5-HT$_{2B}$ antagonist SB 204741 completely blocked the potentiating effect of 5-HT on NMDA-depolarizations. SB 204741 is more than 20- to 60-fold more selective for the 5-HT$_{2B}$ than for the 5-HT$_{2A}$, 5-HT$_{2C}$, and other receptors at which it has been tested (Baxter *et al*., 1995). In contrast, antagonists selective for 5-HT$_{1A}$, 5-HT$_{2A,2C}$, and 5-HT$_4$ receptors were without effect. Additional albeit indirect support is provided by observations that 5-HT$_2$ receptors are present on the dendrites of adult rat spinal motoneurones

(Ridet *et al*., 1994). More specifically, mRNA for 5-HT$_{2B}$ receptors can be detected in mammalian spinal cord tissue (Helton *et al*., 1994).

Second messengers, Ca^{2+} release, and the facilitating actions of α-Me-5-HT

In a number of neural systems cleavage of membrane phospholipids, following activation of 5-HT$_2$ receptors, yields IP$_3$ and diacylglycerol (DAG). IP$_3$ causes a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) by mobilizing Ca^{2+} from internal stores, and DAG is known to activate PKC. However, we could not find evidence that either Ca^{2+} release from internal stores or PKC activation are involved in the α-Me-5-HT facilitation of NMDA depolarization of frog motoneurone. Thapsigargin (which blocks Ca^{2+}/ATPase-dependent Ca^{2+} uptake into intracellular compartments thereby depleting intracellular Ca^{2+} (Treiman *et al*., 1998)) did not prevent the ability of α-Me-5-HT to facilitate NMDA depolarization. Similarly, staurosporine (Tamaoki *et al*., 1986), H9 (Hidaka & Kobayashi, 1992), effective inhibitors of PKC and U73122 (Bleasdale *et al*., 1990), a phospholipase C inhibitor, were without effect on potentiation of NMDA depolarizations. And, as we have previously shown, the phorbol ester PMA (phorbol-12-myristate 13-acetate), a potent activator of PKC, does not enhance NMDA-depolarizations (Holohean *et al*., 1999a).

Although evidence that 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors couple to PI hydrolysis is substantial, such coupling has not been unequivocally established for the 5-HT$_{2B}$ receptor. When expressed heterologously in mammalian cells, 5-HT$_{2B}$ receptors stimulate PI hydrolysis; but they do not do so in the rat stomach fundus where they are native receptors (Cohen & Wittenauer, 1987; Secrest *et al*., 1989; Wainscott *et al*., 1993; Schmuck *et al*., 1994). Moreover, it has not been proven that native 5-HT$_{2B}$ receptors in the CNS couple to PI hydrolysis. Our data would indicate PI is not involved in 5-HT$_{2B}$ enhancement of NMDA responses in frog spinal cord.

Ca^{2+} channels

A role for Ca^{2+} in NMDA facilitation was provided by experiments using dihydropyridine nifedipine, phenylakylamine gallopamil, or KN-62 to block the effects of α-Me-5-HT. Sensitivity to dihydropyridines is considered the hallmark of L-type Ca^{2+} channels. Gallopamil is a methoxy derivative of verapamil which also blocks L-type Ca^{2+} channels (Bournaud *et al*., 1998). KN-62, is a CaM-Kinase II inhibitor with the ability to block L-type Ca^{2+} channels (Sihra & Pearson, 1995). Accordingly, we believe that the Ca^{2+} necessary for the potentiation of NMDA responses enters frog motoneurones by means of L-type Ca^{2+} channels. In contrast to the actions of nifedipine or gallopamil, ω-CgTx, which blocks N-type Ca^{2+} channels, and amiloride, which blocks T-type Ca^{2+} channels, did not affect the augmentation of NMDA responses. In accordance with our results, there is evidence that activation of 5-HT receptors can increase Ca^{2+} currents in mammalian neurons (Nedergaard *et al*., 1988; Berger & Takahashi, 1990) and reptile motoneurons (Perrier & Hounsgaard, 2003). In particular, the contractile response of the rat stomach fundus produced by activation of 5-HT$_{2B}$ receptors is partially sensitive to antagonists of the L-type Ca^{2+} channel (Secrest *et al*., 1989). Opening of L-type Ca^{2+} channels, and the subsequent influx of extracellular Ca^{2+}, plays a major role in 5-HT-induced contraction of the fundus. Perhaps the signal transduction mechanisms coupled to the 5-HT$_{2B}$ receptor differ substantially from those of other members of the 5-HT$_2$ receptor family.

G-proteins

We found that facilitation of NMDA responses by α-Me-5-HT was blocked by GMP-PNP, a hydrolysis-resistant GTP analog that irreversibly activates G-proteins by saturating most GTP-binding sites, and by GP antagonist 2A, an inhibitor of $G\alpha_q$ subunit activation, but not by PTX, which blocks the coupling of some, but not all, $G\alpha_{i,o,t}$ subunits with their receptors (Bokoch *et al*., 1983; Mukai *et al*., 1992). Our finding that the facilitation of NMDA was mediated by G-proteins, presumably by subunits linked to $G\alpha_q$ was not unexpected: nearly all other examples of transmitter modulation of Ca^{2+} currents involve G-proteins (Hille, 1994). We assume that a component of the activated G-protein interacts directly with the L-type Ca^{2+} channel to increase the probability of its opening. We found no evidence that conventional second-messenger systems are responsible for our results. We did not find PI hydrolysis to be involved in the facilitation of NMDA depolarizations by α-Me-5-HT and we have previously reported that elevation of intracellular levels of cAMP and arachidonic acid were without effect on NMDA responses in frog motoneurones (Holohean *et al*., 1999a).

Is it possible that the presumed Ca^{2+} channel opening in frog motoneurones seen in our experiments might not be a direct result of 5-HT$_{2B}$ receptor activation. In earlier studies, we demonstrated that 5-HT$_2$ receptors depolarize frog motoneurones (Holohean *et al*., 1990). 5-HT-depolarization of mammalian motoneurones has been attributed to reduction of K^+ conductances which in turn may unmask an inward Ca^{2+} current (Takahashi & Berger, 1990; Larkman & Kelly, 1992). But as mentioned above, experiments with ketanserin and with elevated $[K^+]_o$ show that motoneurone depolarization is neither necessary nor sufficient to potentiate NMDA responses.

Increased $[Ca^{2+}]_i$ and calmodulin

There is an inexact understanding of where and how Ca^{2+} affects NMDA receptors. NMDA receptors are transiently inactivated and desensitized by increased $[Ca^{2+}]_i$ (Legendre *et al*., 1993; Vyklicky, 1993; Rosenmund *et al*., 1995). In contrast, the elevated $[Ca^{2+}]_i$ that results from the formation of PI derivatives has been postulated to underlie the potentiation of NMDA responses produced by several neurotransmitters (Markram & Segal, 1992; Rahman & Neuman, 1996). These disparities may be reconciled by reports that intracellular Ca^{2+} does not consist of a single homogeneous pool. There are at least two pools of readily releasable Ca^{2+}: the IP$_3$-releasable stores and the Ca^{2+}-induced Ca^{2+} stores (Henzi & MacDermott, 1992). Discrete pools of $[Ca^{2+}]_o$ may influence NMDA receptor function in dissimilar ways. In this regard, we have previously shown in frog motoneurones that whereas caffeine – which induces release of Ca^{2+} from the Ca^{2+}-induced release stores – does in fact reduce NMDA responses, generation of phosphoinositides with subsequent release of $[Ca^{2+}]_o$ by metabotropic glutamate receptors

potentiates the actions of NMDA (Hackman *et al*., 1994; Holohean *et al*., 1999a).

Ca^{2+} exerts most of its intracellular effects *via* interaction with Ca^{2+}-binding proteins. Among the Ca^{2+}-binding proteins, the ubiquitous, multifunctional calmodulin is a major Ca^{2+} receptor. Because W-7, a potent calmodulin inhibitor, reduced α-Me-5-HT-potentiation of NMDA depolarizations, calmodulin appears to be a necessary substrate for the Ca^{2+}-mediated facilitation of such responses. Calmodulin is involved in the activation of many important enzymes, including CaM Kinase II. Of pertinence to the present experiments are findings that NMDA receptors are associated with CaM Kinase II (Husi & Grant, 2001). However, selective inhibition of CaM Kinase II by KN-93 did not prevent α-Me-5-HT-facilitation of NMDA depolarizations. Activation of CaM Kinase II does not appear to be necessary for enhanced NMDA depolarization.

Taken together, our results suggest that the potentiation of NMDA-induced depolarization by α-Me-5-HT is caused by a mechanism that involves: (1) activation of 5-HT$_{2B}$ receptors; (2) activation of a G-protein, presumably, $G\alpha_q$; (3) a transduction mechanism (apparently independent of PI turnover) causing an influx of extracellular Ca^{2+} through L-type Ca^{2+} channels; (4) binding of Ca^{2+} to calmodulin; and (5) reduction of the open-channel block of the NMDA receptor produced by physiological concentration of Mg^{2+} ions. The proposed mechanism for 5-HT$_{2B}$ receptor activation-induced modulation of NMDA depolarization is in contrast to our previous report on ACPD-induced modulation of NMDA-induced activity (Holohean *et al*., 1999a) that depended on Ca^{2+} from IP_3-mediated release of intracellular stores. This subtle difference in the mechanism of Ca^{2+}-mediated NMDA modulation by two different transmitters (5-HT and glutamate) argues for a subcellular compartmentalization of NMDA receptors with specific metabotropic receptors activating different modulatory signaling pathways that have local effects.

The different effects of 5-HT receptors on NMDA receptors may play a role in the functional regulation of spinal cord rhythmicity and locomotion. Activation of both 5-HT and NMDA receptors are necessary for the observed rhythmic actions in the spinal cord (Beato & Nistri, 1998; Wallis *et al*., 1998). Our results indicate that the interactions are concentration dependent and complex. At low 5-HT levels, the 5-HT$_{1A}$ receptor enhances NMDA-induced depolarizations in a non-Mg^{2+}-dependent manner (Holohean *et al*., 1992a). At higher levels of neuronal activity, the increased level of 5-HT released may activate 5-HT$_2$ receptors (Holohean *et al*., 1990). If the NMDA receptor is partially blocked by Mg^{2+} ions 5-HT$_{2B}$ receptors can act to greatly enhance NMDA-induced depolarizations. However, if the NMDA receptor is completely unblocked then the 5-HT$_{2A/2C}$ receptors will act to depress the NMDA-evoked depolarizations and possibly prevent overexcitation of the NMDA receptors (Holohean *et al*., 1992b). Thus, the excitation level of the spinal cord can dictate the 5-HT receptors that predominate the modulation of NMDA receptor activity. Multiple 5-HT receptors activating different modulatory mechanisms may act as switch components within a circuit that modulates motoneurone output.

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