SEROTONERGIC MODULATION OF ION CHANNELS IN THE SPINAL NEURONS OF *XENOPUS* LARVAE

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ABSTRACT

Serotonin modulates both the locomotion motor pattern and sensory transmission in *Xenopus* larvae. I have explored the cellular and ionic mechanisms underlying the serotonergic modulation, by using whole-cell patch clamp recordings from acutely isolated spinal neurons of *Xenopus* larvae. The primary sensory (R-B) neurons possess N, P/Q, L and R type HVA currents and T-type Ca^{2+} currents. 5-HT_{1A} and 5-HT_{1D} receptors, caused purely voltage-independent inhibition of the N and P/Q HVA currents and the T-type currents. The inhibition of HVA currents is mediated by a pertussis toxin-sensitive G-protein acting through a diffusible second messenger. By using non-hydrolyzable GTP analogues and synthetic peptides derived from the conserved cytoplasmic regions of the rat 5-HT_{1A} and 5-HT_{1D} receptors, I have found that 5-HT modulates T-type channels through a membrane-delimited pathway that does not involve G-proteins and involves functional domains of the receptor that are distinct from that which couples to G-proteins. I have found that these channels play a role in spike initiation in R-B neurons. Modulation of T-type channels by 5-HT could therefore modulate the sensitivity of this sensory pathway, by increasing the thresholds of R-B neurons.

In the non-sensory neurons, the 5-HT_{1A} and 5-HT_{1D} receptors produced different forms of inhibition. Whereas the T, L and R channels are not modulated by 5-HT, the N and P/Q channels were differentially modulated. The inhibition of P/Q channels was voltage-independent and the modulation of N channels had both voltage-dependent and -independent components. The 5-HT_{1A} receptors not only preferentially caused voltage-independent inhibition, but did so by acting mainly on the ω-agatoxin-IVA sensitive Ca^{2+} channels. In contrast, the 5-HT_{1D} receptor produced both voltage-dependent and -independent inhibition and was preferentially coupled to ω-conotoxin-GVIA sensitive channels. This complexity of modulation may allow fine tuning of transmitter release and calcium signaling in the spinal circuitry of *Xenopus* larvae. The functional consequences of modulation of N and P/Q channels in both sensory and non-sensory neurons were discussed.

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AUTHOR'S DECLARATION

(i) I, Qian-Quan Sun, hereby certify that this thesis, which is approximately 56,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree. The work is original and carried out by the author except the appendix which was written in collaboration with Dr Nicholas Dale. In conjunction with Dr. Nicholas Dale, the following Chapters have been published: a) Chapter 2 was published in *Journal of Neuroscience* **17**, 6839-6849; b) Chapter 4 was published in *Journal of Physiology* **510,** 103-120; c) The appendix was published in *Journal of Physiology* **507**,257-264. The views expressed in the thesis are those of author and not of the University.

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(ii) I was admitted as a research student in October, 1996 and as a candidate for the degree of Ph.D. in October, 1996; the higher study for which this is a record was carried out in the University of St Andrews between 1996 and 1998.

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(iii) I hereby certify that the candidates has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph. D. in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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Chapter 1

GENERAL INTRODUCTION

Released from the descending fibres of the raphe nucleus, 5-HT has contrasting actions on the sensory and locomotor behaviours of vetebrates: it enhances motor bursts and inhibits sensory transmission. Although several receptor subtypes (mostly 5-HT_1 and 5-HT_2 classes) have been implicated in modulation of neuronal fuction, the cellular and ionic mechanisms underlying serotonergic modulation of sensory and motor circuits are not totally clear. A general consensus is that like other neurotransmitters, such as the muscarinic and adrenergic transmitters, 5-HT receptors mediate their major actions exclusively through G-proteins (but see Hall et al., 1998). Activation of these receptors can alter the gating of various ion channels and change the properties of neurones, such as dentritic space constants, somata firing pattern and the quantal content of release from nerve terminals. Currently, on the mechanistic side, the quest to identify the components and mechanisms underlying the G-protein mediated modulation of ion channels has become a very hot spot and getting hotter (cf. Wess, 1998; Wickman & Clapham, 1996; Dunlap, 1997; Dolphin, 1998). However, much less attention has been given to the physiological role of the modulation by different neurotransmitters (cf. Hille, 1994). This has been hampered because of the difficutlies in cooperating the shifting role of neurones as the stage of animal behaviours. This is particularly true in mammalian preparations, where it is more difficult to obtain robust and easily accessible neurones for patch clamp recordings; and the neural circuits are very difficult to define. The actions of 5-HT therefore need to be studied in a model system where its actions can be characterised in detail and in a functional context. *Xenopus* embryo and larva spinal cord is an ideal model system, in which the role of most of the ion channels, and the neural circuits underlying sensory transmittion and locomotion have been largely elucidated. 5-HT modulates both sensory transmission and locomotion in *Xenopus* larvae. In this chapter, I will summarize the present state of knowledge that forms the background to my work in the following aspects: (1) Neuronal voltage-gated Ca^{2+} channels; (2) GTP-regulatory proteins and G-protein-coupled receptors; (3) Modulation of Ca^{2+} channels by G-protein-coupled receptors; (4) Neuropharmacology of 5-HT receptors; (5) Postsynaptic actions of 5-HT; (6) Serotonergic modulation of motor circuits; (7) Serotonergic modulation of sensory transmission in spinal cord; (8) *Xenopus* embryo and larvae as models for studying the control of locomotion.

1.1 Neuronal voltage-gated Ca^{2+} channels

Voltage-gated Ca^{2+} channels are transmembrane proteins that undergo voltage-sensitive conformational changes in response to membrane depolarisation. When open, they allow passage of Ca^{2+} through the channel pore without significant influx of any other ion. The resulting entry of Ca^{2+} ions into the cell can trigger a series of important physiological functions, such as patterning of neuronal firing (Dale, 1995 a & b), neurotransmitter and hormone secretion (Luebke et al., 1993; Turner et al., 1993; Wheeler et al., 1994), Ca^{2+}-dependent spikes (Huguenard, 1996), gene expression (Duncan et al., 1998), neuronal degeneration and death (Choi, 1988). In section 1.1, I will briefly discuss the following aspects: (1) the structures that control the voltage-dependent gating and selectivity; (2) the molecular and pharmacological diversity of Ca^{2+} channel types; and (3) how Ca^{2+} channels are modulated. Particularly, emphasis will be given to the modulation of the voltage-gated Ca^{2+} channels by G-protein coupled receptors, because in the past few years, tremendous progress has been made in this area, and this also forms the background to a large part of my work.

1.11 Diversity of Ca^{2+} channels

The existence of different types of Ca^{2+} channels was initially documented through kinetic studies, then by pharmacological characterisation, and most recently by molecular cloning (Fox, et al., 1987a & b; Bean, 1989b; Tsien, et al., 1991; Snutch & Reiner, 1992). Currently, at least 6 classes of Ca^{2+} channels have been identificalled T, L, N, P, Q, R type Ca^{2+} channels (Table 1.1). These Ca^{2+} channels can be distinguished by kinetic and pharmacological criteria, and fall into two major categories: the low-voltage-activated (LVA) and high-voltage-activated (HVA) channels. LVA channels were also named T-type channels by Tsien' s group and were initially found in cardiac cells but later also found in neurones (Tsien, 1983; Carbone & Lux, 1984).

T-type channels T-type channels are kinetically distinct from others. They activate at negative voltages near the threshold for action potentials (between -60 mV to -40 mV), and start to inactivate when the resting potential is more positive than -90 mV. They open and inactivate rapidly but close (deactivate) much more slowly than other Ca^{2+} channels. The T channels have a low conductance (5-9 pS) and small unitary currents compared to HVA channels. Another characteristic is that T-type channels are not selective for Ba^{2+} over Ca^{2+}. Y^{3+}, La^{3+}, Ni^{2+} and Zn^{2+} appear to effectively block the T- channels with potency sequence of Y^{3+} >La^{3+} >Zn^{2+} >Ni^{2+} (see Huguenard, 1996; Ertel & Ertel , 1997).

HVA channels of L N P Q & R type The rest of the Ca^{2+} channels were collectively named as high-voltage-activated (HVA) Ca^{2+} channels. These include the L, N, P, Q, and R type Ca^{2+} channels (cf. Birnbaumer, et al., 1994; Table 1.1). Initially, the L-type and N-type Ca^{2+} channels were described on the basis of their pharmacology and kinetics. L channels can be blocked by 1,4-dihydropyridines (DHP), and thus were also called DHP-sensitive channels. The name of L-type channels derives from the fact that these channels typically had long inactivation kinetics and a large unitary conductance that distinguishes them from most other HVA Ca^{2+} channels (Tsien et al., 1987).

In comparison with L-type channels, **N-type channels** are more prone to inactivate and are susceptible to different organic blockers (Nowycky et al., 1985; Fox et al., 1987 a, b). The N-type Ca^{2+} channels were originally identified by their characteristic electrophysiological properties in chick sensory neurones, and named N because their intermediate inactivation kinetics made them neither transient (T) nor long-lasting (L). High sensitivity to ω-conotoxin GVIA is a definitive pharmacological hallmark for the presence of N-type Ca^{2+} channels in mammalian systems. N-type channels are restricted almost entirely to neurones in both peripheral and central nervous systems.

On pharmacological grounds, another subtype called **P -type channels** was first identified in cerebellar Purkinje cells bodies, hence the name P-type (Llinas et al., 1989). P-type channels are sensitive to block by the spider peptide toxin ω-agatoxin

and a polyamine toxin, FTX, from the funnel web spider. The P-type channels of Purkinje neurones are also blocked by ω-conotoxin-MVIIC, but the rate of onset of block is rather slow (Swartz, et al., 1993). More recently Q and R-type Ca^{2+} channels have also been further separated from other HVA Ca^{2+} channels, also according to pharmacology and kinetics.

Q channels are insensitive to DHP and ω-conotoxin-GVIA but, like P channels, are sensitive to ω-agatoxin-IVA. However, Q channels are much less sensitive to block by ω-agatoxin-IVA (K_d around 70 nM) than P channels (K_d around 2 nM). Kinetically, P currents show no decay during long depolarising pulses, but Q-type currents are rapidly inactivating (Randall & Tsien, 1995). However, this designation may by overly simplistic and more evidence is needed to prove the existence of a unique class of Q channels.

R channels were defined as HVA channels which were not blocked by any of the known Ca^{2+} channel blockers, such as dihydropyridine, ω-conotoxin-GVIA, ω-agatoxin-IVA or ω-conotoxin MVIIC (Ellinor et al., 1993). R channels may also be much more subject to inactivation than other HVA channels (cf. Randall & Tsien, 1995; Page et al., 1997).

1.12 Molecular identity of Ca^{2+} channel proteins

Molecular cloning techniques have identified six α1 genes that encode Ca^{2+} channels as well as large number of auxiliary subunits (α2, β,γ, and δ-) that are associated with the α1 subunits and may modify their properties. The deduced amino acid sequences of the Ca^{2+} channel α1 subunits show that they are structurally similar and are evolutionarily related to voltage-gated Na^+ and K^+ channels. The α1 subunit serves as both the pore and the voltage-sensor, and also bears binding sites for three classes of Ca^{2+} channel drugs (Tanabe et al., 1988; Mikami et al., 1989; Perez-Reyes, et al., 1989; Biel et al., 1993). Analysis of the hydrophobicity suggests that it is embedded in the membrane (Catterall, 1991; see Snutch & Reiner, 1992; McCleskey 1994; Varadi

the membrane (Catterall, 1991; see Snutch & Reiner, 1992; McCleskey 1994; Varadi et al., 1995 for review). A significant proportion of the heterogeneity of Ca^{2+} channels is due to the expression of a unique α1-subunit (Table 1.1). In addition to considerable sequence divergence among the different genes, the chromosomal localisation of four genes suggests that Ca^{2+} channel α1 genes represent a multigene family (reviewed by Hofmann et al., 1994; Table 1.1). Complementary DNAs of the α1 subunits have been isolated and the functional expression of cDNAs isolated thus far have revealed properties that fall into the L, N, P/Q, R and T categories (Table 1.1).

Table 1.1 Summary of Ca^{2+} channel diversity and classification

Activation Voltage	Functional type	Anatagonists	α-1subunits class	Chromosomal localisation
	L	DHP	S*	1q32 (human)
	L	DHP	C	12p13.3 (human)
	L	DHP	D	3p14.3 (human)
				14 (mouse)
High-Voltage-Activated	N	ω-Conotoxin-GVIA (< 100nM) ω-Conotoxin-MVIIC; ω-Agatoxin- IIIA	B	2, band A (mouse)
	P	ω-Agatoxin-IVA (<10 nM) ω-Conotoxin-MVIIC	A	n.d.
	Q	ω-Agatoxin-IVA (>70 nM) ω-Conotoxin-MVIIC	A?	n.d
	R		E	n.d
Low-Voltage-Activated	T	Y^{3+}, La^{3+}, Ni^{2+}	G	17q22 (human) 11(mouse)

DHP: dihydropyridine; n.d., not determined. *: all α-subunit genes are found in nervous system, except α1S, which only exists in skeletal muscle.

Table 1.1 Summary of the biophysical and pharmacological and molecular characterisation of neuronal Ca^{2+} channels.

Neuronal L channels are encoded by two distinct classes of α1-subunit: class C and class D. Overall, the **class C** and **D α1-subunits** are more closely related to each other (70-76% amino acid identity) than to the class A and class B subunits (30-44%).The structural similarity between the class C and D proteins is also reflected in their functional properties. However, the class C and D Ca^{2+} channels appear to differ from each other in their current voltage relations as well as pharmacologically. The Class D but not the class C channels are partially and reversibly blocked by 10-15 μM ω-conotoxin-GVIA (Williams et al., 1992). **P/Q channels** are encoded by the **class A α1-subunit** (Mori et al., 1991; Starr et al., 1991; see review by Snutch & Reiner, 1992). The class A α1-gene from rabbit brain encodes a HVA Ca^{2+} channel that is insensitive to both DHP antagonists and ω-conotoxin-GVIA, but is blocked by crude venom from the funnel web spider (Mori et al., 1991). Ca^{2+} channels which were functional expressed from rat brain class A α1 gene produced current kinetically similar to P channels, which were partially blocked by ω-agatoxin-IVA (200 nM) and substantially blocked by ω-conotoxin-MVIIC but unaffected by dihydropyridines and ω-conotoxin-GVIA. This suggests that the α1A channels share some similar properties with both P and Q channels (Stea et al., 1994). Using an antisense oligonucleotide (ODN) against α1A, P-type calcium channel current were reduced and thus suggests that α1A encodes for P channels (Gillard et al., 1997). The **class B α1 subunit** encods for the **N-type channels**. Class B α1 subunits are analogous to class A subunits, and share >80% amino acid identity in the four domains with class 1A subunits. Evidence that links the class B subunits to N-type channels includes: 1) antibodies directed against the class B α1 subunit immunoprecipitate radiolabelled brain ω-conotoxin binding sites; and 2) class B transcripts are selectively localised in the nervous system (Snutch and coworkers 1992; see Snutch & Reiner, 1992 for review). More recent evidence has shown that the class B α1 subunit, along with the α2 and β subunits, contributes to the efficient assembly and functional expression of the N channel complex (Williams et al., 1992a; Witcher et al., 1993; Brust et al., 1993; Stea et al., 1993; Bleakman et al., 1995). Heterogeneity has also been found within the class B α1 subunits. There are at least two subtypes, named α1B-1 and α1B-2. These combined with the herterogeneity in the α2 and β subunits contribute to the existence of many biophysical variants of the N-type Ca^{2+} channel (Williams et al., 1992b).

the α2 and β subunits contribute to the existence of many biophysical variants of the N-type Ca^{2+} channel (Williams et al., 1992b).

T-type channels was originally thought to be encoded by the **α1-E gene** (Bourinet et al., 1996). But more recent evidence suggests that **α1-E channels** are more similar to **R channels** than to T channels (Tsien and coworkers, cf. Ertel & Ertel, 1997; Page et al., 1998). Indeed, very recent work has found the gene that encodes the T-type channels. This has been named **α1G**, and has similar alignments of the putative membrane spanning region to those of the α1C and α1E subunits. Functional expression of α1G in *Xenopus* oocytes produced currents with kinetic characteristics identical to those of T-type channels (Perez-Reyes et al., 1998).

1.13 Structure

Conceptual translation of the α1 cDNAs has revealed a relatively simple generalised secondary structure (Fig 1.4), consisting of four repeating motifs (I-IV), each motif comprising six hydrophobic segments (S1-S6) that are embedded in the membrane. This suggests the same transmembrane topology as that described for Na^+ and K^+ channels (Tsien et al., 1991; Jan & Jan, 1992; see also for review by Hofmann, et al.,1994; Dolphin, 1998). The short N-terminal and the long C-terminal domains of the protein are positioned intracellularly. One transmembrane segment (S4) in each motif contains a positively charged amino acid in every third or fourth position. It has been demonstrated experimentally for Na^+ and K^+ channels that this S4 segment may be the voltage sensor for voltage-gated ion channels (Stuhmer et al., 1989).

Four other peptides α2, β, γ, and δ co-purify with the main subunit from muscle and nerve. The α2 and δ subunits are linked to the α1 subunits by disulphide bridges (Caterall, 1991; Witcher et al., 1993). Although the α1 subunits play a central role in channel function, the auxiliary subunits have been shown to various extents to modulate the activation and inactivation kinetics of the channel. For example, when α1 subunits are expressed alone in frog oocytes, relatively few functional Ca^{2+}

channels are produced, but co-expression with various other subunits greatly increases Ca^{2+} current amplitudes. Thus these other subunits, such as β and α2 must play important but as yet undefined roles, even though they do not form the pore or voltage-sensor (cf. Stea et al., 1993; Berrow et al., 1995).

Ca^{2+} influx via these channels plays prominant role in the propagation and translation of nervous signaling. Thus the modulation of these channels could change the activity of target neurones. So far, most of the Ca^{2+} channels have been found to be the target of modulation by the GTP-regulatory proteins.

1.2 G-proteins and G-protein-coupled receptors

1.21 GTP-regulatory proteins

G-proteins are heterotrimers composed of α, β and γ subunits, and are classified by virtue of their α subunits (Fig 1.1 & Table 1.2). Initially, Gα subclassification was based on adenylyl cyclase regulation. Thus the G_s subclass stimulates and the G_i subclass inhibits adenylyl cyclase (Katada, et al., 1987; Taussig et al., 1993), while the G_0 subclass has no effect (Neer et al., 1984; Sterweis et al., 1984). To date, 23 distinct α subunits encoded by 17 different genes are known and can be subdivided into 4 families based on the degree of primary sequence homology: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$ (reviewed by Simon et al., 1991; Olate & Allende, 1991; Sprang, 1997, as shown in Table 1.2). The cellular concentration of G-proteins belonging to the $G_{i/0}$ family considerably exceeds those of the other families.

By contrast, there seems to be less diversity in the structure of the G-protein βγ subunits. This led to the suggestion that G βγ subunits are interchangeable among G proteins (reviewed by Olate & Allende, 1991; Hepler & Gilman, 1992; Wickman & Clapham, 1995; Clapham & Neer, 1997; Fig 1.1).

Fig 1.1 Schematic graph showing the transmembrane signalling by activation of a G-protein herterotrimer

When the receptor (R) binds its cognate ligand (A), it changes shape and its intracelluular domains catalyse the release of GDP from Gα subunits and its replacement by GTP. GTP-α and Gβγ subunits are set adrift in the plane of the membrane to bind and activate their downstream effector molecules. The process can be persistently stimulated by aluminum fluoride (AlF_4^-) or the non-hydrolysable analogues of GTP, such as GMP-PNP or GTP-γ-S, and can be blocked by GDP-β-S.

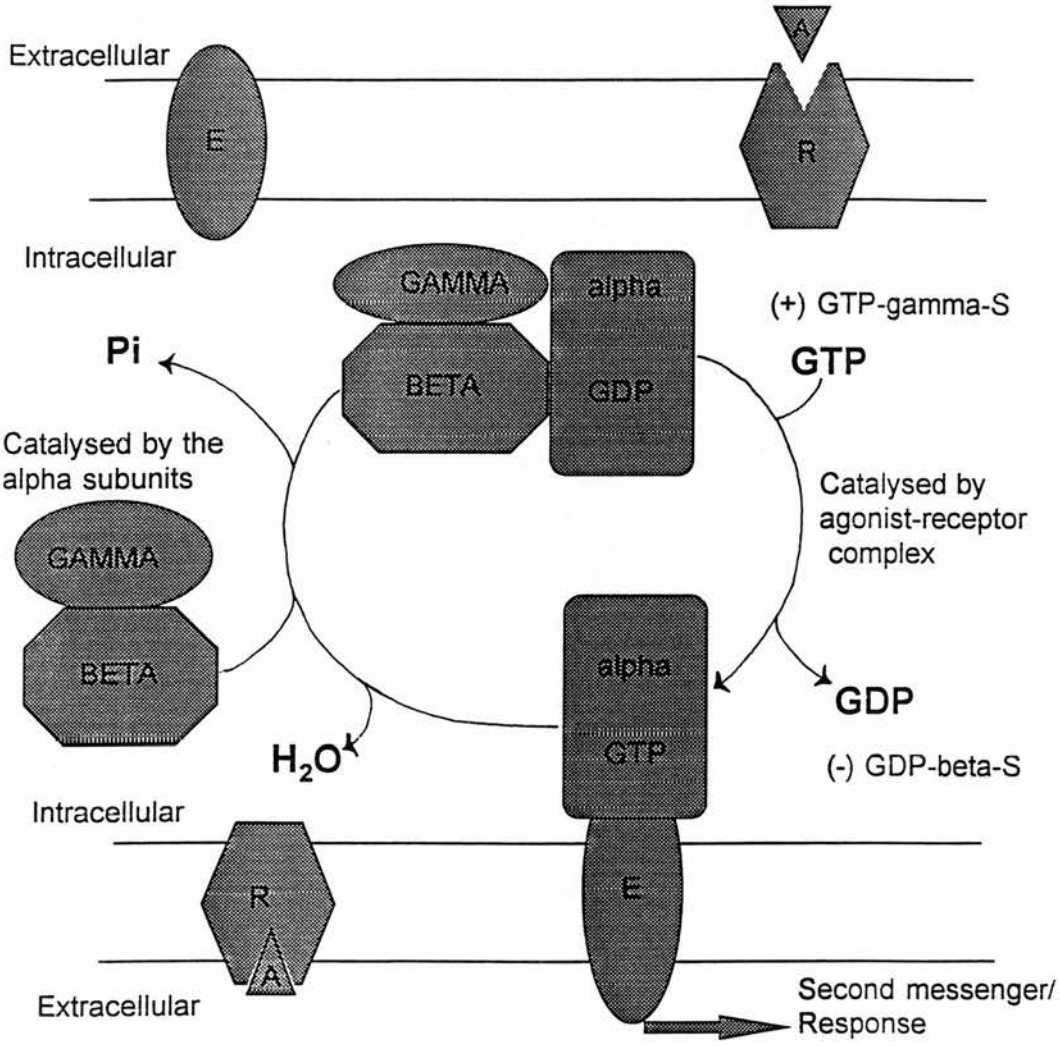

Extracellular
A
E
R
Intracellular
GAMMA
alpha
BETA
GDP
(+) GTP-gamma-S
Pi
GTP
Catalysed by the
alpha subunits
Catalysed by
agonist-receptor
complex
GAMMA
BETA
alpha
GTP
GDP
H_2O
(-) GDP-beta-S
Intracellular
R
A
E
Extracellular
Second messenger/
Response

Table 1.2. Characteristics of cloned mammalian G_α (Adapted from Wickman and Clapham, 1995)

α-subunits	size KDa	Expression	Example of effector	Toxin
G_s Family				
$G\alpha_s$-1	52	Ubiquitous	↑ Adenylyl cyclase	CTX
$G\alpha_s$-2	52			
$G\alpha_s$-3	45			
$G\alpha_s$-4	45	Olfactory neurones		
$G\alpha_{10}$	45			
G_i Family				
$G\alpha_{gust}$	41	Taste buds	↑cGMP-PDE	PTX
				CTX
$G\alpha_t$-1	39	Retin (rod)		
$G\alpha_t$-2	40	Retina (cone)		
$G\alpha_i$-1	41	Brain		
$G\alpha_i$-2	40	Ubiquitous	↓ Adenylyl cyclase	
$G\alpha_i$-3	41			PTX
$G\alpha_0$-1	39	Brain	↓ Ca^{2+} channel	
$G\alpha_0$-2	39			
$G\alpha_z$	41	Brain, platelets	↓ K^+ channel	Neither
G_q Family				
$G\alpha_q$	41	Ubiquitous	↑PLC-β	
$G\alpha_{q11}$	41			
$G\alpha_{q14}$	42	Stroma cells		
$G\alpha_{q15}$	43	B lymphocytes		
$G\alpha_{q16}$	43	Monocytes, T-cells		
G_h family	44	Ubiquitous	?	

PTX, pertussis toxin; CTX, cholera toxin; cGMP-PDE, cGMP-dependent phosphodiesterase; PLC-β, phospholipase C-β.

1.22 G-protein-coupled receptors

Despite the remarkable structural diversity of their active ligands, all G-protein-coupledreceptors are thought to share a common molecular architecture consisting of

7 transmembrane helices (TMI-VII) linked by alternating intracellular (i1-3) and extracellular loops, and to be members of a gene superfamily (Fig 1.2). The bestcharacterised 7 transmembrane domain receptor of this class of G-protein coupled receptors is the β-adrenergic receptor (Kobilka et al., 1992). Mutational analysis of this receptor and other G-protein-coupled receptors has shown that a ligand binding pocket is formed by the 7 transmembrane spanning domains, while the amino acid sequences within the intracellular loops are involved in functional coupling of the receptor to the G-protein (Kobilka et al., 1992, Strada et al., 1994)

The current consensus is that ligand-induced changes in the relative orientation of individual transmembrane helices affect the conformation of the intracellular receptor surface, thus enabling the G-protein to interact with previously inaccessible regions of the receptor protein (cf. Wess, 1997).

In most cases, an individual G-protein-coupled receptor, when activated by the appropriate ligand, can recognise and activate only a limited set of the many structurally closely related G-proteins (as defined by the molecular structure of the α-subunits) expressed within a cell. Understanding how this selectivity is achieved at a molecular level has become the focus of an ever increasing number of laboratories. Besides the structural information encoded by the receptor and G-protein primary sequences, other factors including receptor and G-protein density, and perhaps restricted localisation of specific G-protein heterotrimers and receptors in the plasma membrane may also contribute to the observed degree of coupling selectivity.

Receptor mutational studies (Ostrowski et al., 1992; Hedin et al., 1993; Strader et al.,1994) and experiments using receptor antibodies (Weiss, et al., 1988) or short synthetic peptides that inhibit or mimic receptor interactions with various G-proteins (Konig et al., 1989; Dalman et al., 1991; Okamoto et al., 1991; Luttrell et al., 1993; Munch et al., 1991; Malek et al., 1993) have identified the i2 loop (I2), the amino- and carboxyl-terminal domains of the i3 loop (Ci3), and the membrane-proximal portion of the carboxyl-terminal tail (I4) as receptor domains that participate in receptor/G-protein interactions (Fig 1.2). The conclusion drawn from studies using the chimeric receptor approach generally agree well with biochemical experiments using

short synthetic peptides derived from distinct intracellular receptor segments. Several laboratories have shown that peptides corresponding to the I2, Ni3 and Ci3 region (in some cases also from the I4 segment, Fig 1.2) can mimic or inhibit receptor interactions with G-proteins (Konig et al., 1989; Munch et al., 1991). In agreement with mutagenesis studies (Wong et al., 1990; Wong et al., 1994), investigations with short peptides (Konig et al., 1989) also indicate that these regions act in a cooperative fashion to dictate proper G-protein recognition. The fact that several of these peptides can activate G-proteins directly suggests that these sequences are not accessible to the G-protein heterotrimer in the nonactivated state of the receptor.

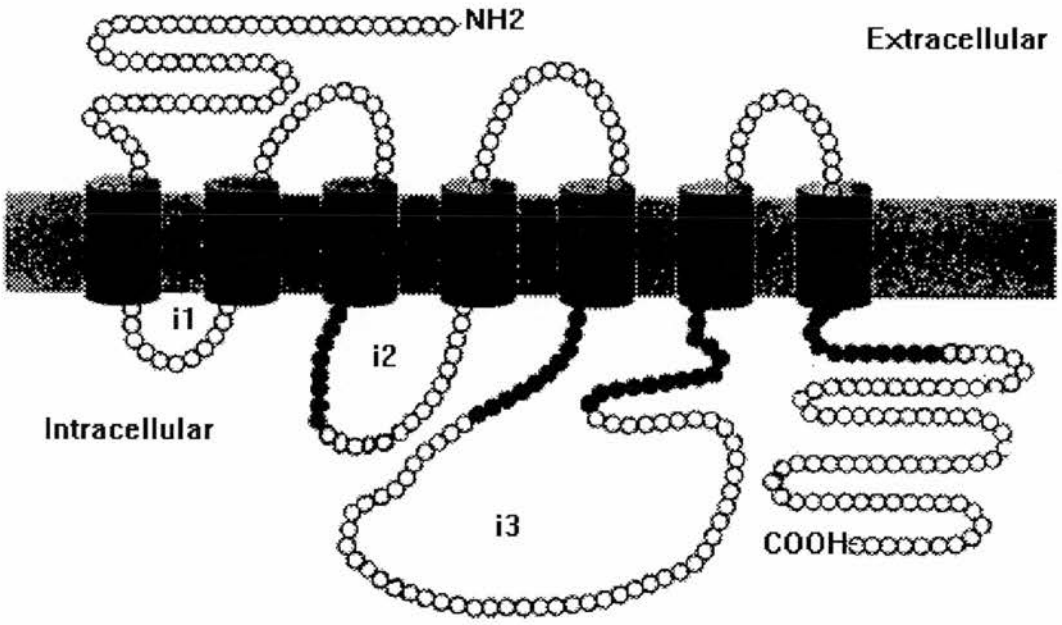


Fig 1.2 Topographical model of 7 transmembrane G-protein coupled receptor. The horizontal band corresponds to the membrane seperating extracellular (top) and intracellular (bottom) spaces. The receptor is a monomeric protein possessing seven transmembrane hydrophobic domains linked by three extracellular and three intracellular loops (i1-i3). The NH2 terminal is extracellular and the COOH terminal is intracellular. Filled circles represent domains which have been found to be involved in receptor-G-protein interaction.

1.23 Activation of G-proteins

Ligand binding to receptors causes conformational changes in the receptor protein that promotes its interaction with distinct classes of G-protein that are attached to the cytoplasmic surface of the plasma membrane. This interaction triggers the exchange of GTP for GDP on the α-subunit, leading to the dissociation of the G-protein from the receptor and to a dissociation of the α-subunit from the βγ complex. The released G-protein subunits, α-GTP and βγ complex, are then able to interact with distinct effector enzymes and ion channels, eventually leading to the desired physiological response (Fig 1.1). Finally, the intrinsic GTPase activity of the α subunits converts the bound GTP to GDP, terminating the activation of α subunits.

Aluminium tetrafluoride (AlF_4^-), together with Mg^{2+}, can interact with α-bound GDP to mimic actions of GTP and thereby strongly activate α subunits (Bigay et al., 1987). Non-hydrolyzable GTP analogues, such as GTP-γ-S or Gpp (NH)p can not be hydrolysed by GTPase activity and thus permanently bind to the Gα subunit and persistently stimulate its activation. In contrast, nonhydrolyzable GDP analogues, such as GDP-β-S can irreversibly inhibit G-proteins (Burch & Axelrod, 1987).

Certain classes of G-proteins (see Table 1.2) have α-subunits which possess specific residues that can be covalently modified by bacterial toxins. Cholera toxin (CTX) catalyses the transfer of the ADP-ribose moiety of NAD to a specific Arg residue in certain α-subunits, and similarly pertussis toxin ADP ribosylates those α-subunits that posses a specific Cys residue near the carboxyl terminus. Modification of α-subunits by CTX constitutively activates these proteins (by inhibiting their GTPase activity) , whereas modification by pertussis toxin prevents receptor-mediated activation of G-proteins (see Table 1.2) (Reviewed by Olate & Allende, 1991; Hepler & Gilman, 1992).

1.3 Modulation of Ca^{2+} channels by G-protein-coupled receptors

Ion channels , particularly, voltage-gated Ca^{2+} channels are often the target of G-protein-coupled receptors. Recent studies have made great progress in understanding the mechanisms that underlie the G-protein-mediated inhibition of ion channel activity. Particularly, there is abundant literature reporting the most common form of inhibition, the voltage-dependent and membrane-delimited inhibition, which plays important roles in modifying the excitability of target neurones and the strength of synaptic connections (Hille, 1994; Wickman & Clapham, 1996; Dolphin, 1998).

1.31. Voltage-dependent and membrane-delimited fast inhibition

Voltage-dependent inhibition manifested as having the following characteristics: (1) the inhibition is never complete; (2) strong depolarisation restores normal fast activation kinetics, and increase the current amplitude, but not to the control level; (3) at some stages, the time course of activation is clearly biphasic; (4) channel activation is shifted to more positive voltages, and the activation curve is less steep; and (5) the mean inhibition varies with membrane potential and has a bell-shaped relation when plotted against membrane voltage (reviewed by Jones & Elmslie, 1997; Dolphin et al., 1998; see Fig 1.3 for example).

(1) Biophysical and molecular mechanisms

The molecular mechanisms underlying the voltage-dependent inhibition have not been fully elucidated. However, there are some clues as to how G-protein binding to the Ca^{2+} channels results in kinetic slowing. Most of these studies have explored the biophysical changes at the whole-cell macroscopic current level. In the original studies, Bean (1989) proposed the ' willing-reluctant' model. In this model, it was assumed that the channel can exist in two modes, in equilibrium. In one mode (willing), channels can be opened readily by a small depolarisation, whereas in the other (reluctant), channels require a much longer or larger depolarisation to open (and

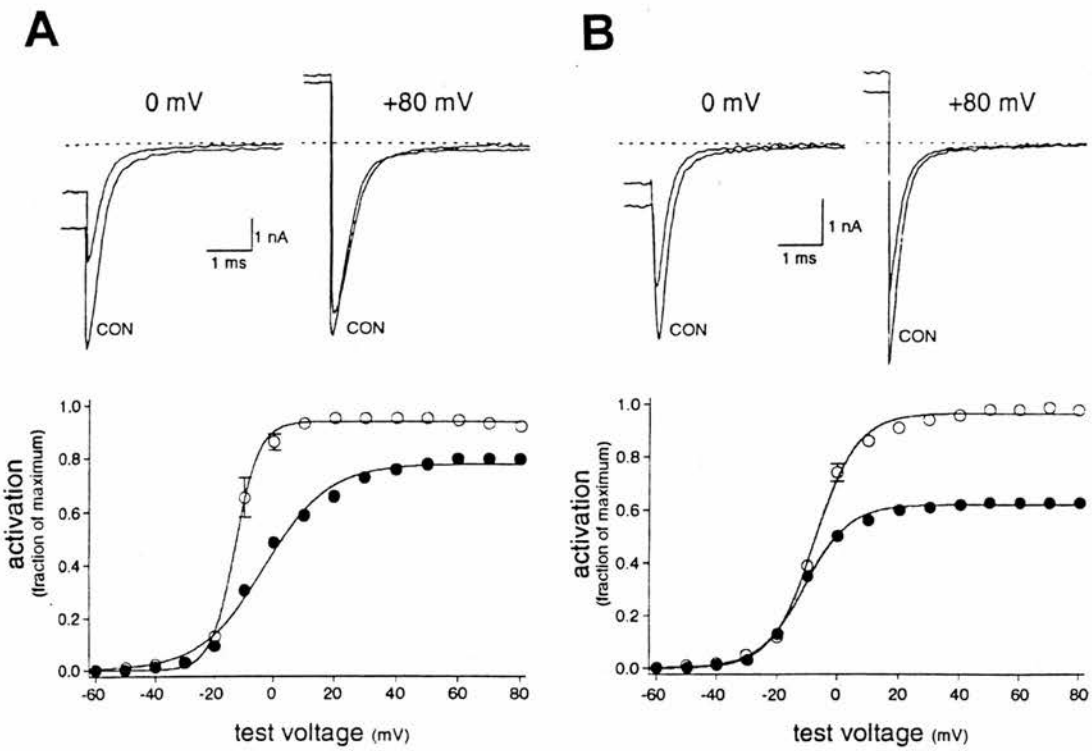


Figure 1.3 Voltage-dependent and -independent inhibition of HVA Ca^{2+} currents **A, B**) Instantaneous current/voltage-relationships in cells with and without kinetic slowing. Tail currents were measured at -80mV following 5 ms step depolarizations to a series of test potentials before (Con) and during application of 100 μM GABA. **A**) A neuron showing prominent voltage-dependent inhibition; **B**) In this neuron, the inhibition is voltage-independent. The smooth curves represent the best-fit single Boltzman relationship for each data set. (Figure is adapted from Luebke & Dunlap, 1994)

have a somewhat less steep voltage-dependence and a slightly smaller maximal probability of being open). In one simple form of this model (Elmslie et al., 1991), normal gating is approximated as a voltage-dependent transition from C (closed state) to O (open state), paralleled by RC (reluctant closed state) to RO (reluctant open state) gating for the modulated channel (Fig 1.4). When the channel is closed, the equilibrium is toward the reluctant state; when it is open, toward the willing state. This scheme can account for the qualitative features of Ca^{2+} channel modulation. The simplest model involves opening only of the free and not the G-protein-bound channels (Dolphin, 1991). As the biochemical evidence suggests that $G\beta\gamma$ bind to $\alpha 1$ subunits of the Ca^{2+} channel, one hypothesis would be that the willing-reluctant transition is G-protein binding itself. Modeling the whole-cell current modulation by agonists has suggested either that the G- protein-bound channels open with different gating properties, or that the G-protein has to dissociate from the channel to allow opening (Bean, 1989; Elmslie et al., 1990). Models have also suggested that more than one activated G-protein may be bound per channel in a co-operative manner (Boland & Bean, 1993).

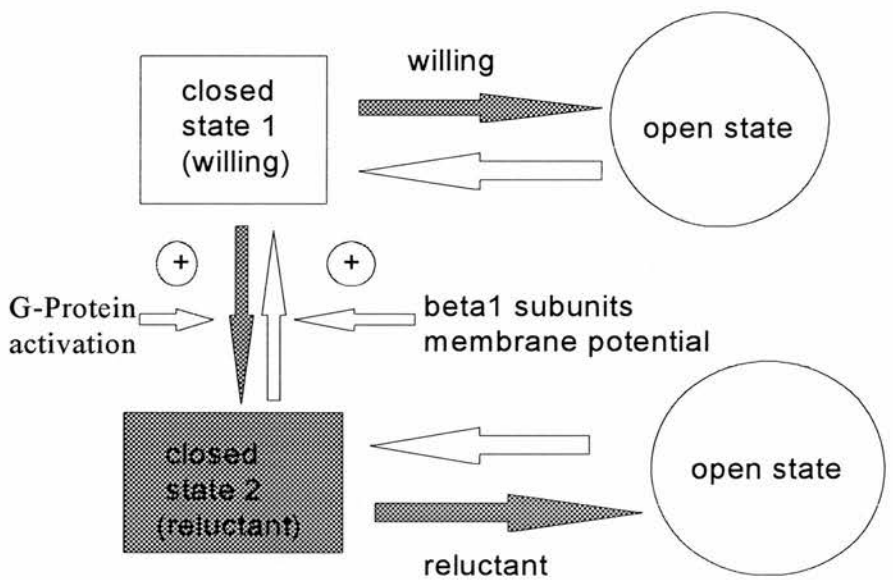


Figure 1.4 A theoretical model based on Bean (1989), showing functional state of HVA channels modulated by neurotransmitter (adapted from Clapham, 1996)

Single channel recordings in the cell-attached mode have identified changes in gating of native single Ca^{2+} channels in sympathetic neurones in the presence of noradrenaline (Delcour & Tsien, 1993; Delcour et al., 1993). In these studies, unitary N-type Ca^{2+} channel currents were recorded from symathetic neurones. The authors have found three distinct patterns of gating, designated low-P0, medium- P0, high-P0 modes according to their probability of being open at morderatly depolarised potentials (-10 mV). When inhibitory neurotransmitter was added into the pipette, it greatly decreased the prevalence of high P0-gating and increased the proportion of time a channel exhibited low-P0 behaviour or no activity at all, which thereby reduced the overall current. Further studies (Patil et al., 1996) have now shown that the only clear effect at the single channel level of muscarinic modulation of cloned 1B channels (expressed in HEK 293 cells together with muscarinic m2 receptors), is a prolonged latency to first opening in the presence of a muscarinic agonist. Once a channel has opened, they observed no effect on subsequent open probability or gating pattern. The delay to first opening may be due to dissociation of the activated G protein species from the channel, allowing it to open. This would imply that the G-protein-bound channel does not open, even with large depolarisation. If this is the case, either the G-protein binding is itself strongly voltage-dependent, or the G-protein binds to a site on the channel that produces a voltage-dependent block. To give a better understanding of the biophysical mechanisms of modulation, more single-channel work is needed but, regrettably, the N and P/Q channels are very unstable in excised patches.

(2) Biochemical mechanisms underlying voltage-dependent inhibition (signal transduction pathways)

The inhibition of Ca^{2+} channels recorded in the cell-attached patch mode only occurs when the receptor agonist is present in the patch pipette, and not when it bathes the remainder of the cell membrane (Forscher et al., 1986). This suggests that the regulation occurs by events confined to the membrane, perhaps involving a direct action of the activated G protein (Hille, 1994). The G-proteins involved in the fast voltage-dependent inhibition of Ca^{2+} currents were most commonly found to be PTX-

sensitive (Hille 1992; Wickman & Clapham, 1995). The use of antisense and antibodies against G- protein subunits has shown that Go is responsible for inhibition in many cases (Ewald et al. 1989; Campbell et al., 1993; Menon-Johansson et al., 1993). However, Gi can also play a role (Ewald et al., 1989) and in many other cases, Gq- or Gs-coupled receptors have been found to produce similar modulation (Shapiro & Hille, 1993; Golard et al., 1994; Zhu & Ikeda, 1994). This led two groups to test the hypothesis that it was the moiety common to all these G proteins that mediated the inhibition, ie, the Gβγ subunit rather than any particular Gα subunit (Ikeda, 1996; Herlitze et al., 1996).

By studying the binding of radiolabelled G-protein subunits to various Ca^{2+}-channel subunits that were expressed as glutathione s-transferase (GST) fusion proteins, Gβ and Gβγ (but not Gα or Gγ alone) were found to bind to the cytoplasmic linker region located between tranfected membrane repeats I and II of some Ca^{2+} channel classes (α1A, α1B, α1E), but not the others (α1C, α1D, α1S).Therefore this inhibitory pathway occurs through a direct interaction between the Ca^{2+} channel complex and the βγ subunits that are released from the activated G-protein heterotrimers (Gαβγ) (Herlitz et al., 1996; Ikeda et al., 1996; De Waard et al., 1997).

Dolphin and her group (cf. Page et al., 1998) have tested the hypothesis that this loop is involved in G-protein-mediated inhibition by making chimeras containing the I-II loop of α1B or α1A inserted into α1E (α1EBE and α1EAE, respectively). They found that although α1B, co-expressed with α2δ and β1b transiently expressed in COS-7 cells, showed both kinetic slowing and steady-state inhibition when recorded with GTP-γ-S in the patch pipette, both could be reversed by a depolarising prepulse, the chimera α1EBE (and, to a smaller extent, α1EAE) showed only kinetic slowing in the presence of GTP-γ-S, and this also was reversed by a depolarising prepulse. These results indicate that the I-II loop may be the molecular substrate of kinetic slowing but that the steady-state inhibition shown by α1B may involve a separate site on this Ca^{2+} channel (see Fig 1.5).

To study further how the activated G-protein inhibits Ca^{2+} channels, Dolphin' s group (Berrow et al; 1995; Cambell et al., 1995b) developed an antisense strategy to deplete the β subunit of Ca^{2+} channels. They found the loss of the β subunit caused smaller Ca^{2+} currents and altered kinetics. Application of baclofen caused much more inhibition than in neurones without antisense injection. They therefore proposed that there is competition between the activated G-protein subunit and the Ca^{2+} channel β subunit for binding a site on the α-subunit of Ca^{2+} channels (cf. Berrow et al; 1995; Cambell et al., 1995b; Dolphin, 1998). This led to the identification of the intracellular segment (called α interaction domain or AID) between transmembrane domains I and II of the the α-subunit of Ca^{2+} channels as the possible binding region of the activated G-protein subunit (Fig 1.5). Evidence obtained from expression of α-subunit of Ca^{2+} channels with massive amount of β-subunits (Rohe et al., 1995) or lack of β-subunits (Bourinet et al., 1996) have supported this idea. The former procedure blockes the G-protein mediated inhibition and the latter enhance it.

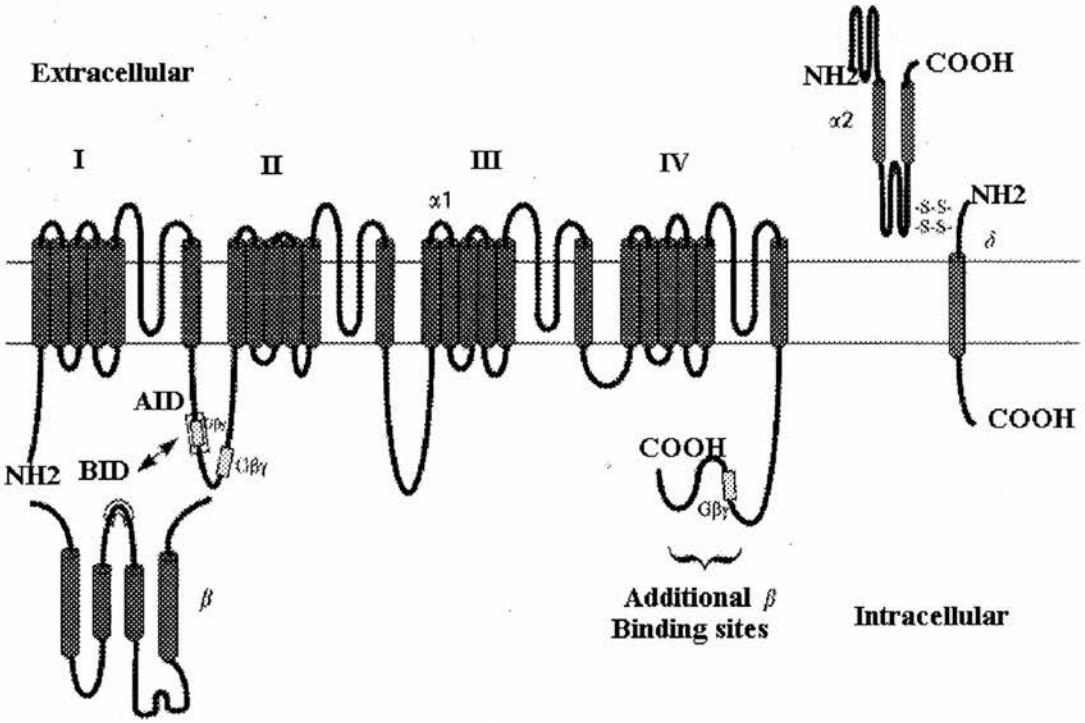


Fig 1.5 The voltage-gated Ca^{2+} channel oligomeric complex

Binding sites on the α1 I-II loop for Ca^{2+} channel β subunit and G βγ are shown. All Ca^{2+} channel α-1 subunits have a binding site for β (α interaction domain, AID) on this loop, but Gβγ biding has only been shown for α 1A, B and 1E. Regions of the C-terminal tail of human α-1E which may be involved in the Ca^{2+} channel β subunit and Gβγ binding are also indicated. BID β interaction domain. (This figure is adapted from Dolphin, 1998.)

1.32 Voltage-independent slow modulation and co-existence of multiple-mechanisms

The voltage-independent inhibition of Ca^{2+} channels may also occur and can be produced either separately or in conjunction with the well-studied voltage-dependent inhibition. N-type channels are inhibited by noradrenaline or 5-HT through both voltage-dependent and -independent mechanisms (Beech et al., 1992; Luebke & Dunlap, 1994; Diversé-Pierlussi et al., 1995; Page et al., 1998). Voltage-dependent and -independent inhibition produced by one neurotransmitter has also been reported for several subtypes of HVA channels in various preparations (Ciranna et al., 1996;

Albillos et al., 1996, Currie & Fox, 1997). Although it remains unclear whether these two forms of modulation represent different signalling pathways or simplely two facets of the same inhibitory mechanism, recent evidence suggests that the former explanation seems more likely (Diversé-Pierluissi et al., 1995; Page et al., 1997).

Voltage-independent inhibition is characterised by an incomplete ability of a depolarising prepulse to reverse the inhibition (Diversé-Pierlussi & Dunlap, 1993; Page et al., 1998) and the continuing presence of inhibition measured from the tail current amplitude at large depolarisations (Diversé-Pierlussi & Dunlap, 1993). However, little is known about the mechanisms underlying the voltage-independent inhibition. Although the whole-cell recordings have shown a reduction in maximum HVA channel conductance occurred (Diversé-Pierlussi & Dunlap, 1993), this could either be produced via a modification of the single channel conductance or through reduction of the number of functional Ca^{2+} channels.

The second messengers underlying the voltage-independent inhibition are not clear either. In some circumstances, this inhibition may be mediated via phosporylation of N-type channels by protein kinase C (PKC; Diversé-Pierlussi & Dunlap, 1993). However, PKC appears to inhibit Ca^{2+} currents only in sensory neurones (Rane et al., 1989; Boland et al., 1991) whereas in sympathetic neurones channel activity is enhanced by PKC activation (Swartz, 1993; Zhu & Ikeda, 1994). Attempts to identify the second messengers in these sympathetic neurones have so far been unsuccessful. Some pieces of evidence surport the involvement of diffusible second messengers: e.g. muscarinic receptors are thought to suppress Ca^{2+} channels in rat sympathetic neurones via a slow, diffusible second messenger (Bernheim et al., 1991); and angiotensin receptors act through a PTX-insensitive G-protein and a diffusible second messenger to suppress HVA currents (Shapiro et al., 1994).

In summary, various mechanisms have been identified by which hormones and neurotransmitters modulate the activity of ion channels. All of the mechanisms involve heterotrimeric G-proteins (with one exception report, cf. Hall et al., 1998).

The interaction of G-proteins with channel proteins or associated regulatory proteins is well established as a major modulatory mechanism of almost all voltage-dependent ion channels, particularly the voltage-gated Ca^{2+} channels. One of the best studied forms of modulation is voltage-dependent inhibition, which involves a direct interaction between $G\beta\gamma$ and the linker region between the I and II domains of Ca^{2+} channel $\alpha 1$ subunits. Biophysical models have also been proposed to explain how this form of inhibition can occur at the molecular level. Another form of inhibition, the voltage-independent inhibition may also be produced in addition to the voltage-dependent inhibition. The mechanisms underlying this form of inhibition are not clear, and the existence of these components remain to be established in a model system, in which both the second messengers and their functional consequences can be investigated.

1.4 Pharmacology and physiology of serotonin receptors in the spinal cord of vertebrate

1.41 Introduction

In 1911, Ramon Cajal firstly described the central or midline (raphe) location of the large multipolar neurones with uncertain projections in the brainstem. From then, investigators had known of a blood-borne chemical that produced vasoconstriction (' serum' factor that affected the blood vessel ' tonus' , hence the name serotonin) and of a substance present in the gut that increases intestinal motility. In the mid-twentieth century, 5-HT, the single compound producing both these effects, was isolated and synthesized, and its molecular structure was elucidated. Almost at the same time, it was found in the central nervous system (Twarog & Page, 1953).

The serotonergic cell bodies appear to be exclusively localised in the brainstem (raphe nucleus). The number of serotonergic neurones constitute approximately one-million of all rat CNS neurones. However it has been estimated that 1/500 of all axonal terminals in the rat cortex originate from these neurones (cf. Jacobs & Azimitia, 1992). The four *descending nuclei* in the primate brain are believed to

innervate the substantia gelatinosa of the dorsal horn, the motor neurones of the ventral horn, and the intermediate autonomic neurones in the spinal cord. The *ascending neuclei* in the primate brain use multiple tracts to innervate most of the subcortical and cortical areas of the forebrain with a distribution pattern highly consistent with that reported in rats. Therefore the relative paucity of cell bodies and their highly branched terminal domain implies that these neurones may be an essential component of the process, by which a wide ranges of brain functions are integrated, such as sleep and arousal; thermoregulation, learning and memory; pain; sex; feeding; neuroendocrine regulations; motor activity; behavior such as aggressive; and biological rhythms (see review by Anwyl, 1990; Jacobs & Azmitia, 1992; Zifa & Fillion, 1992; Jacobs & Fornal, 1995; Millan, 1995). Further investigations revealed that part of the multiplicity of function is due to the existence of multiple serotonergic receptor subtypes (Zifa & Fillion, 1992; Hoyer et al., 1994).

1.42 Neuropharmacology of 5-HT receptors

Gaddum and Picarelli (1957) classified the serotonin (5-HT) receptors in the guinea-pig ileum into D and M types based on the activity of dibenzyline (D) and morphine (M) to block contractions of intestinal smooth muscle caused by 5-HT. The subsequent location of specific ligand binding sites for 5-HT in the brain has led to the identification of ten 5-HT receptor subtypes in rat brain (reviewed by Hoyer et al., 1994). While there is some controversy over the functional importance of many of these receptor subtypes, there is evidence that they fall into two major groups according to the nature of their coupling to secondary messengers or ion channels. Thus the 5-HT_1 and 5-HT_2 receptors are members of the G-protein-coupled receptor superfamily and may be coupled either to adenylate cyclase (most 5-HT_1 subtypes) or phosphatidyl inositol (5-HT_2 subtypes, cf. Baxter et al., 1995). The central "M" receptors (now termed 5-HT_3) appear to be members of the ligand-gated ion channel superfamily (cf. Jackson & Yakel, 1995)

The initial characterisation of 5-HT receptor subtypes relied solely on the use of pharmacological tools. On the basis of the receptor binding profiles, common secondary messenger coupling and the functional activity of ligands, four main

subgroups of 5-HT receptors, termed 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_4 were identified (reviewed by Peroutka, 1988; Zifa & Fillion, 1992; Hoyer et al., 1994). More recently, molecular biological techniques have both confirmed this classification, in that each subgroup has been found to have relatively dissimilar protein structures, and has led through cloning techniques to the identification of novel 5-HT receptors (termed 5-HT_{1F}, 5-HT_5 , 5-HT_6 and 5-HT_7). The cloned receptors have now been expressed in cultured cell lines and their pharmacological and functional properties characterised (reviewed by Zifa & Fillion, 1992; Tecott & Julius, 1993; Hoyer et al., 1994). Therefore, what was previously thought to be a family of three pharmacologically defined classes of 5-HT receptors is now seen to be composed of seven distinct subfamilies, designated 5-HT_{1-7} (Table 1.3). The 5-HT_1, 5-HT_2, and 5-HT_5 subfamilies currently consist of five, three and two subtypes respectively; while the 5-HT_3, 5-HT_4, 5-HT_6, and 5-HT_7 "subfamilies" have at present one subtype each (reviewed by Hoyer et al., 1994).

Table 1.3 Serotonin receptor classifications

Receptor	**5-HT_1**	**5-HT_2**	**5-HT_3**	**5-HT_4**	**5-HT_5**	**5-HT_6**	**5-HT**
Effector	AC↓	PLC↑	Ion channel	AC↑	AC↓	AC↑	AC↑
	(G_i/G_0)	(G_Q/$_{11}$)	$Na^+/K^+/Ca^{2+}$	(G_s)	?	(G_s)	(G_s)
					(G_s)		
Subtypes	5-HT_{1A} 5-HT_{1B}	5-HT_{2A}			5-HT_{5a}		
	5-HT_{1D} 5-HT_{1E}	5-HT_{2B}			5-HT_{5B}		
	5-HT_{1F}	5-HT_{2C}					

Table 1.3 5-HT receptors are currently divided into 7 classes, based on their pharmacological profiles, cDNA-deduced primary sequences and signal transduction mechanisms. With the exception of the 5-HT_3 receptor, which forms a ligand-gated ion channel, all 5-HT receptors belong to the superfamily of G-protein-coupled receptors containing a predicted seven-transmembrane domain structure.

5-HT_1 receptors

At least five 5-HT_1 receptor subtypes have been recognised: 5-HT_{1A}, 5-HT_{1B} (formerly termed 5-$HT_{1D\beta}$), 5-HT_{1D} (formerly termed 5-$HT_{1D\alpha}$), 5-HT_{1E} and 5-HT_{1F}.

The genes for all these receptors have been cloned and the receptors shown to be single-protein structure, varying in size from 365 to 422 amino acids with high sequence homology of more than 60% in transmembrane domains (~40% overall homology). These receptors are all thought to be negatively linked to adenylyl cyclase, presumably via a common or similar G-protein link (G_i or G_0). All these receptors are potently activated by 5-carboxamidotryptamine (5-CT), except 5-HT_{1E} and 5-HT_{1F} (Bradley et al., 1986).

5-HT_{1A} receptors are widely distributed in hippocampus, septum, amygdala and raphe nuclei, particularly the dorsal raphe nuclei. Many of these regions are components of pathways involved in the modulation of emotion and the limbic system. The presence of high densities of the 5-HT_{1A} receptor in the raphe nuclei suggests that 5-HT can modulate the serotonergic neurones, perhaps acting at "autoreceptors" to regulate its own release from these neurones. 5-HT_{1A} receptors are also present in neocortex, the hypothalamus and the substantia gelatinosa of the spinal cord. This suggests that the 5-HT_{1A} receptor could be involved in the functions of the hypothalamus, in the regulation of propioception and in integrative functions of neocortex. Several agonists show selectivity for the 5-HT_{1A} receptor, particularly 8-OH-DPAT (Table 1.4), which acts as a full agonist in most systems. However, most antagonists, such as NAN190, WAY100635 show partial agonist properties in studies of somatodentritic autoreceptor function (cf. Hoyer et al, 1994). To date, the only selective high affinity silent antagonist at this receptor is WAY 100, 635 (Table 1.4).

5-HT_{1B} receptor have been found in basal ganglia, striatum and frontal cortex. The amino acids sequences of this receptor have been found to have an overall identity of 93% and a 96% identity in the transmembrane domains with that of the 5-$HT1_{D\beta}$ receptor, a close homologue found in higher species, with similar distribution and function (cf. Hoyer et al. 1994). Thus it has recently been agreed to classify the receptors as species homologues of the same receptor termed h5-HT_{1B} (formerly 5-$HT1_{D\beta}$) and r5-HT_{1B} with the h and r prefixes referring to the human and rat species respectively. 5-HT_{1B} receptors serve as autoreceptors in rat, guinea-pig and man. They may also act to control the release of other neurotransmitters, such as

acetylcholine, glutamate and dopamine. Agonist CGS12066B possesses over 10 fold selectivity for the 5-HT_{1B} receptor compared to the 5-HT_{1A} receptor, and is full agonist (Schoeffter & Hoyer, 1989; Table 1.4).

5-HT_{1D} receptors have been found in the brain of several non-rodent mammalian species including rabbit, dog, pig, calf and human (cf. Hoyer et al., 1994). 5-HT_{1B} sites appear to be absent in these species, and the 5-HT_{1D} receptor has similar distribution and function to the 5-HT_{1B} receptor described in rodents. The highest density of 5-HT_{1D} receptors is in basal ganglia, substantia nigra and nigrostriatal pathways, with lower densities in hippocampus, raphe nucleus, and cortex (Waeber et al., 1990). The localisation of 5-HT_{1D} receptor mRNA in the raphe nucleus, suggests that it may function also as a 5-HT autoreceptor. It has also been identified as a terminal autoreceptor mediating inhibition of 5-HT release from cortical nerve terminals of guinea-pig brain (Middlemiss et al., 1988). It may also function to modulate release from cholinergic and glutamatergic neurones (Schoeffter & Hoyer, 1990).

Table 1.4 5-HT_1 subtype selective ligands

Subtype	**5-HT_{1A}**	**5-HT_{1B}**	**5-HT_{1D}**	**5-HT_{1F}**
Agonist	8-OH-DPAT	CP 93129	L-694,247	LY334370
	RU 24969	CGS12066B	GR46611	
	MDL 73005EF	Sumatriptan		
Antagonist	NAN-190	GR55562	GR127935	-
	Pindolol	Pindolol		
	Way100635	Isamoltane		
	Pindobind 5-HT_{1A}	Cyanopindolol		
	p-MPPI	SB242289		

5-HT_2 receptor family

The 5-HT_2 receptor family consists of three subtypes termed 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. The latter was previously termed 5-HT_{1C} before its structural similarity to the 5-HT_2 family members was recognised (cf. Hoyer et al., 1994). All three are single protein molecules of 458-471 amino acids with an overall homology of

approximately 60%-80% in the seven transmembrane domains. These receptors are coupled to phosphoionositol hydrolysis via G-proteins of the Gq subtype.

Centrally, the **5-HT$_{2A}$** receptor is principally located in the cortex, claustrum (a region connected to visual cortex) and basal ganglia (Hoyer et al., 1994). In the cortex, lesion and binding experiments suggest that the 5-HT$_{2A}$ receptor could be located on intrinsic somatostatin-containing neurones. Functionally, this receptor mediates neuroexcitation in guinea-pig cortical pyramidal neurones and rat raphe cell bodies (cf. Hoyer et al, 1994). Selective agonists and antagonists are listed in Table 1.5.

5-HT$_{2B}$ There is only a low level of expression of 5-HT$_{2B}$ receptors found in the brain. They are found in the amygdala, septum, hypothalamus and cerebellum. In rodents, Northern blot analysis has not revealed the presence of this receptor in the brain (cf. Hoyer et al, 1994).

5-HT$_{2C}$ receptors are mainly found in CNS, with high density present in choroid plexus and lower levels in cerebral cortex, hippocampus, striatum and substantia nigra of rat and man. It may play a role in a variety of processes such as locomotion, feeding and adrenalcorticotrophic hormone release, regulation of cerebro-spinal fluid production, and in anxiety and anorexia nervosa. In general, compounds claimed to be 5-HT$_{2A}$ receptor selective show similar affinity for the 5-HT$_{2C}$ receptor (Hoyer, 1988a). This is not surprising, given their structural similarities (Table 1.5).

Table 1.5 5-HT$_2$ subtype selective ligands

Subtype	5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_{2c}$
Agonists	α-Me-5-HT	α-Me-5-HT	α-Me-5-HT
		BW 723C86	MK212
			mCPP
Antagonists	Ketanserin	Rauwolscine	SB 206553
	MDL 11, 939	SB 206553	RS 102221
	MDL 100,907	SB 204741	SB 221284
	Spiperone	LY 266097	

5-HT_3 receptor (ligand gated receptor)

5-HT_3 receptors are distributed widely in the peripheral nervouse system, such as in dosal root ganglian, vagus nerve, superior cervical ganglian and the myenteric plexus. In the central nervous system, they are rarely observed and are present on only some 5 to 10% neurones in the hippocampus and striatum (cf. Jackson & Yakel, 1995). The precise function of this receptor has been difficult to identify, but it is clear that they are involved in emesis and anxiety. The clone receptor is a 487-amino acid polypeptide with 27% amino acid identity with the Torpedo nicotinic receptor α subunit, 22% identity with the β1 subunit of the bovine $GABA_A$ receptor, and 22% identity with the rat glycine receptor (Maricq et al., 1991.) The most closely related ligand-gated channel is the neuronal nicotonic receptor α7 subunit, with 30% amino acid homology. Thus the 5-HT_3 receptor belongs to the nicotonic/GABA receptor gene family. The four hydrophobic putative transmembrane segments, found in other ligand-gated channel sequences and traditionally denoted as M1 through M4, are also present in the deduced amino acid sequence of the 5-HT_3 receptor. The M2 segment shows particularly strong holmology with other ligand-gated channels. In the nicotinic receptor, a variety of studies have indicated that the M2 segment contains residues that line the pore through which ions flow. High conservation in this region is consistent with the function of 5-HT_3 receptor as a cation-selective channel. Although the 5-HT_3 receptor channel is permeable to monovalent cations, it discriminates poorly amongst inorganic monovalent cations. (see review by Jackson & Yakel, 1995).

5-HT_4 receptor

The highest 5-HT_4 receptor densities are found in the limbic system. 5-HT_4 receptors are also found in components of other neuronal pathways, including the cortico-striatal-tectal pathway and septo-hippocampal-habenulo-interpeduncular pathway. The location of the 5-HT_4 receptor in the limbic system (the islands of Calleja,

olfactory tubercle, frontal cortex, fundus striatus, ventral pallidum, septal region, etc) and several lines of evidence suggests a role for the receptor in memory and learning and other cognitive processing. Other functions may involve modulation of dopamine transmission and anxiolysis (cf Eglen et al., 1995). 5-HT_4 agonist includes SC53116, ML10302, or RS67506. Antagonists such as GR113808 and GR125487 have been extensively used for characterisation of 5-HT_4 receptors.

Two **5-HT_5 receptors** (5-HT_{5A} and 5-HT_{5B}) identified from rat cDNA and subsequently cloned were found to have 88% overall sequence homology, yet were not closely related to any other 5-HT receptor family. The cloned 5-HT_{5A} receptor has high affinity for 5-CT and 5-HT. The mRNA coding for the receptor is located in rat cortex, hippocampus, olfactory bulbs and cerebellum, but mainly expressed on glial cells. The receptor is negatively linked to adenylyl cyclase via Gs proteins and may act as a terminal autoreceptors in the mouse frontal cortex (cf. Hoyer et al., 1994).

5-HT_6 receptors have a unique pharmacological profile: they have high affinity for methiothepin; and various ergolines (e.g. metergoline). They have been found in striatum, olfactory tubercle, cerebral cortex and hippocampus. It is positively linked to adenylyl cyclase, but the whole cell function of this receptor has not been defined (cf. Hoyer et al., 1994).

The 5-HT_7 receptor is located in the hypothalamus and thalamus, and to a lesser extent in other forebrain regions. This receptor is positively linked to adenylyl cyclase. At present, no selective agonist or antagonists are known, but this receptor has pharmacological features similar to the 5-HT_1 receptor with significant affinity for 8-OH-DPAT (reviewed by Hoyer et al., 1994).

1.43 Postsynaptic actions of 5-HT

After release from its presynaptic terminals, 5-HT interacts with the postsynaptic receptor subtypes to activate subsequent intracellular second messengers. These second messengers are then able to target various ion channels. The nature of the

postsynaptic actions evoked by 5-HT are diverse (excitatory, inhibitory or modulatory), depending on the different receptor subtypes present and the intracellular biochemical mechanisms that are coupled to each.

The predominant postsynaptic actions of 5-HT in the forebrain, especially in the neocortex and dorsal hippocampus are inhibitory, although excitation is also seen (cf. Jacobs & Azmitia, 1992). The inhibitory actions of 5-HT in these areas are mediated via a 5-HT_{1A} receptor (Andrade & Nichol, 1987; Colino & Halliwell, 1987; Wu et al., 1988). It seems that the inhibition is attributable to membrane hyperpolarisation, which is produced via an increase in a resting potassium conductance that is not Ca^{2+}-dependent (Andrade & Nichol, 1987; Colino & Halliwell, 1987; Wu et al., 1988).

5-HT can also produce inhibitory actions in the somatosensory neurones of the spinal cord, especially dorsal horn cells involved in nociception. Current evidence from mammalian (El-Yassir et al., 1988) and lower vertebrate preparations (Sun & Dale, 1997) suggests that 5-HT_1 receptors, particularly 5-HT_{1A} and 5-HT_{1B} (or 5-HT_{1D}) receptors may be responsible for this. Another CNS site where 5-HT has strong inhibitory postsynaptic actions is the raphe nucleus. The actions of 5-HT in these serotonergic neurones reduces the release of 5-HT, a phenomenon known as autoinhibition. This has been extensively studied and is mediated by 5-HT_{1A} receptors (Penington et al., 1991; Bayliss et al., 1997; reviewed by Jacobs & Azmitia, 1992). The actions of 5-HT were mediated either via an increased potassium channel conductance (Aghajanian & Lakoski, 1984), or a reduction of Ca^{2+} influx via N and P/Q type voltage-gated ion channels (Penington et al., 1991; Bayliss et al., 1997).

The excitatory actions of 5-HT are in the brainstem and spinal cord (except for neurones involved in nociception). Here, 5-HT can either produce direct excitation or potentiate the action of other excitatory transmitters. The most frequently reported postsynaptic excitation is directed at motor neurones (Jacobs & Fornal, 1997). The direct excitation on motor neurones is attributable to a depolarisation, probably mediated by a decreased potassium conductance (Wang & Dun, 1990) and via 5-HT_2

receptor pharmacology (Wang & Dun, 1990). 5-HT can also have excitatory effects on motor neurones via 5-HT_1 receptors through a reduction of an apamin-sensitive K-Ca-channels, which contributes to regulation of the neuronal firing and to the termination of the locomotor bursts in motor neurones (El Manira et al., 1994).

1.44 Serotonergic modulation of motor circuits and motor behavior

(1) 5-HT and initiation of patterned motor activity

In the adult lamprey, *Xenopus* embryo and larvae, neonatal rat and other mammalian preparations, rhythmic output from a central pattern generator (CPG) can occur without modulation by sensory afferents and can generate what is termed ' fictive locomotion' . Preparations of isolated brainstem-spinal cord that are placed in a bath and partitioned at the spinomedullary junction allow pharmacological agents to be applied either to the brain stem or to the spinal cord. Recordings using suction electrodes from a ventral root or from a group of roots innervating a single limb reveal patterned discharges, and have suggested excitatory amino acids (EAA) neurotransmitters are the principal mediators for initiation of patterned activities (Grillner et al., 1981; Dale & Roberts, 1984; Barbeau & Rossignol, 1991).

Nevertheless, parallel routes of activation using different transmitters (for example, noradrenaline, acetylcholine and 5-HT are a possibility (cf. Bareau & Rossignols, 1991). In neonatal rat (one to six days old), stimulation of brainstem regions can elicit patterned EMG discharge in rat limb muscles, equivalent to stepping movements. Cazalets et al., (1992) have shown that bath-applied 5-HT can initiate an alternating pattern of right and left action potential bursts. The period of this rhythm was dose-dependent and the effects of 5-HT were blocked by a 5-HT_1 antagonist (propanolol) and by 5-HT_2 antagonists (ketanserin, cyproheptadine, mianserin). 5-HT_3 antagonists were ineffective. Therefore the effects of 5-HT are mediated via 5-HT_{2A} or 5-HT_{2C}, (and possibly 5-HT_1) receptors. Bath application of NMDA or acetylcholine similarly elicit rhythmic activity in the same in vitro spinal preparations. However, these substances seems to preferentially activate specific patterns (Clowley & Schmidt, 1994). Further studies in neonatal rat preparations

have found that the 5-HT-sensitive oscillatory network, capable of producing a locomotor-like pattern of activity, is distributed throughout the supralumbar region of the spinal cord and mediates descending rhythmic drive to lumbar motor centres (Clowley & Schmidt, 1997). These results suggested the involvment of 5-HT receptors in the initiation of the motor programme.

(2) Serotonergic modulation of locomotion pattern and central pattern generators

5-HT has been found to modulate locomotor patterns in a variety of animal preparations, ranging from lower vertebrates such as the lamprey (Matsushima & Grillner, 1992; Schotland & Grillner, 1993; Wikstrom et al., 1995; Zhang et al., 1996), and *Xenopus* (Sillar et al., 1992), to mammalian preparations, such as neonatal rat (Sqalli-Houssaini et al., 1993; Kiehn & Kjaerulff, 1996), rabbits (Viala & Buser, 1969), and cats (Barbeau & Rossignol, 1990). The effects of 5-HT on motor pattern generation are rather general: it increases the intensity and duration of each burst of motorneuron activity (reviewed by Sillar et al., 1997).

In the lamprey, the actions and mechanisms of 5-HT on locomotion have been studied in detail. Serotonin is known to affect the pattern of fictive locomotion induced by NMDA receptor activation, by increasing spike rate within a ventral root burst, increasing burst duration, and increasing the intersegmental delay (Matsushima & Grillner, 1992). Both the effects of 5-HT on the intersegmental delay (Matsushima & Grillner, 1992) and burst duration (Zhang et al., 1996) occured in a rostrocaudal graded fashion. The changes in duration and intensity of motor bursts in each cycle increase the force of muscle contraction. With changes in locomotor frequency during application of 5-HT, the phase relationships between motor neurones pools must be modified to accomodate the concomitant changes in burst duration.

Two cellular mechanisms contribute to these effects of 5-HT in this system: the after-hyperpolarisation (AHP) following individual action potentials is reduced and

the depolarising plateau induced by NMDA is prolonged (Matsushima & Grillner, 1992; Schotland & Grillner, 1993; Wikstrom et al., 1995; Zhang et al., 1996). Lamprey spinal motor neurones have an AHP that consists of both a fast and a slow phase (sAHP). The sAHP is produced by an apamin-sensitive Ca^{2+}-activated potassium conductance (El Manira et al., 1994). These apamin-sensitive channels may contribute to the regulation of the neuronal firing and to the termination of the locomotor bursts in lamprey neurones (El Manira et al., 1994). The prolongation of the depolarising plateau by 5-HT also serves as an important burst-terminating factor in the locomotor network (Matsushima & Grillner, 1992; El Manira et al., 1994).

In *Xenopus* tadpoles, bath-applied 5-HT enhances the duration of motor activity on each cycle of fictive swimming, an effect similar to that in lamprey (Sillar et al., 1992). However, the responsible underlying ionic mechanisms may be different from these in lamprey, because the apamin-sensitive Ca^{2+}-activated K^{+} currents of *Xenopus* differ from those in the lamprey and are unlikely to contribute to burst termination. Instead, they are involved in regulating the length of episodes of fictive swimming (Wall & Dale, 1995). 5-HT also modulates the locomotor pattern of chronic spinal cats walking on a treadmill. The 5-HT mediated action, generated by administration of 5-hydroxytryptophan, increases step length and augments contraction of hind-limb extensors and flexors (Barbeau & Rosignol, 1990; 1991). In lightly anaesthetised rabbits, 5-HT released by treatment with 5-hydroxytryptophan increases flexor-burst amplitude during fictive locomotion and induces locomotion in acute spinal rabbits via receptors that were not characterised (Viala & Buser, 1969).

(3) 5-HT and voltage oscillations in CPG motor neurones

The NMDA receptor-mediated intrinsic oscillations in membrane potential are present in the spinal motor neurones in a wide range of vertebrate species: amphibian embryo and larvae (Sillar & Simmers, 1994), lamprey (Matsushima & Grillner, 1992; Schotland & Grillner, 1993), adult turtles (Guertin & Hounsgaard, 1998) and neonatal rat (Sqallihoussaini et al., 1993; Hochman et al., 1994; MacLean et al., 1997). However, the oscillation in amphibian and turtle spinal network differ from oscillations in mammals.

In mammals, the voltage-oscillation can be caused by bath-applying NMDA or 5-HT alone. In visually identified neurones surrounding the central canal in 300 μM transverse slices of lumbosacral spinal cord from 7- to 14-day-old rats, activation of the NMDA receptor alone can elicit low- and high-frequency membrane voltage oscillations. These neurones may be involved in generation of the locomotor pattern (Hochman et al., 1994). Membrane depolarisation produced by oscillations may activate other intrinsic conductances which generate plateau potentials, thereby providing the neuron with enhanced voltage sensitivity, compared to that produced by NMDA receptor activation alone. These oscillatory events may have a role in the regulation of motor output in a variety of rhythmic behaviours including locomotion (MacLean et al., 1997). This evidence therefore suggests that 5-HT or NMDA alone can cause voltage oscillations in mammalian CPG neurones. However 5-HT did increase the stability of the period from one cycle to another when applied together with NMDA compared with the activity induced by bath application of NMDA or 5-HT alone (Sqallihoussaini, et al., 1993).

Unlike in the mammal CPG, in amphibians and turtles, the oscillations appear to rely on co-activation of 5-HT_{1A} and NMDA receptors. In larvae of the amphibian. *Xenopus laevis*, spinal neurones which are active during fictive swimming also display tetrodotoxin-resistant membrane potential oscillations following the co-activation of NMDA and 5-HT receptors. The enabling function of 5-HT may involve the facilitation of the voltage-dependent block of the NMDA ionophore by Mg^{2+} through activation of receptors with $5HT_{1a}$-like pharmacology (Scrymgeour-Wedderburn et al., 1997). In adult turtles, the existence of intrinsic oscillations in single neurones are also dependent upon NMDA and 5-HT (Guertin & Hounsgaard, 1998). These results suggest that in this simple, developing vertebrate locomotor system, the activation of 5-HT receptors on spinal cord neurones in turn modulates NMDA receptor activation to enable the expression of intrinsic oscillatory membrane properties which could contribute to the generation of locomotor behaviour (cf. Sillar & Simmers, 1994).

(4) Sources of 5-HT

There is a large population of serotonergic neurones located in the raphe regions of the ventral medulla. These neurones possess axonal projections that extend into the dorsal and ventral horns of the spinal cord. Unit recordings from these neurones show that they are normally tonically active and exhibit an increased firing rate during motor behaviour. A subset of serotonergic neurones displays rhythmic activity that is phase-coupled to that of the spinal motor pattern generators. Activation of the remainder of the serotonergic neurones appears to be related to the level of tonic motor activity (reviewed by Jacobs & Fornal, 1997). Although the serotonergic neurones in the raphe region appear to be the main source of 5-HT acting on locomotor circuitry in mammals, in lower vertebrates such as lamprey (Van Dongen et al., 1985), mudpuppy (Jovanovic et al., 1996) and turtle (Kiehn & Harriswarrick, 1992) there are also populations of serotonergic neurones that exist in the spinal cord, which also play an important role in modulating locomotion.

(5) Pharmacological profile of the serotonin receptor subtypes involved

In newly hatched *Xenopus laevis* (stage 37/38), the 5-HT-containing neurones in the raphe nuclei are in the process of extending their axons down the spinal cord (Van Mier et al., 1989). In *Xenopus* tadpoles, the effects of 5-HT can be mimicked by the non-selective 5-HT_1 agonist 5-CT, and the 5-HT_{1A} agonist R(+)-8-OH-DAPT, and blocked by NAN-190. Therefore these results suggest involvement of 5-HT_{1A} receptor (Sillar et al., 1992b). However, the effect of R(+)-8-OH-DPAT was much smaller than that of 5-CT, and another 5-HT_{1A} agonist, buspirone, had very little effect on swimming motor pattern. This suggests the involvement of other unidentified 5-HT_1 receptor subtypes in the modulation of locomotion pattern in *Xenopus*. In adult lamprey, the effect of 5-HT on locomotion occurred via the activation of possibly more than one receptor subtype (Van Dongen et al., 1986; Wallen et al., 1989; Grillner et al., 1991). Although a 5-HT_{1A}-like receptor may be involved (Wikstrom et al., 1993), the detailed receptor subtypes have not been identified. There is also evidence for a similar involvement of 5-HT_1 receptors in the locomotory function of other vertebrates, including mammals (Cazalets et al., 1992;

Wang & Dun 1990). For example, in neonatal rat motor neurones, 5-HT_{1A} and 5-HT_2 receptors produced different responses, one hyperpolarising and the other depolarising (Wang & Dun, 1990). In adult frog spinal motor neurones, a hyperpolarisation was caused by 5-HT_{1A} receptor-activation (Holohean et al., 1990). In spinal cats, 5-HT_{2A} 5-HT_{2C} and also 5-H_{1A} receptors are all found to be involved in modulating motor pattern (Barbeau & Rosignol 1990; 1991).

In summary, after being released from the serotonergic neurones of the raphe region, 5-HT activates multiple metabotropic, postsynaptic receptors to mediate its actions on motor behaviour in the following aspects: (1) initiation of patterned motor activity in a parallel routes with EAA-mediated initiation of motor activity; (2) modulation of motor pattern in a wide range of vetebrate locomotion systems, where the amine' s principal effect is to enhance the duration and intensity of motor bursts in each cycle; and (3) elicit oscillatory membrane properties of motor neurones. The roles of the 5-HT_1 and 5-HT_2 receptor subtypes have also been discussed. Although 5-HT has been widely documented to modulate the motor pattern, little is known about the underlying mechanisms involved, particularly ionic and cellular mechanisms. Therefore, a major goal of my thesis is to gain further understanding of the 5-HT actions in a simple vertebrate model system.

The descending 5-HT system also plays an inhibitory role in modulating sensory pathways. This dual action of 5-HT on motor and sensory circuits has led to the proposal that intraspinal 5-HT might be released simultaneously onto both rhythm-generating and sensory-processing networks, facilitating motor output while depressing somatosensory inputs. In this way, the responsiveness of an organism to cutaneous inputs could be reduced during periods of intense locomotor output, such as in response to injury or during escape from danger (cf. Hultborn & Illert, 1991; Sillar & Simmers, 1994).

1.45 Serotonergic modulation of sensory transmission in spinal cord

(1) Actions of 5-HT on sensory transmission in spinal cord

Nociception involves the detection of painful, potentially threatening stimuli. In the periphery, unmyelinated C fibres (C polymodal nociceptors) or line myelinated A delta fibres are excited by noxious stimulation, either direct mechanosensitive or indirectly through inflammatory processes. Nociceptive afferent fibres terminate in the superficial laminae of the dorsal horn of the spinal cord where their outputs are integrated and controlled. These first synapses are modulated by excitatory amino acids (glutamate and aspartate) and by many peptides (e.g. substance P, CGRP, CCK, endogenous opiods). Although nociceptive signals are modulated at all levels of their transmission, the more extensively studied controls are located at the spinal level, where they can be inhibited following activation of bulbospinal descending inhibitor pathways and release of serotonin, noradrenaline and, indirectly, endogenous opiods (see review by Guirimand & Lebars, 1996; Millan, 1995). Although the mechanisms of inhibitory action of 5-HT on somatosensory processing are not totally clear, the demonstration that monoaminergic projections often show axonal varicosities, without obvious conventional synaptic spinalisations, has led to the proposal that the amines may be liberated in a paracrine fashion onto spinal circuitry (Hultborn & Illert, 1991), thereby influencing a diverse range of possible target neurones simultaneously. The available evidence suggests that 5-HT exerts various pre- and postsynaptic actions to modulate somatosensory processing in the dorsal horn.

Presynaptic mechanisms Endogenously released 5-HT can inhibit the excitatory synaptic transmission from primary afferents to dorsal horn neurones (Mjellem et al., 1992; Sillar et al., 1992; Clarke et al., 1996; Lopezgarcia & King, 1996). 5-HT may also excite local spinal inhibitory interneurones that contain GABA (Alhaider et al., 1991) or encephalins (Jessel & Kelley, 1991). These interneurones can then presynaptically inhibit the terminals of the primary afferents.

Postsynaptic actions: The dorsal horn neurones are themselves richly endowed with 5-HT receptors (Yoshimura & Nishi, 1991). Current evidence suggests that the predominant effects of 5-HT on nociception are in the deep dorsal horn (cf. Lopezgarcia & King, 1996). In adult frogs (*Rana pipiens*), 5-HT produced direct changes in the membrane potential of dorsal horn neurones. 5-HT also appeared to

affect spinal dorsal horn neurones indirectly, because it evoked both EPSPs and IPSPs. In addition, the amine appeared to suppress sensory transmission in the dorsal horn because it decreased the size of dorsal root-evoked EPSPs in some cells, and increased the stimulation threshold for dorsal root-evoked action potentials in others. These diverse actions of 5-HT are dependent upon the activation of several different receptor subtypes which may all contribute to the amine's inhibition of nociceptive signal processing in the spinal dorsal horn (Tan & Miletic, 1992). Similar results are seen in the mammalian dorsal horn neurone membrane (Gjerstad et al., 1996 & 1997). Other lines of evidence include 5-HT-induced depression of the NMDA-evoked response at the postsynaptic site on dorsal horn neurones (LopezGarcia 1998), and an inhibitory effect on the release of somatostatin, which is involved in nociceptive transmission in the dorsal horn from the central terminals of primary sensory neurones (Kuraishi et al., 1991).

(2) Pharmacological profile

The roles of 5-HT receptors have been studied by intrathecal administration of selective agonists, or antagonists, and observing the (anti)nociceptive effects in the increasing temperature hot-plate test and changes in NMDA-receptor mediated behaviour (Ali et al., 1994). *In vitro* preparations of spinal cord have also been used, and responses to dorsal root stimulation (DR-EPSP) and to droplet application of N-methyl-D-aspartic acid (NMDA) and α-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMPA) were obtained by means of intracellular recordings from dorsal horn neurones (LopezGarcia, 1998).

The role of 5-HT in the regulation of nociception depends on the 5-HT receptor subtypes involved and on long-term functional changes in the 5-HT receptors. Current evidence suggests the involvement of the 5-HT_1, 5-HT_2, 5-HT_3 receptor classes. However, in most of these reports, 5-HT_1 receptors (particularly 5-HT_{1A} and 5-HT_{1B}) appear to play the more important roles.

5-HT_1 receptors: Spinal 5-HT_1 receptor subtypes may either facilitate or inhibit nociceptive input depending upon the type of nociceptor that is activated, as opposed

to the type of receptor subtype that is stimulated (Murphy et al., 1992). Several reports suggest a facilitatory role for 5-HT_{1A} receptors on nociceptive responses and support the involvement of the 5-HT_{1B} receptor subtype in the antinociceptive action of serotonin in mammalian dorsal horn (Alhaider & Wilcox, 1993; Ali et al., 1994; Millan et al., 1997). However, many other researchers have obtained contradictory results for the role of the 5-HT_{1A} receptor. For example, stimulation of 5-HT_{1A} receptors inhibits the activity of many dosal horn neurones (Gjerstad et al., 1996), suggesting that the activation of spinal 5-HT_{1A} receptors could be antinociceptive (Crisp et al., 1991; Mjellen et al., 1992; Xu et al., 1995; Oyama et al., 1996; Clarke et al., 1996; 1997; LopezGarcia, 1998; but see also Millan et al., 1994). Similar effects have also been noted on 5-HT_{1B} receptors, where stimulation of the 5-HT_{1B} receptors may have both pro and antinociceptive effects on a wide range of neurones in the dorsal horn following repeated electrical stimulation (Gjerstad et al., 1997). Information on 5-HT_1 receptor subtypes other than 5-HT_{1A} and 5-HT_{1B} receptors is sparse. However available evidence suggests that 5-HT_{1D} receptors (cf. Clarke et al., 1997) as well as the 5-HT_7 receptor (Clarke et al., 1997) may be involved, and probably play a role in antinociception in vertebrate spinal cord (cf. Clarke et al., 1997).

5-HT_2 receptors When these are located in supraspinal structures they may inhibit descending nociceptive neurotransmission. However when these receptors are located in the spinal cord they modulate or inhibit nociception (Crisp et al., 1991; Xu et al., 1994; Clarke, et al., 1996). Several reports have also suggested that the 5-HT_2 receptor facilitate the actions of 5-HT_{1A} receptors on the nociceptive response (Crisp et al., 1991; Millan et al.,1997). 5-HT_2 receptors are also responsible for the attenuating effect of 5-HT on the noradrenaline- or morphine-induced antinociception at the spinal cord level (Crisp et al., 1991; Zhang et al., 1995).

5-HT_3 receptor: In the spinal cord of rodents, activation of 5-HT_3 receptors may evoke release of GABA, which may in turn inhibit nociceptive transmission at a site postsynaptic to terminals of primary afferent fibres (Alhaider et al., 1991). The 5-

HT_3 receptor has also been reported to facilitate nociception in the spinal cord (Oyama et al., 1996), but some researchers have questioned the involvement of the 5-HT_3 receptor in spinal nociceptive transmission (cf. Xu et al., 1995; Bardin et al., 1997).

In summary, stimulation of 5-HT_1 receptors, as well as of the 5-HT_2 and 5-HT_3 receptors, may reduce nociceptive sensitivity. In addition, activation of 5-HT_1, 5-HT_2 and 5-HT_3 receptors may enhance nociceptive sensitivity. Up- or down-regulation of the 5-HT receptors may result in long-lasting changes (plasticity) in the 5-HT systems (cf. Eide & Hole, 1993). Perhaps one reason for the apparently diverse or even contradictory actions of certain 5-HT receptor subtypes on nociception is that many of the pharmacological experiments were carried out in *in vivo* preparations and the effect of 5-HT receptor subtypes were tested through intrathecal administration of selective drugs. This could lead to the activation of multiple unknown pathways and non-specific receptor-mediated responses. It would be helpful to obtain more results with direct recordings from dorsal horn neurones and knowing whether certain effects of drugs were produced via pre vs. post-synaptic sites.

1.50 The *Xenopus* embryo and larvae as a model for studying control of locomotion

1.51 The simple neuroanatomy of the spinal cord

The spinal cord of *Xenopus* at stage 37/38 is very simple and contains only eight anatomical classes of neurones, as revealed by horse radish peroxidase (HRP) studies (Roberts & Clarke, 1982). These comprise: 1) The mechanosensory Rohon-Beard neurones (R-B neurone); 2) Dorsal lateral commisural interneurones (DLC) which

are sensory interneurones that relay excitation from R-B neurones to the rest of the CNS (Roberts & Sillar, 1990) 3) Dorsolateral ascending interneurones (DLA), which perform a similar role to DLCs; 4) Descending interneurones which are the major excitatory interneurones and use glutamate as a neurotransmitter (Dale & Roberts, 1985); 5) Commisural interneurones which are immunoreactive for glycine (Dale et al., 1986) and are responsible for reciprocal inhibition during locomotion activity (Dale et al., 1990) and are readily identifiable under phase contrast microscopy (Dale, 1991); 6) Motorneurones which lie in the ventral part of the cord (Roberts & Clarke, 1982); 7) Ascending interneurones and 8) Kolmer-Agduhr cells, which both show GABA immunoreactivity, although their functions remain unknown (Fig 1.6).

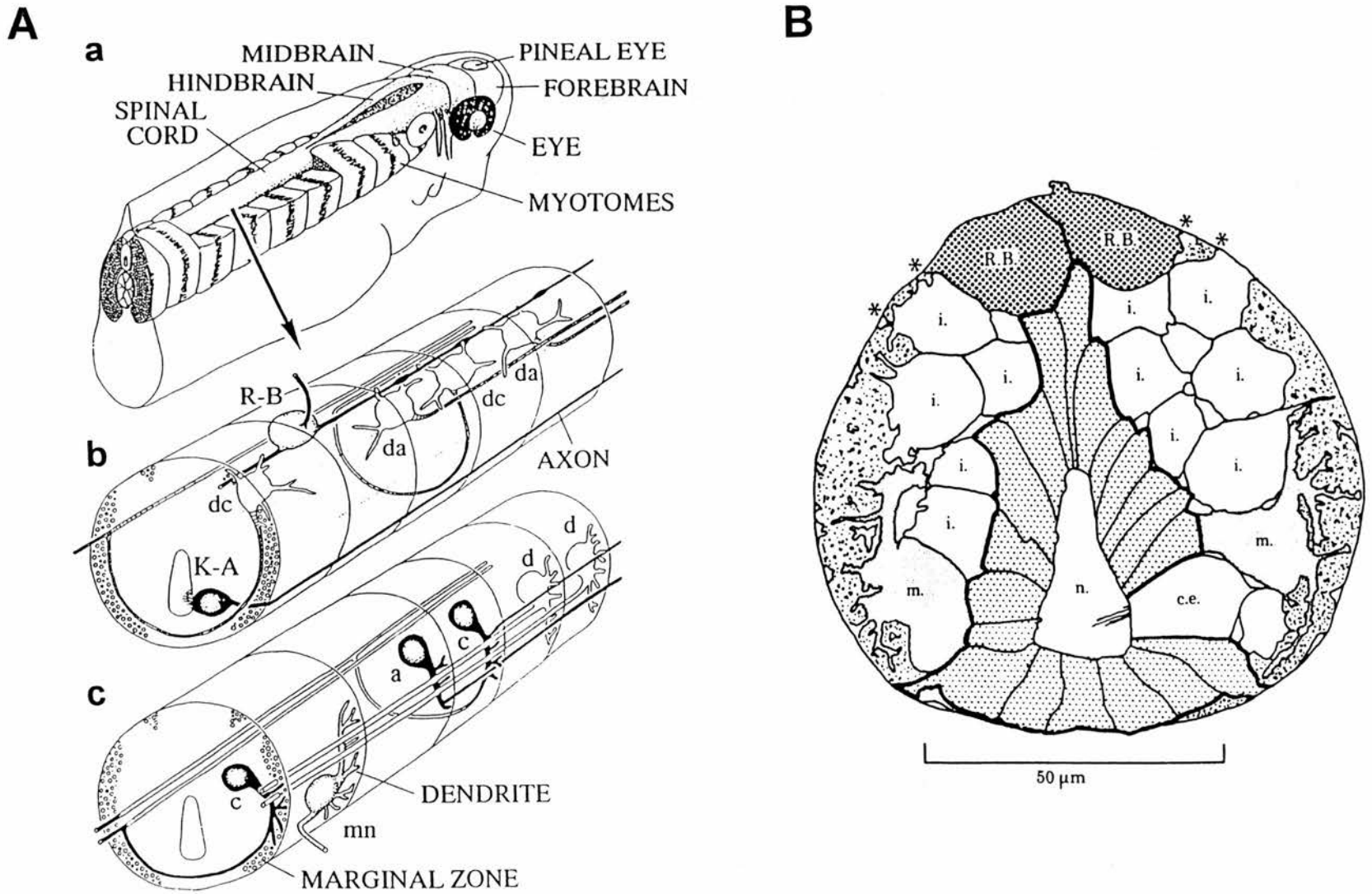
1.52 Sensory pathways of *Xenopus* embryo

The trunk skin of the *Xenopus* embryo is innervated by the free nerve endings of R-B neurones, which respond to transient indentation with one or a few impulse (Clarke & Roberts, 1984). These R-B neurones have naked peripheral neurites, usually under 1μm in diameter, which form a loose network under the skin. The narrow neurites run into the skin between the cells (Roberts & Hayes, 1977). Since R-B neurones are unmyelinated and possess free nerve endings which innervate the trunk skin; use glutamate as a transmitter (Sillar & Roberts, 1988) and substance P as a co-transmitter (Clarke et al., 1984); and have capsaicin receptors (Kuenzi & Dale, 1996), they are analogous to human C fibres of the pain pathways. The acutely isolated R-B neurones have a distinctive morphology, maintain similar membrane properties to their *in vivo* counterparts and can easily be recognised *in vitro* (Dale, 1991). The R-B neurones can therefore be used as a highly advantageous model for studying the neuromodulation of sensory pathways.

The R-B neurones have cell bodies and axons in the dorsal spinal cord, where they form synapses with two classes of sensory interneurones with ascending projections

Fig 1.6 The neuroanatomy of *Xenopus* embryo spinal cord.

A) (i) *Xenopus* embryo nervous system. (ii) Spinal cord. Neurones in the sensory system: Rohon-Beard neurones (R-B) which act as skin touch detectors, Kolmer-Agduhr neurones (K-A), dorsal lateral commisural interneurones (dc) and dorsal lateral ascending interneurones (da). (iii) neurones of the motor system: motor neurones (mm) innervating the myotomes, descending interneurones (d), commisural interneurones (c) and ascending interneurones (a). All neurones have longitudinal axons in the marginal zone on the outside of the cord. B) Diagram of cellular organization of the spinal cord at stage 35/6 in transverse section R.B. Rohon-Beared cells; i., interneurons; m., motor neurons; c.e., ciliated ependymal cell n. neurocoel. (This figure is adapted from Roberts & Clarke, 1982)


A
a
MIDBRAIN
PINEAL EYE
HINDBRAIN
FOREBRAIN
SPINAL CORD
EYE
MYOTOMES
R-B
da
dc
da
AXON
b
dc
K-A
d
d
c
a
c
c
DENDRITE
mn
MARGINAL ZONE
B
R.B.
R.B.
i.
m.
n.
c.e.
50 μm

(Fig 1.6). The excitation of these interneurones by R-B neurones is produced by excitatory amino acid neurotransmitters acting at NMDA and non-NMDA receptors (Clarke & Roberts, 1984; Sillar & Roberts, 1988b; 1990). The first of these classes, dorsolateral commisural (DLC) interneurones, is excited by R-B cells innervating the skin of the same side of animal (Clarke & Roberts, 1984; Sillar & Roberts, 1988b), but not those on the opposite side (Roberts & Sillar, 1990). DLC interneurones in turn relay excitation across the spinal cord via their commissural axons to contralateral motorneurones (Roberts & Sillar, 1990). The second class of sensory interneurones is also excited by R-B cells on the same side as their somata, but they differ from the DLCs in that they are excited by skin stimulation on both sides of the animal (Roberts & Sillar, 1990; Fig 1.6).

1.53 The locomotion motor pattern and underlying neural circuitry have seen largely elucidated

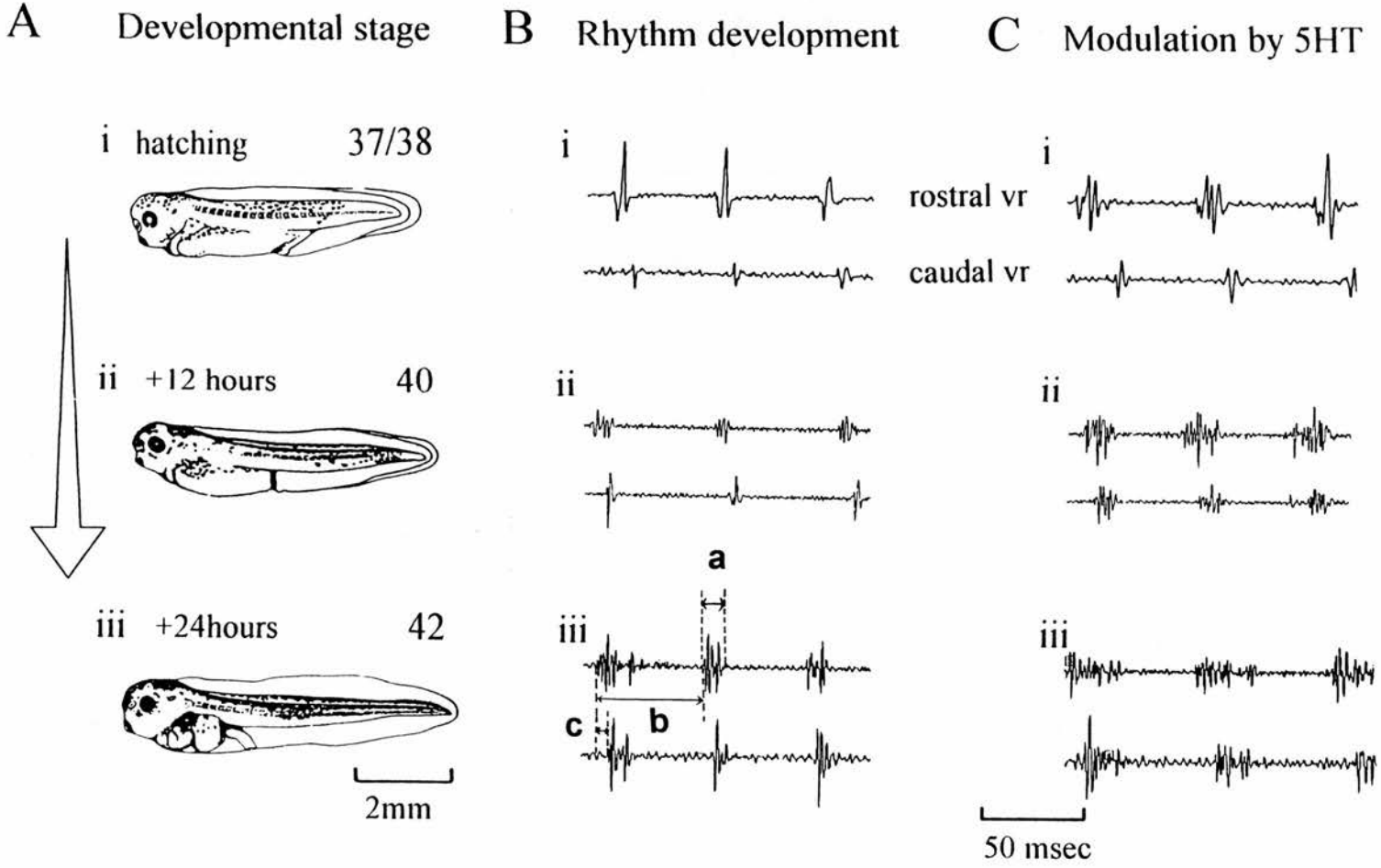
The *Xenopus* embryo exhibits simple and well defined motor behaviours (Roberts & Hayes, 1977; Clarke et al., 1984; Soffe, 1991). The motor pattern for swimming consists of brief alternating bursts of spikes in the ventral roots on either side of the body, the discharge on one side being midcycle to discharge on the other. During this activity, motorneurones (and all rhythmically active interneurones) fire one spike in phase with the ipsilateral ventral root. Neurones receive midcycle inhibition during the discharge of the contralateral ventral root (see reviews by Arshavsky et al., 1993; Dale & Kuenzi, 1997; Fig 1.7).

The excitatory tonic drive for the pattern of activity is mainly provided by a population of glutamatergic interneurones, which excite all rhythmically active neurones on the same side of the spinal cord via NMDA and non-NMDA receptors (Dale & Roberts, 1984; 1985). Recent evidence suggests that spinal neurones possess nicotinic and muscarinic acetylcholine receptors, which also produce excitatory synaptic drive during fictive swimming (Perrins & Roberts, 1993).

The midcycle inhibition present during swimming occurs in phase with the firing of neurones on the opposite side (Soffe & Roberts, 1982b). This reciprocal inhibitory

Fig 1.7 Developmental changes of *Xenopus* motor pattern are mimicked by bath application of 5-HT

A) Time sequences of development of *Xenopus* ambryo and larvae; B) development of rhythmic motor pattern recorded from ventral root burst. The ventral burst developes from a single compund impulse in each cycle (37/38) to prolonged discrete bursts of activity (42). In the intermediate stage (40), only the rostral ventral root burst changes to larvarl like and the caudal part remains embryonic. C) Modulation of ventral root discharge by 5-HT. 5-HT increases burst duration in stage 42 (iii) and 40 (ii) *Xenopus*, but only the rostral burst in stage 3738 (i) is increased. The following aspects of motor pattern were changed and were shown in the figure: a, burst duration; b, cycle period; c, rostral caudal delay (The figure is kindly supplied by Dr. K.T. Sillar)


A
Developmental stage
i hatching 37/38
ii +12 hours 40
iii +24hours 42
2mm
B
Rhythm development
i
ii
iii
a
b
c
C
Modulation by 5HT
rostral vr
caudal vr
i
ii
iii
50 msec

synaptic drive is strychnine-sensitive Cl-conductance and therefore provided by commisural glycingergic interneurones on the two opposite sides of the spinal cord (Dale, 1985; Soffe 1987; reviewed by Arshavsky et al., 1993; Dale & Kuenzi, 1997).

1.54 Ion channels and the control of swimming in *Xenopus* embryo and larvae

Ultimately, the understanding of how neural circuits operate not only requires the identification of specific neurones and their synaptic connectivity, but also knowledge about the properties of various ion channels expressed in the membranes of neurones (Dale & Kuenzi, 1997). Dale's lab has performed extensive investigations to address the roles of individual voltage- and ligand-gated ion channels in controlling the motor pattern for swimming (Dale, 1993; 1995a; 1995b; Wall & Dale, 1995; 1996; Dale & Gilday, 1996; Kuenzi & Dale, 1997; 1998; Sun & Dale, 1998a; reviewed by Dale & Kuenzi, 1997a; b).

Xenopus embryo spinal neurones possess at least 7 classes of ion channel: a fast Na^+ channel (Dale, 1995a); a mixture of kinetically similar Ca^{2+} channels (Dale, 1995a); a fast K^+ channel (Dale, 1995a; Kuenzi & Dale, 1998), a slow K^+ channel (Dale, 1995a; Kuenzi & Dale, 1998); a Na^+-dependent K^+ channel (Dale, 1993); and a slowly-activating Ca^{2+}-dependent K^+ channel (SK; Wall & Dale, 1995). The Na^+ current is TTX sensitive and very fast , and activates with time constants ranging from 0.1 to 0.25 ms depending on voltage. It is also subject to rapid inactivation with time constants ranging from 0.3 to 8 ms (Dale, 1995a). The Ca^{2+} currents are kinetically similar, and activate with time constants ranging from 0.3 to 0.8 ms (Dale, 1995a). Pharmacological investigation revealed that around 65% of whole cell Ca^{2+} conductance were via ω-conotoxin-GVIA-sensitive (N- type) channels (Wall & Dale, 1994). The channels carrying the remainder of the current have not been identified in the embryo. The outward currents are hard to separate either pharmacologically or through differences in voltage-dependence. The delayed rectifier consists of varying proportions of two currents: a fast-activating K^+ current (with time constant of activation ranging from 0.6 to 2 ms) and a slow K^+ current

(with time constants of activation ranging from 5 to 25 ms). The slow current is occasionally seen in isolation (Dale, 1995a). About 30% of the outward K^+ current is Na^+-dependent (Dale, 1993). Together, the fast I-Ki and sodium-dependent I-KNa constitute about 80% of the outward current, and the slow I-Ks constitutes the remainder. The fast potassium currents are selectively blocked by catechol (IC_{50} approximately 10 μM) and the block is voltage-dependent, with partial unblocking occurring at positive voltages. α-Dendrotoxin and dendrotoxin-I selectively blocked the slow potassium current (Kuenzi & Dale, 1998). The Ca^{2+}-sensitive K^+ current is very slow to activate and has a mean time constant of activation of 437 ms at 0 mV. The toxin apamin (10 nM to 2 μM) selectively blocked the Ca^{2+}-dependent K^+ current without affecting voltage-gated K^+ currents. This current may be analogous to a small-conductance Ca^{2+}-dependent K^+ (SK) current; however, unlike some SK currents, the Ca^{2+}-dependent K^+ current is also sensitive to 500 μM tetraethylammonium chloride (Wall & Dale, 1995).

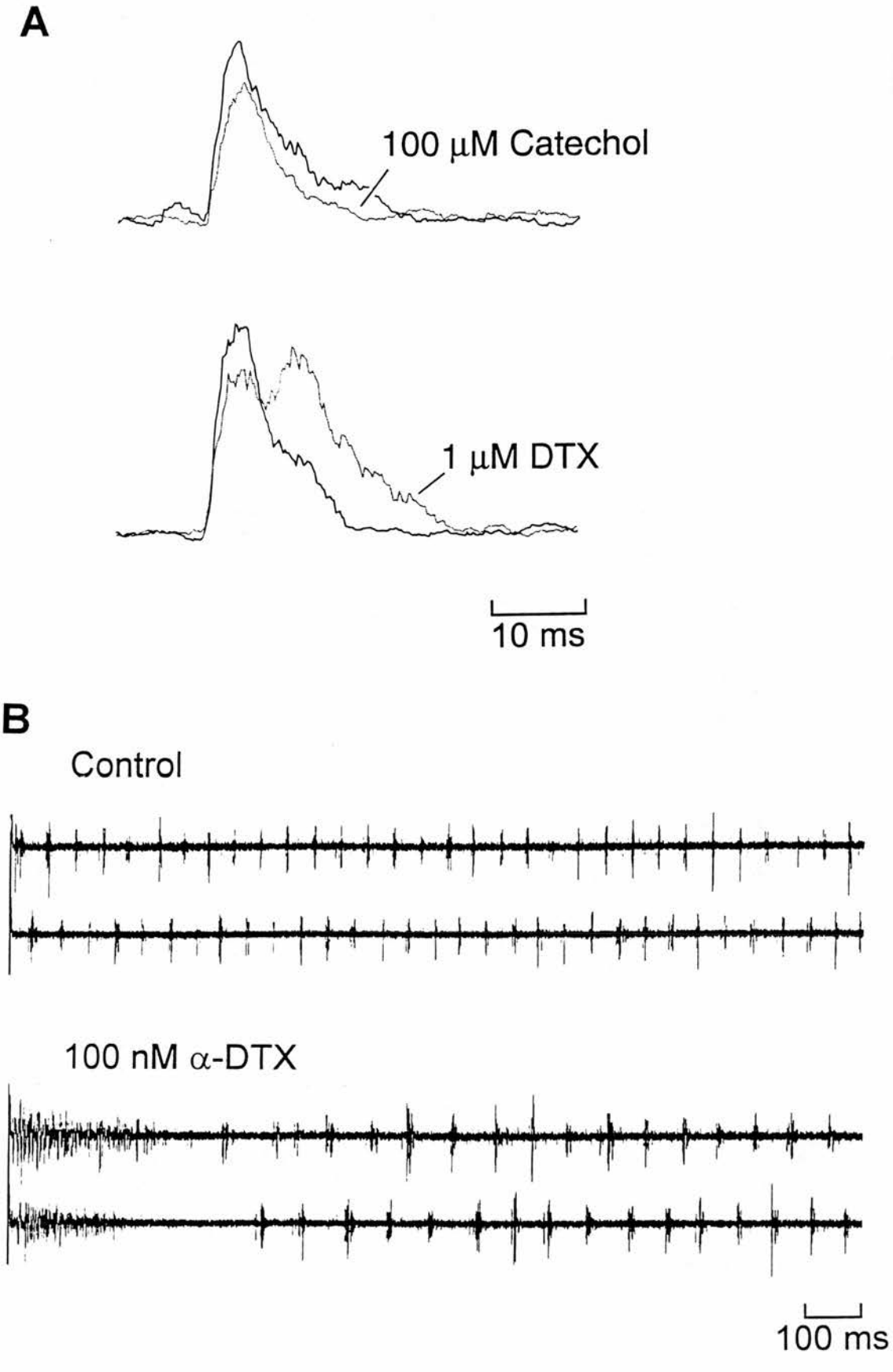
The membrane potential of primary sensory neurones (R-B neurones) of *Xenopus* embryo is more negative than non-sensory neurones (around -90 mV, Spitzer and Lamborghini, 1976). The membrane of R-B neurones also seems to have express different ion channels from those in the non-sensory neurones, *e.g.* R-B neurones have different proportions of slow and fast K^+ channel (Kuenzi & Dale, 1998); larger T-type Ca^{2+} currents (Sun & Dale, 1997), and lack of Ca^{2+}-dependent K^+ currents (Q. Q. Sun, & N. Dale, unpublished observations).

The roles of the voltage-gated currents in determining neuronal firing properties and operation of the locomotor circuitry have been examined both pharmacologically and in realistic computer simulations. Dale (1995a; 1995b) developed simulations of *Xenopus* embryo spinal neurones, which were endowed with Hodgkin-Huxley-style models of voltage-dependent Na^+, Ca^{2+}, slow K^+ and fast K^+ currents together with a Na^+-dependent K^+ current. Model neurones fire repetitively in response to current injection, which is consistent with the observations that most of the isolated spinal neurones can fire repetitively (Q.Q. Sun & N. Dale, unpublished observations). The Ca^{2+} current seems essential for repetitive firing. The conclusions draw from simulations have been confirmed by the observations that reduction of Ca^{2+} currents

by the Ca^{2+} channel blocker ω- conotoxin-GVIA markedly reduces the number of spikes in response to current injection in isolated neurones (Wall & Dale, 1994). GABA, which also inhibits the N type Ca^{2+} currents, have similar effects on the neuronal firing (Wall & Dale, 1994). The fast K^+ current appears mainly to control spike width, whereas the slow Kf current exerts a powerful influence on repetitive firing. Both the model network and the real spinal locomotor circuit appear to tolerate a wide variation in the relative strengths of the component synapses, but are very sensitive to the magnitudes of the voltage-gated currents (Wall & Dale, 1994). In particular, the slow K^+ current, despite being a small component of the total outward current, plays a critical role in stabilising the motor pattern. These predictions from the model have been confirmed by the use of specific pharmacological blockers of the fast and slow K^+ currents and other ion channels involved (Fig 1.8; Kuenzi & Dale, 1998; reviewed by Dale & Kuenzi, 1998). In initial experiments, Wall & Dale (1994) found that the balance between inward and outward currents is essential for the correct operation of the swimming motor circuit. Even small reductions in the K^+ currents disturb this balance and lead to the firing of action potentials at aberrant times in the swimming cycle (Wall & Dale, 1995). Later experiments using selective blockers of the fast and slow K^+ currents, catechol and the dendrotoxins, show that these two channels have different effects on membrane excitability. Catechol causes spike broadening but has little effect on repetitive firing, whereas both dendrotoxins markedly increases repetitive firing without affecting spike width. By applying these agents to the whole embryo, the role of the fast and slow currents in motor pattern generation were examined. Catechol has little effect on fictive swimming (Fig 1.8), suggesting that the fast K^+ currents are not critical to circuit operation. However, dendrotoxin disruptes swimming early in the episode and increases the duration of ventral root bursts. The slow K^+ current, which is a minor component of the total outward current, thus appears to play an important role in motor pattern generation (Fig 1.8; Kuenzi & Dale, 1998). The removal of apamin-sensitive SK channels does not perturb the motor pattern for swimming. However, the cycle periods over which the locomotor rhythm generator could generate appropriate motor activity were lengthened by about 10% and the mean duration of swimming episodes was increased by approximately 40%. Therefore the

Fig 1.8 Effects of specific K^+ channel blockers on fictive swimming and burst duration.

A) Dendrotoxins (DTX, 1 μM) but not catechol (100 μM) increases the burst duration durig ficitive swimming; the thick line is control and the thin line is in the treatment indicated; B) DTX disturbs the swimming pattern of the *Xenopus* embryo; animals were spinalised and extracellular recordings of motor neurone activity were made from ventral roots on the left and right sides (top and bottom traces of each pair, respectively). α-Dendrotoxin (α-DTX) at 100 nM blocks approximately 40% of slow-potassium currrents and distrupts the swimming pattern (modified from Kuenzi & Dale, 1998).


A
100 μM Catechol
1 μM DTX
10 ms
B
Control
100 nM α-DTX
100 ms

Ca^{2+} -dependent K^+ current plays an important role in the self-termination of motor activity (Wall & Dale, 1995).

24 h after hatching, the swimming pattern of *Xenopus* develops from single compound impulse to discrete bursts of activity (Fig 1.7; Sillar et al., 1991). The properties of the neurones involved in the control of locomotion also undergo maturation (Cf. Sillar, et al., 1991; Sun & Dale, 1998a). The density of total outward current in the larval and embryonic neurones remaines the same from stage 37/38 to stage 42. Almost all neurones at stage 42 expresse a fast-activating Ca^{2+}-dependent K^+ current (I-KCa) that is largely absent from embryonic neurones. Whereas I-KCa become larger and more prevalent during development, the delayed rectifier K^+ currents are down-regulated. About 53% of I-KCa is selectively blocked by iberiotoxin. Therefore changes in expression of outward current closely correlate with the maturation of the motor pattern during development (Sun & Dale, 1998a). The developmental changes in other types of outward and inward currents have not been examined, but the developmental changes in neuronal firing properties found in *Xenopus* larvae (Q.Q. Sun & N. Dale, unpublished observations) are unlikely to be produced via a changes in expression of only one type of ion channels. The role of progressive expression of I-KCa and down-regulation of voltage-gated K channels was examined using iberiotoxin as a specific blocker of the BK currents. The firing properties of neurones isolated from embryos were unchanged by iberiotoxin, which is a BK channel blocker. However the toxin greatly increased the frequency of firing in larval neurones. Iberiotoxin extendes the duration of ventral root bursts during active swimming in larvae at stages 41 and 42, but had no effect at stage 40. Therefore changes in expression of outward current closely correlate with the maturation of the motor pattern during development (Sun & Dale, 1998a).

Like many other rhythmic motor patterns, swimming in the *Xenopus* embryo is episodic; it undergoes rundown and self-termination even in the absence of sensory inputs. Current investigations have revealed two components in control the 'run-down'. The slow Ca^{2+}-dependent K^+ current plays a role in the self-termination of swimming (Wall & Dale, 1994). Therefore a progressive increase in intracellular

Ca^{2+}, produced by an activity-dependent increase in Ca^{2+} influx via voltage-gated ion channels and NMDA receptors (cf. Dale & Roberts, 1985), could contribute to the run-down of swimming. However, intrinsic modulation mediated by the release of ATP and production of adenosine in the extracellular space appears to be a very powerful determinant of run-down of the motor pattern (Dale & Gilday, 1996). ATP is released during swimming, reducing K^{+} currents and thus increasing the excitability of the spinal circuits. The ATP is then slowly converted to adenosine in the extracellular space by the action of ATPase. As adenosine builds up it reduces the Ca^{2+} currents and thus damps down the excitability of the circuit. Swimming ends when the inhibitory effects of adenosine exceed the excitatory effects of ATP (Dale & Gilday, 1996; Dale, 1998).

1.6 The aim of this project

In 1.5 section of this chapter, I have summarised why *Xenopus* embryo and larvae become a ideal model for studying neural control of motor behavior and sensory pathways. Serotonin has also been know to modulate both locomotion and sensory transmission in *Xenopus* larvae (Sillar et al., 1992 & 1994; Sillar & Simmers, 1994). Therefore my project aims to investigate and answer the following questions:

(1) Which 5-HT receptor subtypes are possessed by spinal neurones of *Xenopus* larvae (Chapter II, IV) ?

(2) Which types of Ca^{2+} channels, and Ca^{2+} dependent potassium channels are expressed in the spinal neurones (Chapter II, IV & Appendix I)?

(3) How does 5-HT modulate different subtypes of Ca^{2+} channels (Chapter II, III , IV) ?

(4) What are the possible biophysical and biochemical mechanisms underlying the serotonergic modulation of Ca^{2+} channels ? (Chapter II, III , IV)

(5) What are the possible functional consequences of the modulation of Ca^{2+} currents (Chapter II, IV)?

Chapter 2

SEROTONERGIC INHIBITION OF THE T-TYPE AND THE HVA CA^{2+} CURRENTS IN THE PRIMARY SENSORY NEURONES OF *XENOPUS* LARVAE

2.1 Summary

The primary sensory Rohon Beard (R-B) neurons of *Xenopus* larvae are highly analogous to the C fibers of mammalian pain pathway. I explored the actions of 5-HT by studying the modulation of Ca^{2+} currents. In around 80% of the acutely isolated R-B neurons, 5-HT caused a mean inhibition of 16% (n=29) of the HVA currents and 24% (n=41) of the T-type currents. The modulation of the T-type and the HVA currents was mimicked by selective 5-HT_{1A} and 5-HT_{1D} agonists: 8-OH-DPAT and L-694,247. The effects of the agonists were blocked by their respective 5-HT_{1A} or 5-HT_{1D} antagonists: p-MPPI and GR127935, suggesting that both 5-HT_{1A} and 5-HT_{1D} receptors were involved. Around 70% of the actions of 5-HT on HVA currents was occluded by ω-conotoxin-GVIA (N-type channel blocker), while the rest of the modulation (~30%) was occluded by less than 100 nM ω-agatoxin-TK (P/Q-type channel blocker). This suggests that 5-HT acts on N- and P/Q- type Ca^{2+} channels. Neither the modulation of the T-type nor that of the HVA currents was accompanied by changes in their voltage-dependent kinetics. Cell-attached patch clamp recordings suggest that the modulation of the T-type channel occurs through a membrane-delimited second messenger. I have studied the functional consequences of the modulation of T-type Ca^{2+} channels and have found that these channels play a role in spike initiation in R-B neurons. Modulation of T-type channels by 5-HT could therefore modulate the sensitivity of this sensory pathway, by increasing the thresholds of R-B neurons. This is a new and potentially important locus for modulation of sensory pathways in vertebrates.

2.2 Introduction

Serotonin (5-HT) released from the descending fibers of the raphe nucleus plays an important role in limiting the access of nociceptive information from the spinal cord to higher centers (Millan, 1995).The receptors and mechanisms by which 5-HT exerts this antinociceptive action in the spinal cord have been incompletely studied. 5-HT_{1A} (Eide et al., 1990; Crisp et al., 1991; Lucas et al., 1993; Del Mar et al., 1994), 5-HT_{1B} (Eide et al., 1990) and 5-$HT_{2A/2C}$ (Xu et al., 1995) receptors have been implicated in modulation of nociception. However the roles of $5HT_{1D}$ receptor in modulation of sensory transmission remain unknown.

The cellular actions of serotonin on sensory neurons are also incompletely understood. One possible action is to inhibit Ca^{2+} entry into sensory neurons (Del Mar et al., 1994). In other CNS neurons, N-type, or P/Q -type HVA calcium channels can be modulated by serotonin, mostly through 5-HT_{1A} receptor and membrane-delimited G-protein pathways (Pennington et al., 1991; Koike et al., 1994; Bayliss et al., 1995; Foehring et al., 1996). These calcium channels are known to be involved in triggering synaptic transmission (Leubeke et al., 1993; Leubeke and Dunlap, 1994; Wall and Dale, 1994; Wheeler et al., 1994; see Dunlap 1997, for review). By contrast, T-type channels are not involved in transmitter release, but instead influence the firing properties of CNS neurons (Llinas and Yarom, 1981; White et at., 1989; Suzuki and Rogawski, 1989; Crunelli et al., 1989; Zhang et al., 1993). The effects of 5-HT on T -type channels are variable. In some CNS neurons, 5-HT has no action on T-type channels (e.g.: motorneurones, Bayliss et al., 1995); while in others it acts to increase the T-type current (Berger and Takahashi, 1990; Fraser and MacVicar, 1991). However, neither the functions of T-type channels in sensory transmission nor its modulation by 5-HT has been described.

Previous reports have demonstrated that 5-HT mediates presynaptic inhibition of transmitter release from *Xenopus* primary sensory neurons: Rohon-Beard (R-B) neurons (Sillar et al., 1994). We therefore examined the effects of 5-HT on voltage-dependent Ca^{2+} channels in acutely isolated R-B neurons. Our aims were to characterize the voltage-dependent Ca^{2+} channels modulated by 5-HT, identify the types of receptors involved, and explore some of the possible functional consequences. We found that 5-HT inhibits both the N-, P-type HVA currents and the T-type currents. While the modulation of HVA currents could contribute to the presynaptic inhibition of transmitter release from R-B neurons (Sillar et al., 1994), the modulation of T-type currents suggests an additional and important locus for modulation of sensory pathways.

2.3 Materials and Methods

2.31 Preparation of the acutely isolated spinal neurons.

Acutely isolated spinal neurons were prepared by methods based on those described by Dale (1991). In accordance with the UK Animals (Scientific Procedure) Acts 1986, stage 40-42 *Xenopus* larvae (Nieuwkop & Faber, 1956) were anaesthetized in a solution of MS222 (0.5 mg/ml, tricaine, Sigma); pinned to a rotatable Sylgard® table in HEPES saline with following composition (in mM): 117.4 Na^+, 3 K^+, 1 Mg^{2+}, 2 Ca^{2+}, 2 NO_3^-, 2.4 HCO_3^-, 124 Cl^-, 10 HEPES, and 10 Glucose at a pH 7.4; and their spinal cords were carefully removed, transferred to a dish containing 0.1-0.3 mg/ml DNase in HEPES saline and incubated at room temperature for 3 minutes. After this, the spinal cords were placed in a dish containing 8 mg/ml pronase (Sigma) in a low chloride trituration saline, composed of (in mM) 117.4 Na^+, 115 $MeSO_3^-$, 3 K^+, 1 Mg^{2+}, 2 Ca^{2+}, 2 NO_3^-, 2.4 HCO_3^-, 9 Cl^-, 10 HEPES, and 10 Glucose at pH 7.4, and incubated at room temperature for 2 minutes. They were then transferred to a dish of dissociation saline, composed of (in mM): 115 Na^+, 115 $MeSO_3^-$, 3 K^+, 2 EDTA, 3 Cl^-, 10 HEPES, 10 Glucose, and 10 piperazine-N, N'-bis (2-ethanesulfonic acid, PIPES) at pH 7.0 for 1 minute, and then to a dish of

PIPES saline , composed of (in mM): , 115 Na^+, 115 $MeSO_3^-$, 3 K^+, 0.1 Mg^{2+}, 0.1 Ca^{2+}, 3 Cl^-, 20 Glucose, and 10 PIPES, at pH 7.0 for 3 minutes. The spinal cords were then gently triturated in a saline containing 3 mg/ml DNase in a microfuge tube until the cords had dissociated. The cells were finally transferred to 35 mm poly-D-lysine-coated dishes in HEPES saline and allowed to settle and stick to the substrate for at least 1 hour before recording.

2.32 Patch clamp recordings

Owing to their unique morphological characteristics, Rohon-Beard neurons were readily identifiable under phase contrast microscopy, based on the criteria of Dale (1991): a large spherical soma (mean diameter 23 μm), a large nucleus (mean diameter 12 μm) and dark nucleolus. Whole-cell calcium currents and unitary calcium channel recordings were obtained as described by Hamill et al., (1981). Electrodes were fabricated using a Sutter Instrument P97 puller from capillary glass obtained from World Precision Instruments (TW 150F) and Clark Electromedical Instruments (GC150F-10) and coated with Sylgard® and fire polished. A List L/M-PC amplifier together with a DT2831 interface (Data Translation) was used to record and digitize the voltage and current records. Data were acquired to the hard disk of an IBM-compatible PC , while an optical disk was used for long-term storage of experimental records. The sampling and analysis software are written by Dale (1995). The whole-cell recordings had access resistances ranging from 4 to 12 MΩ. Between 70 and 85% of this access resistance was compensated for electronically. The adequacy of the voltage clamp was accessed by studying the I-V relations obtained from a series of voltage steps separated by 5 mV. The criterion for effective space clamp was a smoothly activating current. For recording of Ca^{2+} currents, external solutions were composed of (in mM) 57.5 Na^+ , 57.5 TEA, 2.4 HCO_3^-, 3 K^+, 10 Ca^{2+}, 1 Mg^{2+}, 10 HEPES, 1 4 -aminopyridine(4-AP), pH 7.4, adjusted to 260 mosmol l^{-1}, and TTX (140 nM) . The pipette solution contained (in mM) 100 Cs^+, 1 Ca^{2+}, 6 Mg^{2+}, 20 HEPES, 5 ATP and 10 EGTA, pH 7.4, adjusted to 240 mosmol l^{-1}. For unitary channel recordings (cell-attached mode) were made by methods described by Chen and Hess (1990): the membrane potential outside the bath was zeroed with the following external

solution: 110 mM K-$MeSO_3$, 10 mM EGTA, 10 mM HEPES, titrated to pH 7.4 with KOH; the pipette solution contained 110 mM $BaCl_2$ and HEPES 10 mM, pH 7.5. Leak subtraction was performed on unitary channel and whole cell recordings by either of two methods. For one method, the current of interest was blocked (Y^{3+} 30 μM or Cd^{2+} 120 μM) and the remaining leak currents subtracted from the equivalent experimental records from the same cell. In the other method, a scaled negative version of the experimental pulse protocol was given to the same cell. This was subsequently scaled up and added to the experimental records. In both cases, the leak currents were obtained immediately before or after each set of experimental records. Drugs were applied through a multibarrelled microperfusion pipette that was positioned within 1 mm of the cell. All experiments were performed at room temperature, 18-22°C. Experiments using nifedipine were carried out in dark conditions.

2.33 Chemicals used

Serotonergic agonists and antagonists: 7-trifluoromethyl-4- (methyl-1-piperazinyl) pyrrolo[1, 1-a] -quinoxaline dimaleate (CGS12066B, Tocris cookson), 5-Carboxamidotryptamine (5-CT, RBI), 5-hydroxytryptamine (5-HT, RBI), N- [4-methoxy-3- (4-methyl-1-piperazinyl) phenyl] -2' -methyl-4' - (5-methyl-1, 2, 4-oxadiozol-3-yl) [1, 1-biphenyl} -4-carboxamide (GR127935, Glaxo Wellcome Research and Development Ltd.), R (+) -8-OH-DPAT (DPAT, RBI), ketanserin tartrate (RBI), α-Methyl-5-hydroxytryptamine (α-M-5HT, Tocris cookson), 2- [5-[3- (4-Methylsulphonylamino) benzyl-1,,4-oxadiazol-5-yl] -1 H-indole-3-yl]ethylamine (L-694,247, Tocris cookson), 1- (2-Methoxyphenyl)-4-[4-(2-phthalimido) utyl] piperazine (NAN-190, RBI), 4- [Iodo-N- [2- [4-(methoxyphenyl) -1-piperazinyl]ethyl] -N-2-pyridinyl-benzamide (*p*-MPPI, RBI), N-demsmethly-Clozapine (RBI). 5-CT, L-694,247, clozapine, and NAN-190 were initially dissolved by a few drops of dimethylsulphoxide and stored in freezer.

Ion channel blockers: tetrodotoxin (TTX, Sigma), ω-Agatoxin IVA and ω-Agatoxin-TK (agatoxin, Alomone labs), ω-Conotoxin GVIA (ω-CgTx, Bachem Califonia), ω-Conotoxin MVIIC (Alomone labs), Nifedipine (Sigma), tetraethylammonium chloride (TEA, Aldrich), Yttrium nitrate (Y^{3+}, Sigma).

2.34 Statistics

All data presented as mean ± standard deviation (SD) unless otherwise stated. Analysis by Student' s t-test was performed for paired and unpaired observations. Differences in frequency of occurrence were assessed by using 2×2 contingency table and χ^2 parameter. P values of less than 0.05 were considered as significant level.

2.35 Fitting

The Levenberg-Marquardt algorithm was used to fit the Hill equation to dose response data. This gave the best fitting parameters and their standard errors. For all other fitting procedures, the simplex algorithm was used.

2.4 Results

2.41 Serotonin reversibly reduced both the HVA and T-type Ca^{2+} currents

Whole-cell Ca^{2+} currents recorded from acutely isolated R-B neurons possess both T-type and HVA currents. These can be distinguished by their voltage-dependence of activation and inactivation. T-type currents were elicited at test potentials above -60 mV and almost totally inactivated in a 100 ms test pulse. HVA currents were evoked at potentials of -20 mV or more, and came to dominate the whole-cell current at potentials above -10 mV (Fig 2.1A). Using a twin pulse from a holding potential of -90 mV, both the T-type and the HVA currents were elicited, and the

Figure 2.1 Both the HVA and the T-type Ca^{2+} currents were reduced by 5-HT in acutely isolated R-B neurones

A, R-B neurones possess both the HVA and the T-type Ca^{2+} currents. A_1, Whole-cell Ca^{2+} currents recorded using steps from holding potential of -80 mV to test potentials between -60 mV and +30 mV in a stage 42 *Xenopus* R-B neurone. A_2, I/V curve measured from the peak (showing the T-type and HVA currents) and end (showing only the HVA currents) of the Ca^{2+} currents (symbols correspond to measurements in A_1). B_1, T-type and HVA currents, were elicited in the same neurone by test steps of -30 and +10 mV , respectively, from a holding potential of -90 mV. Both were reduced by 1 μM 5-HT (*), unlabeled traces are control and wash. B_2, Courses of the effects of 5-HT showing that they were totally reversible on both currents in the same neurone (symbols correspond to measurements in B_1).

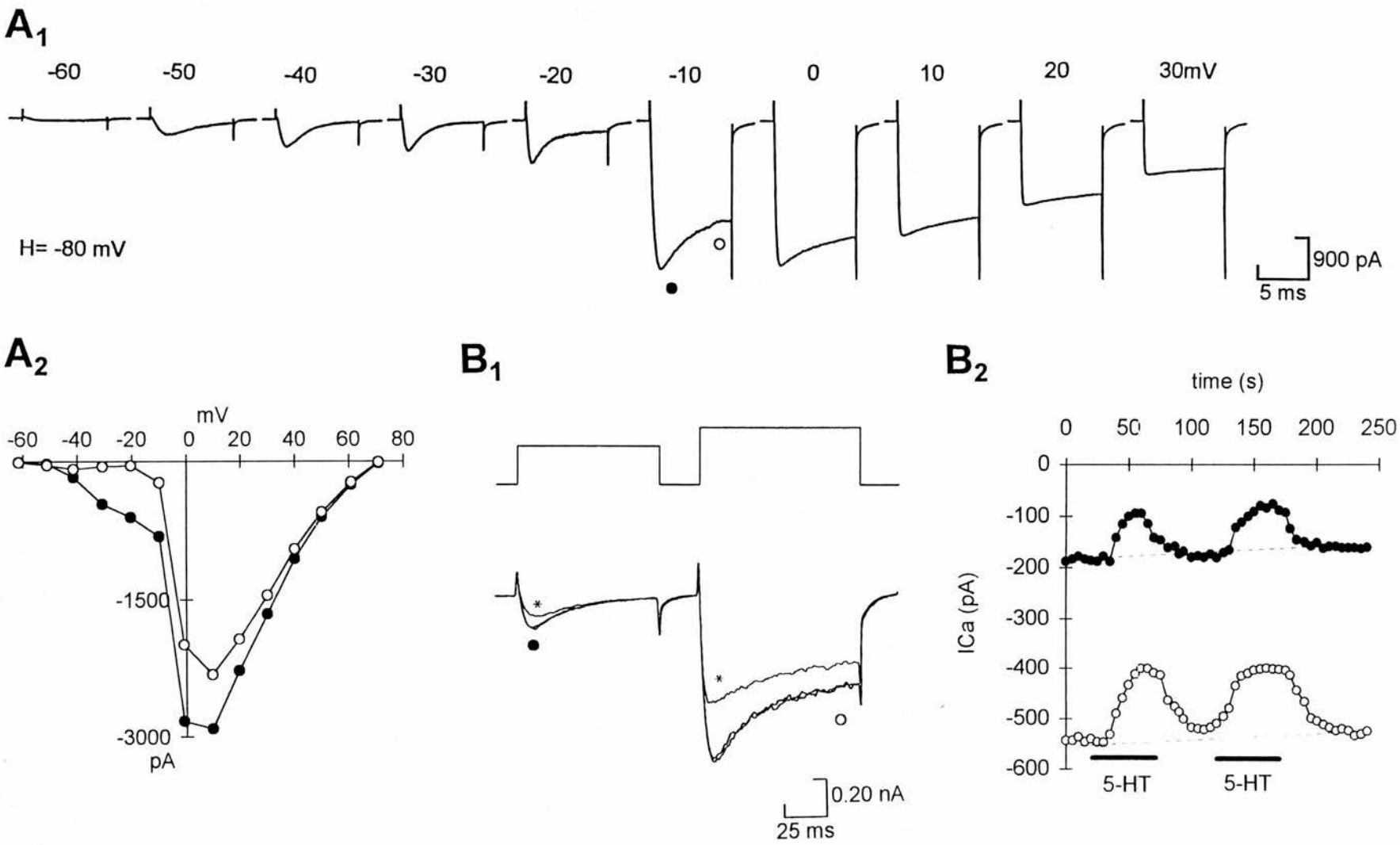
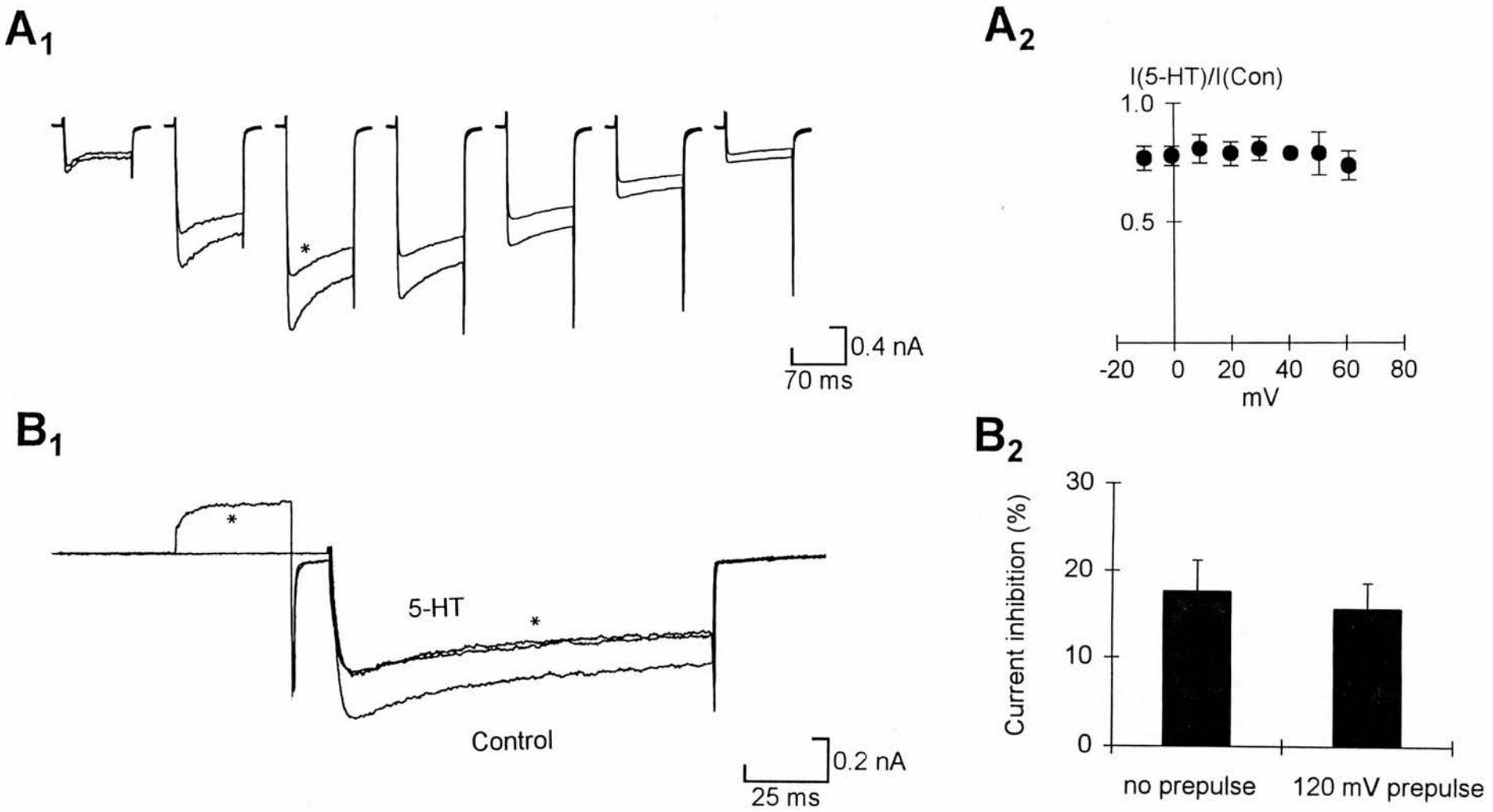

A1
-60
-50
-40
-30
-20
-10
0
10
20
30mV
H= -80 mV
900 pA
5 ms
A2
mV
-60
-40
-20
0
20
40
60
80
-1500
-3000
pA
B1
0.20 nA
25 ms
B2
time (s)
0
50
100
150
200
250
0
-100
-200
-300
-400
-500
-600
ICa (pA)
5-HT
5-HT

Figure 2.2 Voltage-independent inhibition of the HVA Ca^{2+} currents by 5-HT

A_1, Ca^{2+} currents elicited by steps from holding potential of -80 mV (*: 1μM 5-HT); A_2, The inhibition of HVA currents by 5-HT (1 μM) was independent of membrane potential (n=5); $I_{5\text{-}HT}$: current in presence of 5-HT, I_{Con}: current in the control. B_1, Example showing the inhibition of the HVA currents in RB neurones was not changed by applying a +120 mV prepulse (*: trace elicited by test pulse with 120 mV prepulse in 1 μM 5-HT); B_2, Summary showing no significant difference between the mean inhibition of the HVA currents before and after applying a 120 mV prepulse in 5 R-B neurones.


A1
0.4 nA
70 ms
A2
I(5-HT)/I(Con)
1.0
0.5
-20
0
20
40
60
80
mV
B1
5-HT
Control
0.2 nA
25 ms
B2
Current inhibition (%)
30
20
10
0
no prepulse
120 mV prepulse

effects of 5-HT on both were examined. In around 80% of the neurons examined, 5-HT reversibly reduced both the T-type and the HVA Ca^{2+} currents (Fig. 2.1B).

2.42 Inhibition of the HVA currents by 5-HT was not voltage-dependent

The modulation of the HVA currents by serotonin was examined in R-B neurons. 5-HT never caused slowing of activation of the Ca^{2+} currents (Fig. 2. $2A_1$) that usually occurs during direct G-protein modulation (see review by Hille, 1994). In 5 neurons there was no voltage-sensitivity to the block by 5-HT (Fig. $2.2A_2$). In most cases, voltage-dependent G protein inhibition can be relieved by giving a positive prepulse immediately preceding the test pulse. I therefore tested whether prepulses could lessen the block of HVA currents by 5-HT. In 5/5 neurons tested, the inhibition of HVA currents by 5-HT was not, even partially, relieved by a very positive prepulse (Fig. 2. 2B), suggesting the inhibition of HVA currents by 5-HT occurred only through voltage-independent mechanisms.

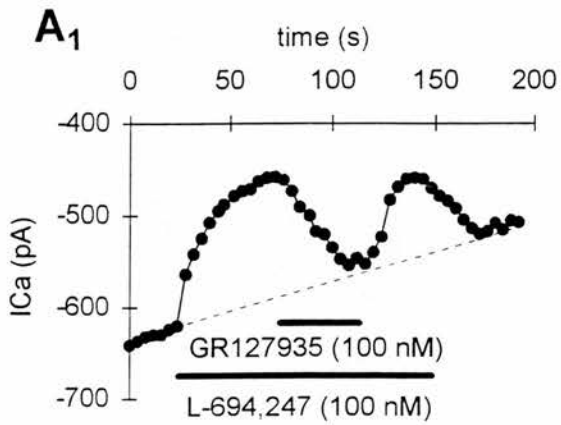
2.43 5-HT receptor subtypes involved in the inhibition of the HVA currents

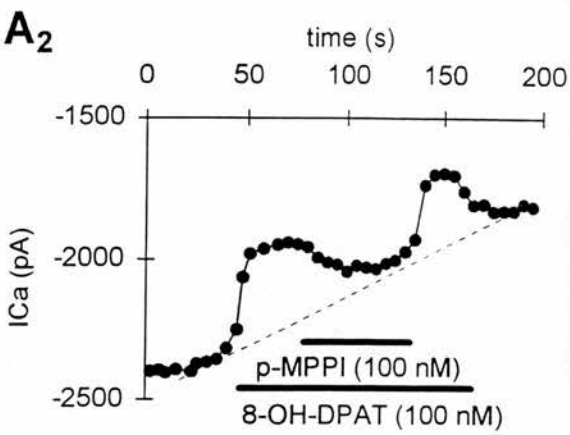
To identify the serotonin receptors involved in the modulation of HVA calcium currents, selective agonists and antagonists were applied. The effects of 8-OH-DPAT (100 nM), a selective 5-HT_{1A} agonist (Middlemiss and Fozard, 1983), were fully blocked by the selective $5HT_{1A}$ antagonist p-MPPI (100 nM, Kung et al., 1994), in 6 examined neurons (Fig. $2.3A_2$ & B). The actions of L-694,247, a specific 5-HT_{1D} agonist (Beer et al., 1993), were not blocked by the selective 5-HT_{1A} antagonists NAN-190 (100 nM) and p-MPPI (100 nM), but were blocked by GR127935 (100 nM), a selective 5-HT_{1D} antagonist (Skingle et al., 1993) in 6 examined neurons (Fig. $2.3A_1$ & B). This suggests that both $5HT_{1A}$ and $5HT_{1D}$ receptors were involved in the inhibition of the HVA Ca^{2+} current.

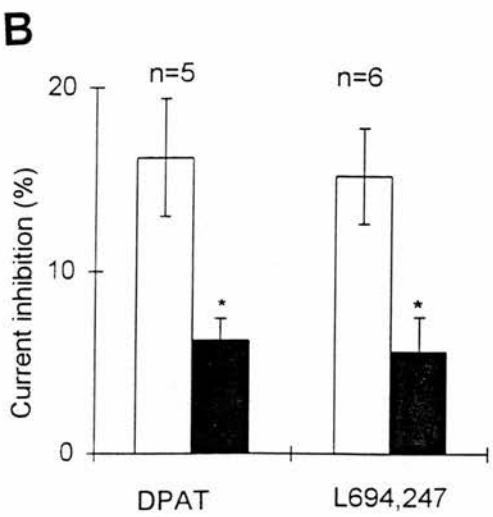
In those neurons that responded, addition of 5-HT produced a dose-dependent reversible reduction of HVA currents with a half block concentration (IC_{50}) of 40.8±20.4 nM (Fig. 2.3C). The 5-HT_{1D} agonist, L694,247, had a similar IC_{50} (38.1

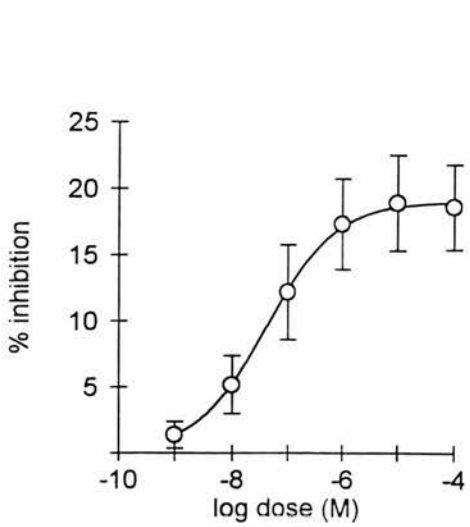
Figure 2.3 The inhibition of HVA Ca^{2+} currents by 5-HT is dose-dependent and mediated through 5-HT_{1A} and 5-HT_{1D} receptors

A_1, The selective 5-HT_{1D} agonist, L-694,247 (100 nM), inhibited HVA currents. The effects of, L-694,247 (100 nM) were blocked by the selective 5-HT_{1D} antagonist, GR127935 (100 nM). A_2, The selective 5-HT_{1A} agonist, 8-OH-DPAT (100 nM) also inhibited the HVA currents. The effects of 8-OH-DPAT (100 nM) were totally blocked by the selective 5-HT_{1A} antagonist, p-MPPI (100 nM). B, Summary of the antagonist effects on the agonists of DPAT and L-694,247 (*: $p<0.05$ vs. agonists alone). C, The inhibition of HVA currents by 5-HT was dose-dependent (n=7-39 for each dose). The smooth line is the best fitting Hill equation.


A1
time (s)
0
50
100
150
200
-400
-500
-600
-700
ICa (pA)
GR127935 (100 nM)
L-694,247 (100 nM)


A2
time (s)
0
50
100
150
200
-1500
-2000
-2500
ICa (pA)
p-MPPI (100 nM)
8-OH-DPAT (100 nM)


B
n=5
n=6
20
10
0
Current inhibition (%)
*
*
DPAT
L694,247


C
25
20
15
10
5
% inhibition
-10
-8
-6
-4
log dose (M)

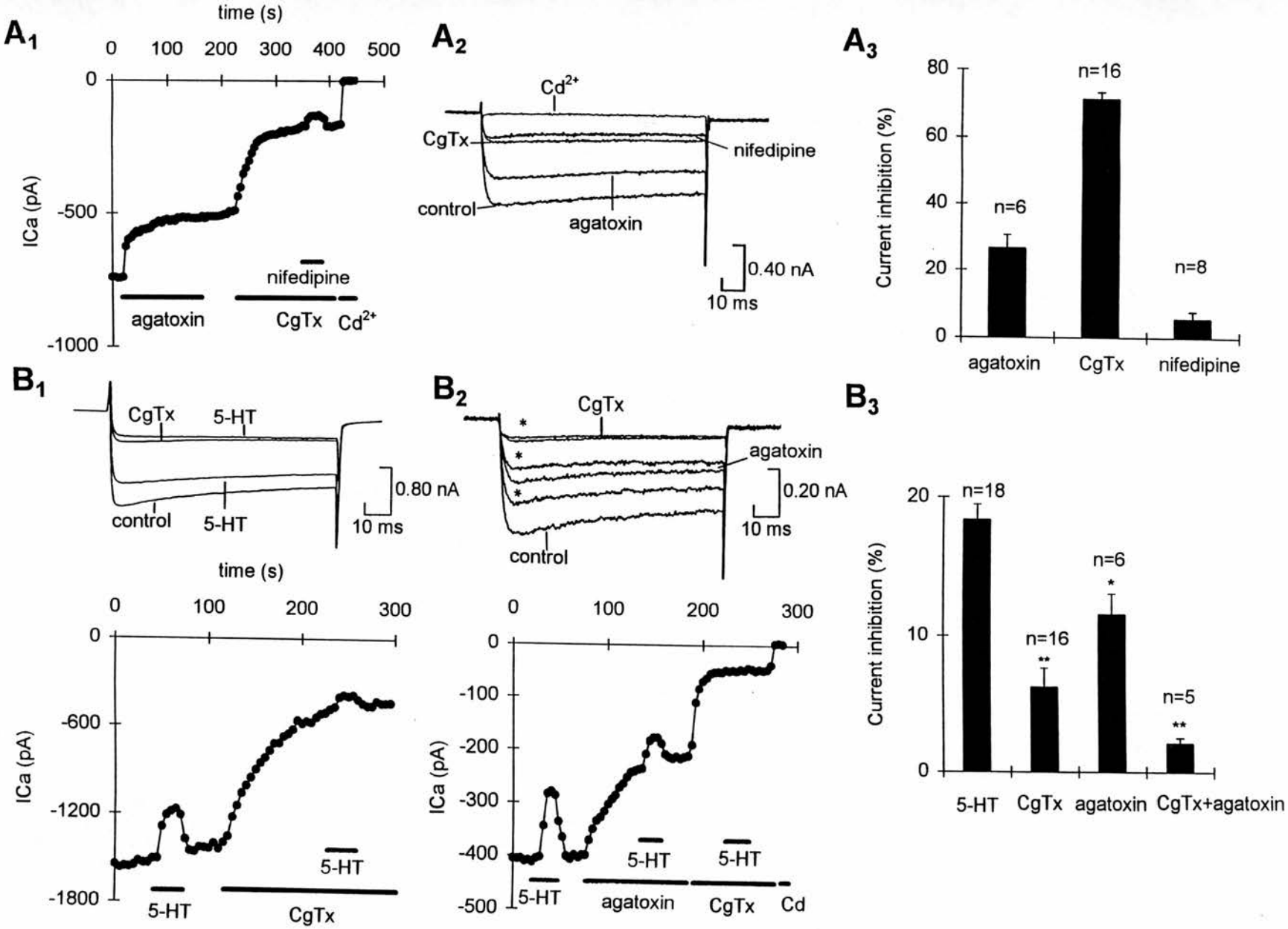
±10.4 nM, n=16) for inhibition of HVA currents. The maximum reduction ever observed of HVA currents was 29%, while the mean maximum inhibition by 5HT (1 μM) was 16.2±2.4 % (n=39). Recovery from the effects of 5-HT on the HVA currents was rather slow on washout. In 6 cells the time course for wash was fitted with single exponential curve. This gave a mean time constant of 22.5±3.4 s (n=6) for recovery of the HVA current from inhibition by 5-HT.

2.44 HVA calcium channel identity

To identify further the HVA channel types possessed by *Xenopus* R-B neurons, I used ω-conotoxin, fraction GVIA (ω-CgTx), which is a selective N-type Ca^{2+} channel blocker (Feldman et al., 1987), ω-agatoxin-TK, which is P/Q type channel blocker (Teramoto et al., 1995), and nifedipine, a selective blocker of L-type channels. ω-CgTx (1μM) irreversibly blocked 70.4± 2.8% (n=16) of the HVA currents (Fig. 2.4A & B). Thus in R-B neurons, the HVA calcium currents were mostly carried through ω-CgTx-sensitive (N-type) channels. ω-agatoxin-TK (200 nM), irreversibly blocked 25.5±3.2% (n=6) of the total HVA currents (Fig. 2.4A). The actions of ω-agatoxin-TK on HVA currents were probably saturated at around 40 nM, since the higher dose of 200 nM did not produce further block. The ω-agatoxin sensitive current did not show significant inactivation (e.g. Fig. 2.4 A_2), suggesting that R-B neurons possess many more P channels than Q-type channels (Taramoto et al., 1995). Nifedipine (10 μM) did not block the HVA currents in 3/8 R-B neurons, and blocked only very small amounts of the HVA current in the other 5 neurons (mean block 5.5±1.8%, $p>0.1$ vs. control, Fig. 2.4A), suggesting that R-B neurons possess only a very small number of L-type channels. In 5 neurons examined, 100 μM Cd^{2+} blocked the remainder of the HVA current following combined treatment with ω-CgTx and ω-agatoxin (around 5%, Fig. 2.4A), suggesting R-B neurons also possess very small amount of R type channels. (Birnbaumer, et al., 1991). Thus the calcium influx through HVA currents was mostly carried by N- and P-type channels in the sensory neurons of *Xenopus*.

Figure 2.4 The identity of the calcium channels inhibited by 5-HT in RB neurones

A_1, Time courses of the effects of calcium channel blockers: ω-agatoxin-TK (100 nM), ω-CgTx (1 μM), nifedipine (10 μM),and Cd^{2+} (100 μM), on the HVA currents. A_2, Calcium currents elicited by steps to 10 mV from a holding potential of -50 mV during the application of the blockers (same neurone as A_1). A_3, Summary of the effects of calcium channel blockers on the HVA currents in R-B neurones. B_1, Recording showing that the inhibition of HVA currents by 5-HT (1 μM) was mostly occluded by ω-conotoxin (1 μM) in a R-B neurone. B_2, Recording showing that the inhibition of HVA currents by 5-HT (1 μM) was partially occluded by ω-agatoxin-TK (100 nM), while the remaining inhibition was totally occluded by 1 μM ω-conotoxin (*: HVA currents recorded in 5-HT on top of Ca^{2+} channel blockers). B_3, Summary of the inhibition of the HVA currents by 5-HT alone, and additional inhibition on top of calcium channel blockers (*:$p<0.05$, **:$p<0.01$, vs. 5-HT alone).


A1
time (s)
0 100 200 300 400 500
0
-500
-1000
ICa (pA)
agatoxin
nifedipine
CgTx
Cd2+
A2
Cd2+
CgTx
nifedipine
control
agatoxin
0.40 nA
10 ms
A3
Current inhibition (%)
80
60
40
20
0
n=16
n=6
n=8
agatoxin
CgTx
nifedipine
B1
CgTx
5-HT
control
5-HT
0.80 nA
10 ms
time (s)
0 100 200 300
0
-600
-1200
-1800
ICa (pA)
5-HT
5-HT
CgTx
B2
CgTx
agatoxin
0.20 nA
10 ms
control
0 100 200 300
0
-100
-200
-300
-400
-500
ICa (pA)
5-HT
5-HT
5-HT
agatoxin
CgTx
Cd
B3
Current inhibition (%)
20
10
0
n=18
n=16
**
n=6
*
n=5
**
5-HT
CgTx
agatoxin
CgTx+agatoxin

I next characterized the identity of the HVA channels modulated by 5-HT in R-B neurons by applying 5-HT alone and in the presence of ω-CgTx (1 μM) or ω-agatoxin-TK (100 nM), to determine whether the blocking action of the two drugs was additive or occlusive. 5-HT inhibited the HVA current by 18.4±1.4% (n=18, P<0.01 vs. control) when applied alone. In the presence of ω-CgTx (1 μM), 5-HT only produced 6.2±1.3 % further inhibition (n=16, P<0.01 vs. 5-HT alone, Fig. 2.4B_1& B_3), Therefore, around 65% of the inhibition of HVA currents was occluded by ω-CgTx. This substantial occlusion suggests that N-type Ca^{2+} channels were the predominant target of 5-HT. In 6 neurons, the effects of 5-HT were also significantly attenuated by 100 nM agatoxin: 5-HT on top of agatoxin only blocked a further 11.6±1.2% inhibition of HVA currents, which is significantly less than 5-HT alone (p<0.05 vs. 5-HT alone, Fig. 2.4B_2 & B_3), suggesting that P-type channels account for around 30% of the inhibition of HVA currents by 5-HT. On top of both ω-CgTx and ω-agatoxin-TK, 5-HT produced almost no further inhibition (2.1±0.4%, n=5, Fig. 2.4 B_2 & B_3). This suggests that N- and P/Q- type calcium channels together account for almost all of the inhibition of HVA currents by 5-HT.

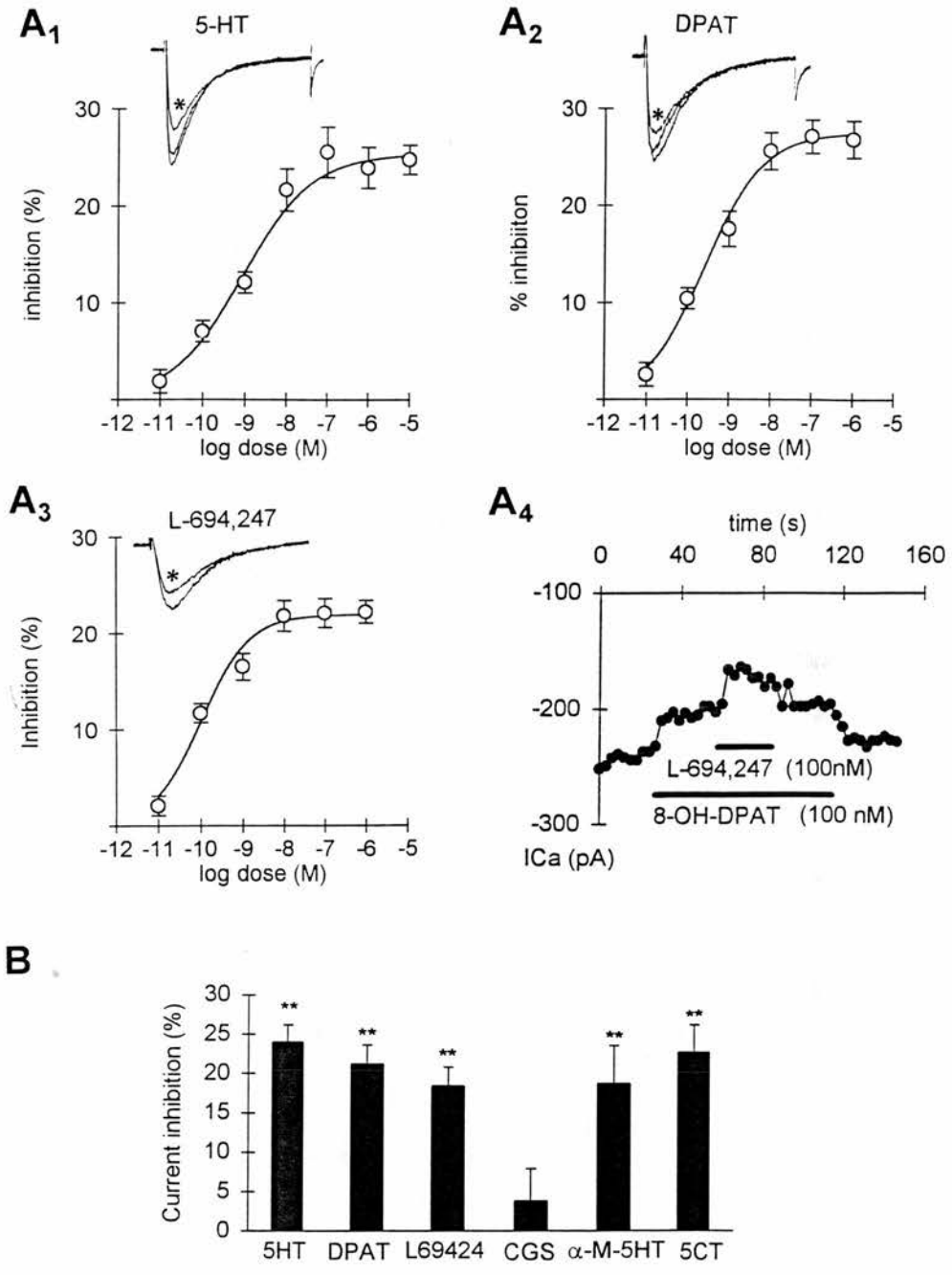
The downstream functional consequences and the signaling pathways for the modulation of the HVA channels, such as N-type and P/Q type channels, by G-protein coupled receptors have been widely described (reviewed by Hille, 1994; Wickman and Clapham, 1995; Dunlap, 1997). The most novel aspects of our results are the modulation of neuronal T-type channels, which differ in their kinetic properties from the HVA channels. These differences mean that HVA and T-type channels performed different roles in the control of neuronal excitability. I have therefore concentrated on characterizing modulation of the T-current in more detail.

2.45 Serotonin inhibits T-type Ca^{2+} currents via 5-HT_{1A} and 5-HT_{1D} receptors

The effect of 5-HT on T-type Ca^{2+} current was examined using a repeated pulse protocol with a test potential of -30 mV from holding potential of -90 mV. In

Figure 2.5 Inhibition of the T-type calcium currents by serotonergic agonists

A, Representative traces (*: represent recordings in 100 nM selective agonists, unlabeled traces are control and wash) and dose-response relations showing the block of T-type currents by selective serotonergic agonists: 5-HT (A_1), 8-OH-DPAT (A_2), L-694,247 (A_3), n=7-41 for each point. The solid line is the best fit of Hill equation. A_4, The inhibition of T-type currents by L-694,247 (100 nM) and 8-OH-DPAT (100 nM) was additive. B, Summary of the mean inhibition of T-type currents by selective serotonergic agonists at maximum dose in R-B neurones. 5-HT (1 μM, n=41), 8-OH-DPAT (100 nM, n=23), L-694,247 (100 nM, n=22), CGS-12066B (1 μM, n=8), α-M-5-HT (1 μM, n=7), 5-CT (100 nM, n=5), (*:$P<0.05$, **:$p<0.01$ vs. control). The individual effects of 8-OH-DPAT (100 nM) and L694,247 (100 nM) when added together are larger than that of a maximal dose of 5-HT. This may mean that both receptors are competing for the same components of signal transduction pathways that act as ' bottle neck' to limit the effect of 5-HT. Alternatively there may be some cross actions of both agonists on both 5-HT_{1A} and 5-HT_{1D} receptors.


A1
5-HT
inhibition (%)
log dose (M)
A2
DPAT
% inhibiiton
log dose (M)
A3
L-694,247
Inhibition (%)
log dose (M)
A4
time (s)
L-694,247 (100nM)
8-OH-DPAT (100 nM)
ICa (pA)
B
Current inhibition (%)
5HT
DPAT
L69424
CGS
α-M-5HT
5CT

acutely isolated R-B neurons, addition of 5-HT produced dose-dependent inhibition (Fig. 2.5A_1). The maximum inhibition was 54% of the control T-type Ca^{2+} current, while the mean inhibition for 5-HT (1 μM) was 24.0±2.2% (n=41).

The time course for wash of the serotonergic modulation of T-type currents could be fitted with single exponential curve. This gave a mean time constant of 9.8±2.6 s (n=6) for recovery from inhibition by 5-HT, which was half that for the recovery time course seen during modulation of the HVA currents measured in the same neurons. This large difference in the speed of recovery from inhibition for the T-type and HVA currents suggests that different underlying second messengers may be involved. To identify the 5-HT receptors involved in modulation of the T-type current, specific agonists and antagonists were applied to the R-B neurons.

5-HT Agonists. A range of selective 5-HT_1 agonists including 8-OH-DPAT, L694,247, 5-CT(Beer et al., 1992; Hoyer et al., 1994), and a 5-$HT_{1/2}$ agonist, α-methyl-5HT (Ismaiel et al., 1994), reversibly inhibited the T-type Ca^{2+} currents (Table 2.1, Fig. 2.5A & B). However, the 5-HT_{1B} receptor agonist, CGS-12066B (Neale et al., 1987), from 10 nM to 1 μM (n=8), had almost no effect on T-type Ca^{2+} currents (Table 2.1 & Fig. 2.5B). The effects of 5-HT, 8-OH-DPAT and L-694,247 were very potent with IC_{50}' s less than 1 nM (Table 2.1 & Fig. 2.5A). Thus, a diversity of agonists which act on 5-HT_1 receptors (except $5HT_{1B}$) inhibited T-type Ca^{2+} currents by similar amounts at very low concentrations (<10 nM). In 3/3 examined R-B neurons, the actions of saturating doses of L-694,247 (100 nM) and 8-OH-DPAT(100 nM) were additive (Fig. 2.5A_4), strongly suggesting that they act on different receptors.

5-HT Antagonists To confirm the identities of the serotonin receptor subtypes involved in the inhibition of the T-type Ca^{2+} current, specific antagonists were used (Fig. 2.6, Table 2.1). Our results are consistent with an involvement of 5-HT_{1A} and 5-HT_{1D} receptors. In brief, the effects of 5-HT, 8-OH-DPAT, 5-CT and α-methyl-5HT were blocked by the 5-HT_{1A} antagonist, p-MPPI (Kung et al., 1994; Fig. 2.6 & Table 2.1). In addition a second 5-HT_{1A} antagonist, NAN-190 (100 nM, Liau et al., 1991) also reduced the effect of 8-OH-DPAT and 5-CT by similar amounts

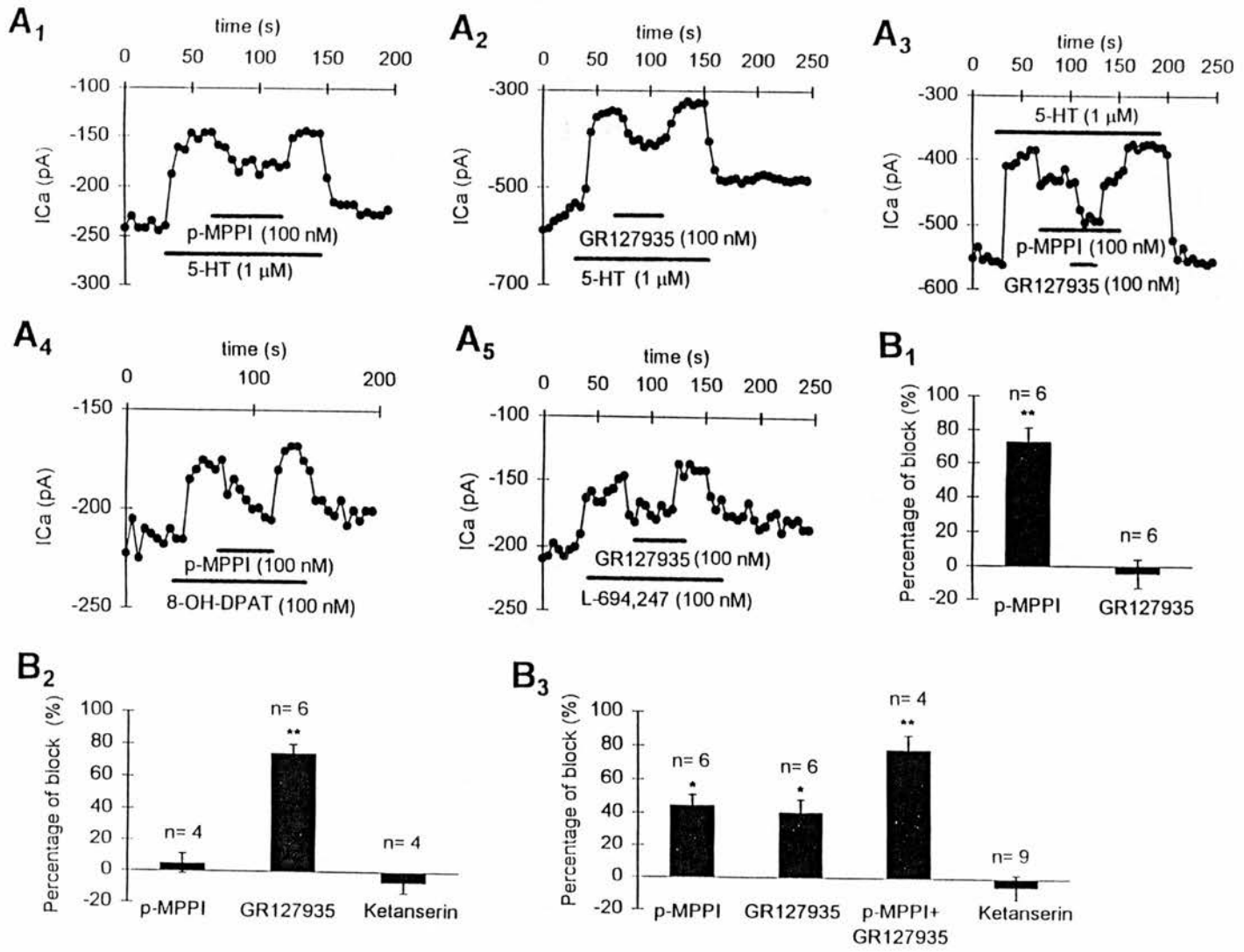
Table 2.1 Effects of selective serotonergic agonists and antagonists on the T-type Ca^{2+} currents in acutely isolated *Xenopus* R-B neurons

Agonist	Current Inhibition	5-HT_{1A} Antagonists† (% block of agonist)	5-HT_{1D} Antagonist‡ (% block of agonist)	$5\text{-HT}_{2A/2C}$ Antagonist*f* (% block of agonist)
5-HT	33.9±1.6% (n=102) **	44.6±5.2% (n=6) **	40.5±4.7% (n=5) **	3.4±6.1 (n=15)
5-CT	29.6±3.4% (n=6) **	49.4±7.1% (n=5) **	—	—
8-OH-DPAT	26.9±3.1% (n=31) **	72.5±4.7% (n=6) **	-4.4±5.4% (n=6)	—
L-694,247	29.6±2.5% (n=46) **	4.9±6.2% (n=4)	74.5±3.9% (n=6) **	—
CGS12066B	5.2±1.6% (n=6)	—	—	—

†p-MPPI ‡ GR127935 *f* Ketanserin ** P<0.01

Figure 2.6 Effects of selective antagonists on the inhibition of T-type calcium currents by selective agonists in *Xenopus* R-B neurones

Both p-MPPI (100 nM, A_1) and GR127935 (100 nM, A_2) partially blocked the effect of 5-HT (1 μM). (A_3) p-MPPI (100 nM) and GR127935 (100 nM) produced an additive block of the effect of 5-HT (1 μM). p-MPPI blocked the effect of 8-OH-DPAT (100 nM, A_4), while GR127935 (100 nM) blocked the effect of L-694,247 (100 nM, A_5). B_1, Summary of the block by the selective antagonists on 8-OH-DPAT (100 nM); (**; $p<0.01$ vs. 8-OH-DPAT). B_2, Summary of the block by antagonists on L-694,247 (100 nM); (**; $p<0.01$ vs. L-694,247). B_3, Summary of the block by selective antagonists on 5-HT (1 μM), (*, $P<0.05$, **; $p<0.01$; vs. 5-HT).


A1
time (s)
0 50 100 150 200
-100 -150 -200 -250 -300
ICa (pA)
p-MPPI (100 nM)
5-HT (1 μM)
A2
time (s)
0 50 100 150 200 250
-300 -500 -700
ICa (pA)
GR127935 (100 nM)
5-HT (1 μM)
A3
time (s)
0 50 100 150 200 250
-300 -400 -500 -600
ICa (pA)
5-HT (1 μM)
p-MPPI (100 nM)
GR127935 (100 nM)
A4
time (s)
0 100 200
-150 -200 -250
ICa (pA)
p-MPPI (100 nM)
8-OH-DPAT (100 nM)
A5
time (s)
0 50 100 150 200 250
-100 -150 -200 -250
ICa (pA)
GR127935 (100 nM)
L-694,247 (100 nM)
B1
Percentage of block (%)
100 80 60 40 20 0 -20
n= 6
**
n= 6
p-MPPI
GR127935
B2
Percentage of block (%)
100 80 60 40 20 0 -20
n= 4
n= 6
**
n= 4
p-MPPI
GR127935
Ketanserin
B3
Percentage of block (%)
100 80 60 40 20 0 -20
n= 6
*
n= 6
*
n= 4
**
n= 9
p-MPPI
GR127935
p-MPPI+ GR127935
Ketanserin

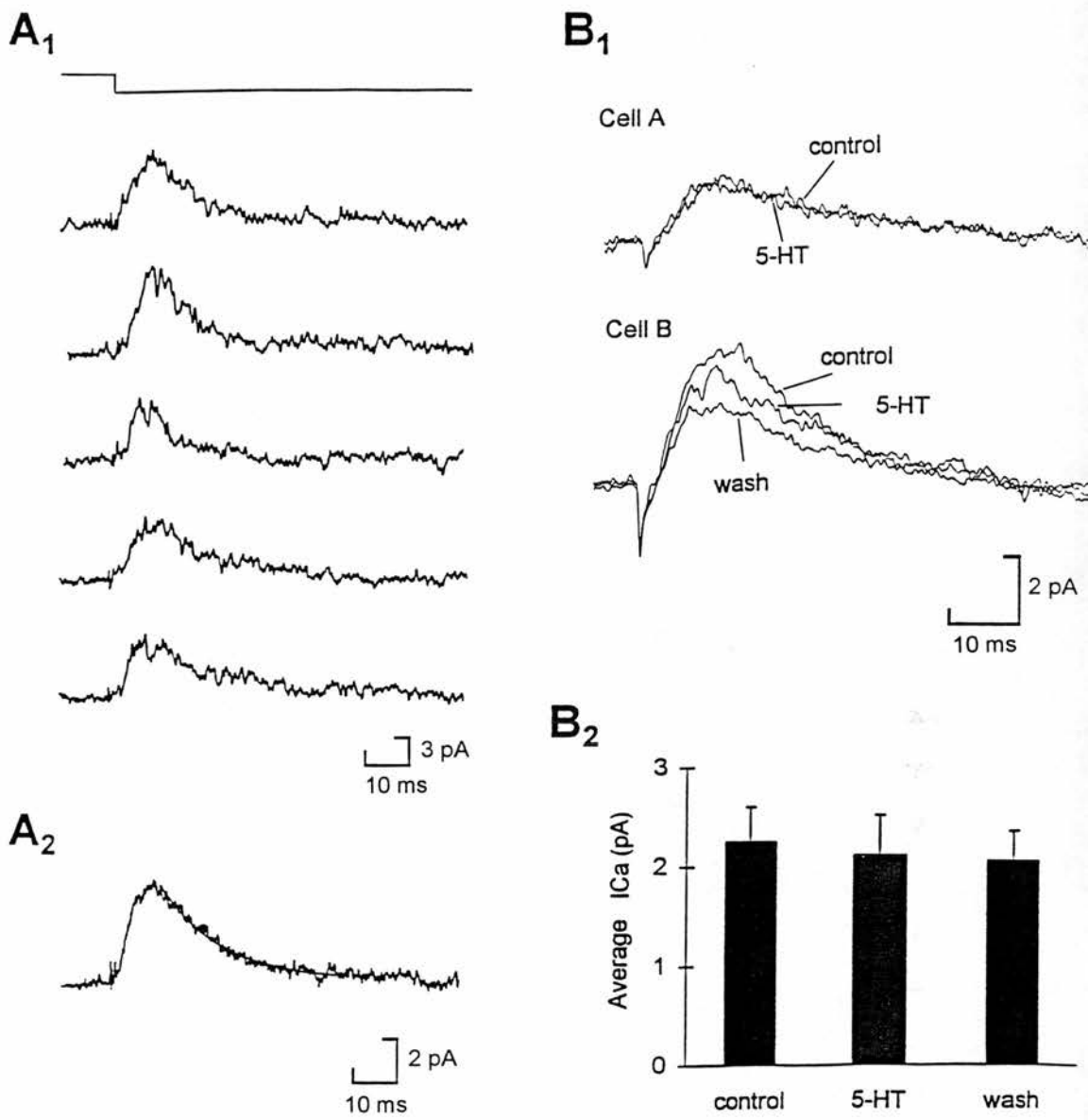
(68.5±5.6 %, n=5, and 52.1 ±3.3%, n=6, respectively), but strangely had no effect on the actions of 5HT (not illustrated). Neither p-MPPI nor NAN-190 blocked the effects of L694,247 (Table 2.1 & Fig. 2.6B). However, $5HT_{1D}$ antagonist GR127935 (Skingle et al., 1993), blocked the effects of 5-HT and L-694,247 without affecting 8-OH-DPAT (Table 1 & Fig. 2.6 B). 5-HT_2 receptors were unlikely to be involved since neither ketanserin nor clozapine, which are both 5-$HT_{2A/2C}$ antagonists (Awouters, 1985, Kuoppamaki et al., 1993), had any effects on 5-HT or α-methyl-5HT. Thus both 5-HT_{1A} and 5-HT_{1D} receptor are involved in the inhibition of T-type current, a conclusion further strengthened by the additive effects of the specific antagonists (Fig. 2.6A_3 and B_3)

2.46 Modulation of T-type channel does not occur through a freely diffusible second messenger pathway

To test whether the modulation of T-type channels was mediated via a freely diffusible second messenger or by a membrane-delimited pathway, I examined the effects of 5-HT on T-type channels activity recorded in the cell-attached mode. The membrane potential was zeroed with high potassium extracellular saline. In 16/55 patches recorded, T-type unitary channel activities were elicited by repeated test steps of -50 or -60 mV from holding potentials of 50 to 90 mV. The high Ba^{2+} levels will screen surface charge on the membrane, therefore the absolute membrane potential experienced by the channels is likely to be shifted to more negative potentials by as much as 20 to 30 mV. Thus the test protocol is similar but not be exactly equivalent to those used to evoke T-type currents in the earlier whole-cell recordings performed with normal levels of divalent cations (Hille, 1992). In 8 patches located near the neuronal process but not the nucleus, quasi-macroscopic T-type currents were elicited by almost every test pulse (Fig. 2.7A), suggesting these patches contained many T-type channels. In patches from 6 neurons which had a large number of T-type channels, the averaged currents were not modulated by 5-HT (Fig. 2.7B). Given the reliability of the modulation of the whole-cell T -type currents by 5-HT (nearly 90% cells responded to 5-HT), I would expect to see at least 5 of these patches being modulated if 5-HT were acting through a freely

Figure 2.7 Effects of 5-HT on T-type semi-macroscopic channel recordings

A_1, Consecutive unitary T-type Ba^{2+} currents were elicited by steps from potentials equivalent to membrane potential of approximately -90 mV to -30 mV (allowing for the screening effect of high Ba^{2+} levels on membrane surface charge) in a cell-attached patch. All traces are leak subtracted. A_2, Average semi-macroscopic T-type Ba^{2+} current record was obtained by averaging of 50 consecutive recordings in the same patch. The solid line was the best fit of single exponential equation, time constant (τ)=23.5 ms. B_1, 5-HT (1 μM) did not modulate the average (50 to 100 consecutive traces) semi-macroscopic T-type Ba^{2+} currents recorded in cell-attached patches. In cell A, there were no changes in the T-type current. In cell B, however, the averaged currents were reduced, but this did not reverse upon wash, suggesting the reduction may result from a ' run-down' of channel activity. B_2, Summary of the effects of 5-HT (1 μM) on T-type semi-macroscopic channel recordings in 6 R-B neurones (In this figure and other figures relating to cell-attached patch-clamp recordings, inward Ca^{2+} currents are shown in the upward direction. This is contrary to convention.)


A1
3 pA
10 ms
A2
2 pA
10 ms
B1
Cell A
control
5-HT
Cell B
control
5-HT
wash
2 pA
10 ms
B2
Average ICa (pA)
3
2
1
0
control
5-HT
wash

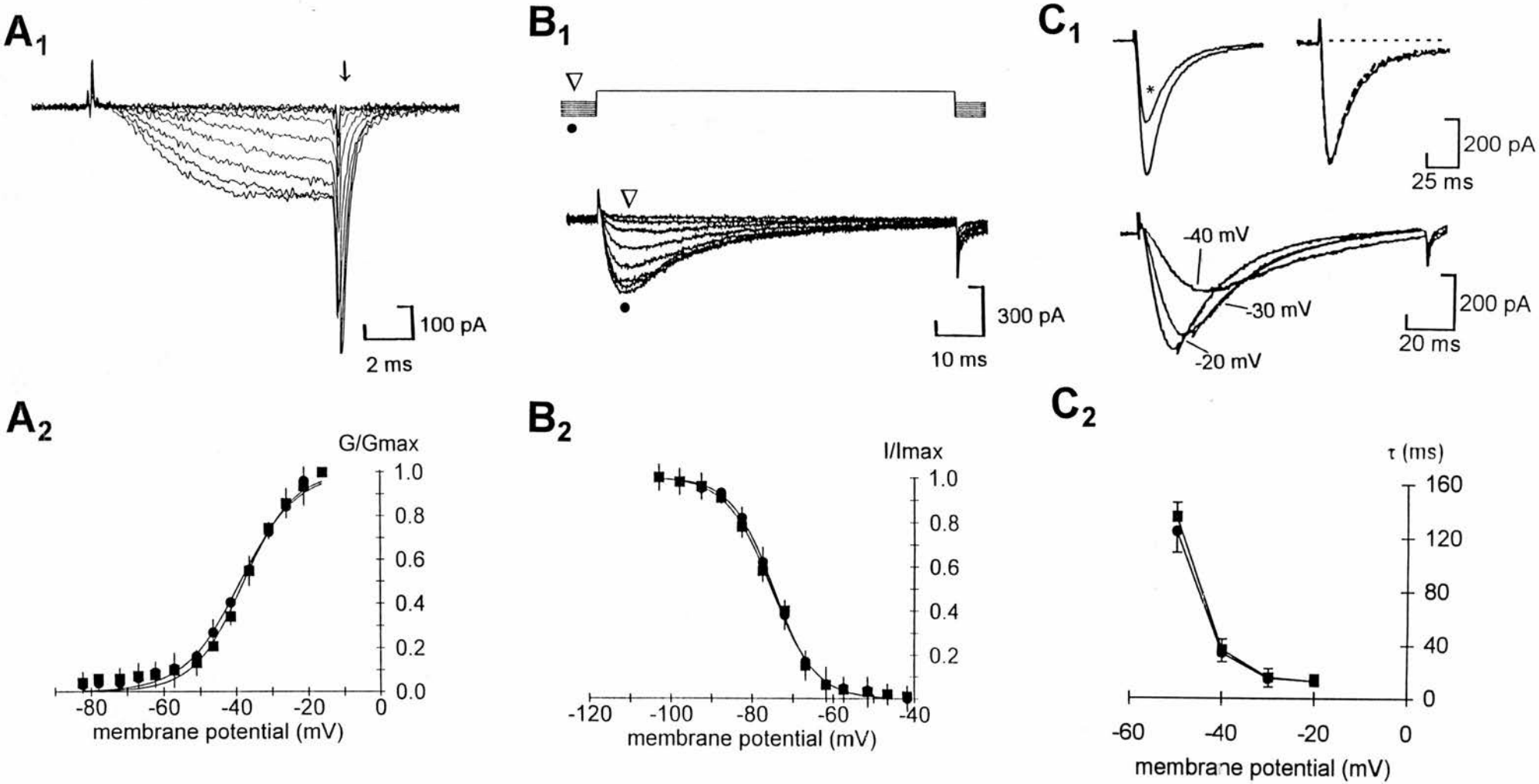
diffusible second messenger pathway. The lack of response therefore suggests that 5-HT cannot act through a freely diffusible second messenger but may instead utilize a membrane-delimited pathway, such as direct modulation by G-proteins.

2.47 5-HT did not change the kinetics of the T-type currents

I explored whether the inhibition of T-type channels by 5-HT might be voltage-dependent. The amount of inhibition of T-type currents remained constant from membrane potentials of -50 mV (mean inhibition 26.0 ± 4.2%, n=4) to -20 mV (mean inhibition 25.2 ± 3.7). In 5 cells in which T-type tail currents were reduced, 5-HT did not change the voltage-dependence of T-type current activation (Fig. 2.8A). Similarly, the voltage-dependence of steady-state inactivation remained unchanged (Fig. 2.8B). In 6/6 neurons, when the reduced T-type currents were scaled up to the same magnitude as control, the currents elicited by test potentials from -50 mV to -30 mV in 5-HT overlapped with their corresponding control traces (Fig. 2.8C_1). However, at membrane potentials more positive than -30 mV, where the HVA currents start to develop, the scaled traces did not overlap in 3 neurons(e.g. Fig. 2.5 A). This was probably due to contamination of the T-type currents by the HVA currents and was not seen in neurons that had been pretreated with 1 μM ω-conotoxin-GVIA (not illustrated). This therefore suggests that 5-HT reduced the T-type currents without altering the macroscopic kinetics. This was confirmed by looking at the time course of inactivation at a range of voltages. Once again 5-HT had no effect on the kinetics of inactivation (Fig. 2.8C). Thus, unlike most examples of G-protein mediated modulation of HVA channels, serotonin reduced the T-type currents without significantly altering the voltage-dependent or time-dependence of channel gating.

Figure 2.8 5-HT did not alter the kinetic characteristics of the whole-cell T-type calcium currents

A_1, Leak subtracted T-type tail currents (shown by arrow) were elicited by series of test pulses (-80 to -20 mV in 5 mV steps) from holding potential of -100 mV in control. A_2, Summary showing that 5-HT (1 μM) had no effect on the activation of the T-type currents in 5 R-B neurones. The data were fitted with Boltzmann relation (solid line): $I/I_{max}= \{1+\exp[(V+V_{1/2})/K]\}^{-1}$. Squares: control, ($V_{1/2}$=-37.2 mV, K= -7.6); Filled circle: 5-HT (1 μM), ($V_{1/2}$=-38.8 mV, K= -7.3). B_1, T-type channels measured from the peak of the currents undergo steady-state inactivation. B_2, 5-HT (1 μM) had no effect on the steady-state inactivation of the T-type currents in 4 R-B neurones. The data were fitted with Boltzmann relation (solid line). Squares: control, (where $V_{1/2}$= -74.7 mV, K=5.2); filled circle: 5-HT (1 μM), (where $V_{1/2}$= -75.2 mV, K=5.4), C_1, top left: T-type currents recorded in control and 1 μM 5-HT (*). top right: the T-type currents recorded in 5-HT in were scaled up to the same peak magnitude as control to show that the two traces almost completely overlap. The time-dependent inactivation of the T-type currents was measured by fitting with a single exponential (solid line). C_2, 5-HT (1 μM) had no effect on the time-dependent inactivation in 3 R-B neurones. Square: control; filled circle: 5-HT (1 μM).


A1
100 pA
2 ms
B1
300 pA
10 ms
C1
200 pA
25 ms
-40 mV
-30 mV
-20 mV
200 pA
20 ms
A2
G/Gmax
1.0
0.8
0.6
0.4
0.2
0.0
-80
-60
-40
-20
0
membrane potential (mV)
B2
I/Imax
1.0
0.8
0.6
0.4
0.2
-120
-100
-80
-60
-40
membrane potential (mV)
C2
τ (ms)
160
120
80
40
0
-60
-40
-20
0
membrane potential (mV)

2.48 The reduction of T-type currents by serotonin increased R-B neuron firing threshold

I next explored the function roles of the T-type currents, and the consequences of modulation of this current by 5-HT for sensory transmission. The trivalent cations, Y^{3+}, La^{3+} and other lanthanides have been reported to differentially block the T-type and HVA Ca^{2+} channels. The smaller cations are more potent T-type antagonists, the IC_{50} for Y^{3+} blocking of T-type channels is around 0.1 nM (Mlinar and Enyeart, 1993). We tested the dose responses of Y^{3+} on T-type and HVA currents. At doses of 10 nM or less, Y^{3+} could reversibly block the T-type currents while having no effect on the HVA currents in R-B neurons (Fig. 2.9). I therefore used Y^{3+} (1 to 10 nM) as a selective T-type channel blocker to test possible functional roles of T-type currents.

Under current clamp, R-B neurons were injected with repeated 5 ms depolarizing command currents (365.5±15.9 pA at resting potential of around -50 mV, n=10) to evoke repeated action potentials (Fig. 2.10A_1). To examine whether the T-type channels could be involved in setting spike threshold, we carefully measured the threshold current that could just evoke spikes in the R-B neurons and observed the effects of Y^{3+} (1 to 10 nM) and 5-HT(10 nM) at this threshold level of current injection. This low concentration of 5-HT greatly blocks the T-type current but has little effect on the HVA current (compare Fig 2.5A_1 and 3C). At a resting membrane potential of -48.5±6.4 mV, n=10, at which T-type channels were inactivated (see Fig. 2.10B), neither Y^{3+} nor 5-HT had any effects on the neuron firing (Fig. 2.10B_1). By injecting a negative holding current (-22.5±4.2 pA, n=10), the membrane potentials of these neurons were set to a more negative value of around -90 mV (-90.5±4.8 mV, n=10). At these membrane potentials, Y^{3+} (10 nM) clearly reduced the R-B neuron firing probability (Fig. 2.10B_2 & C), suggesting that the T-type channels played a role in spike-initiation. 5-HT also reduced the probability of R-B neuron firing (Fig. 2.10B_2 & C). Therefore modulation of T-type channels by 5-HT could increase the firing threshold of R-B neuron and potentially modulate the sensitivity of this sensory pathway.

Figure 2.9 Differential block of the T-type and HVA Ca^{2+} currents by Y^{3+} in R-B neurones

A, Time series measurements in an R-B neurone showing the dose-response for Y^{3+} on T-type currents (A_1) and HVA currents (A_2) recorded in the same neurone. A_3, Example of the HVA and LVA currents traces (*: 10 nM Y^{3+}) recorded simultaneously in the same neurone as shown in A_1 and A_2 .B, Summary of the effects of Y^{3+} (10 nM) on the T-type currents and HVA currents. (**; $p<0.01$ vs. control).

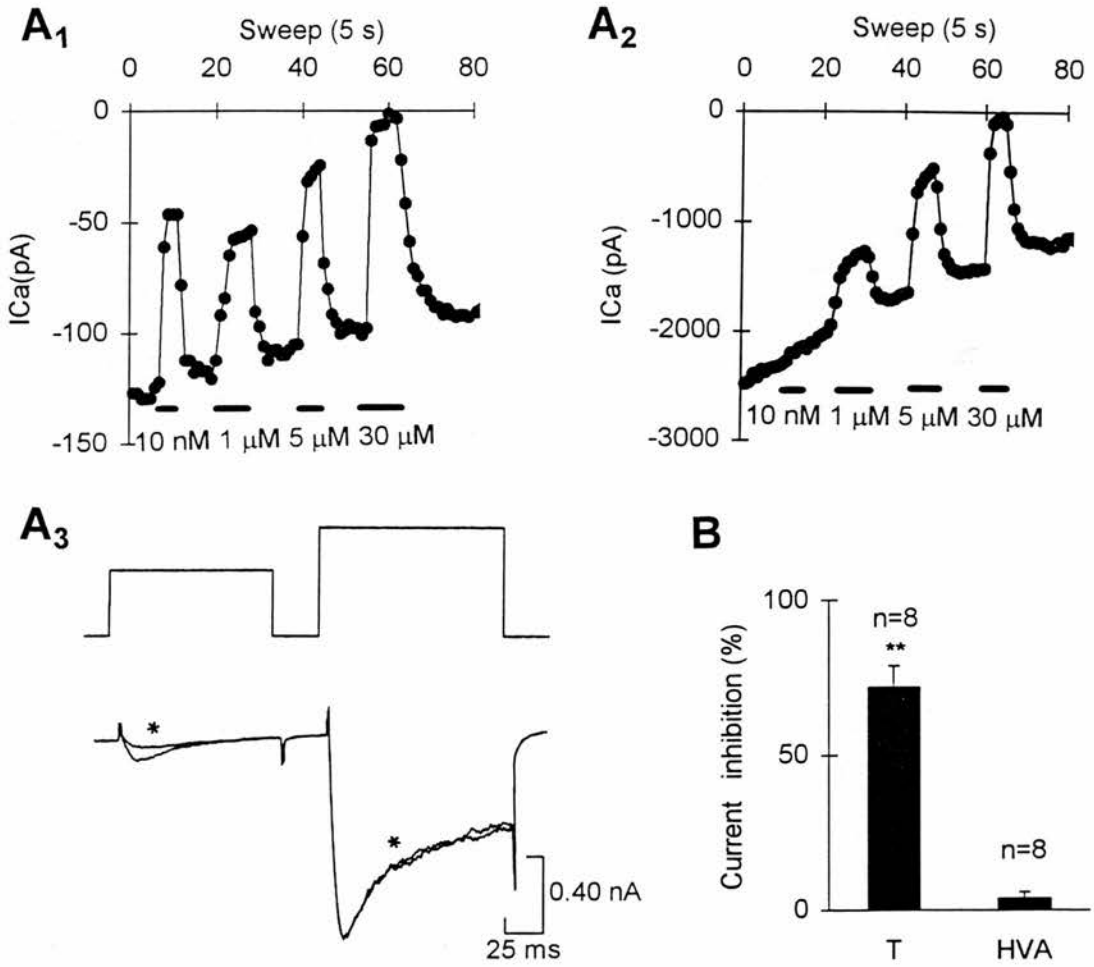
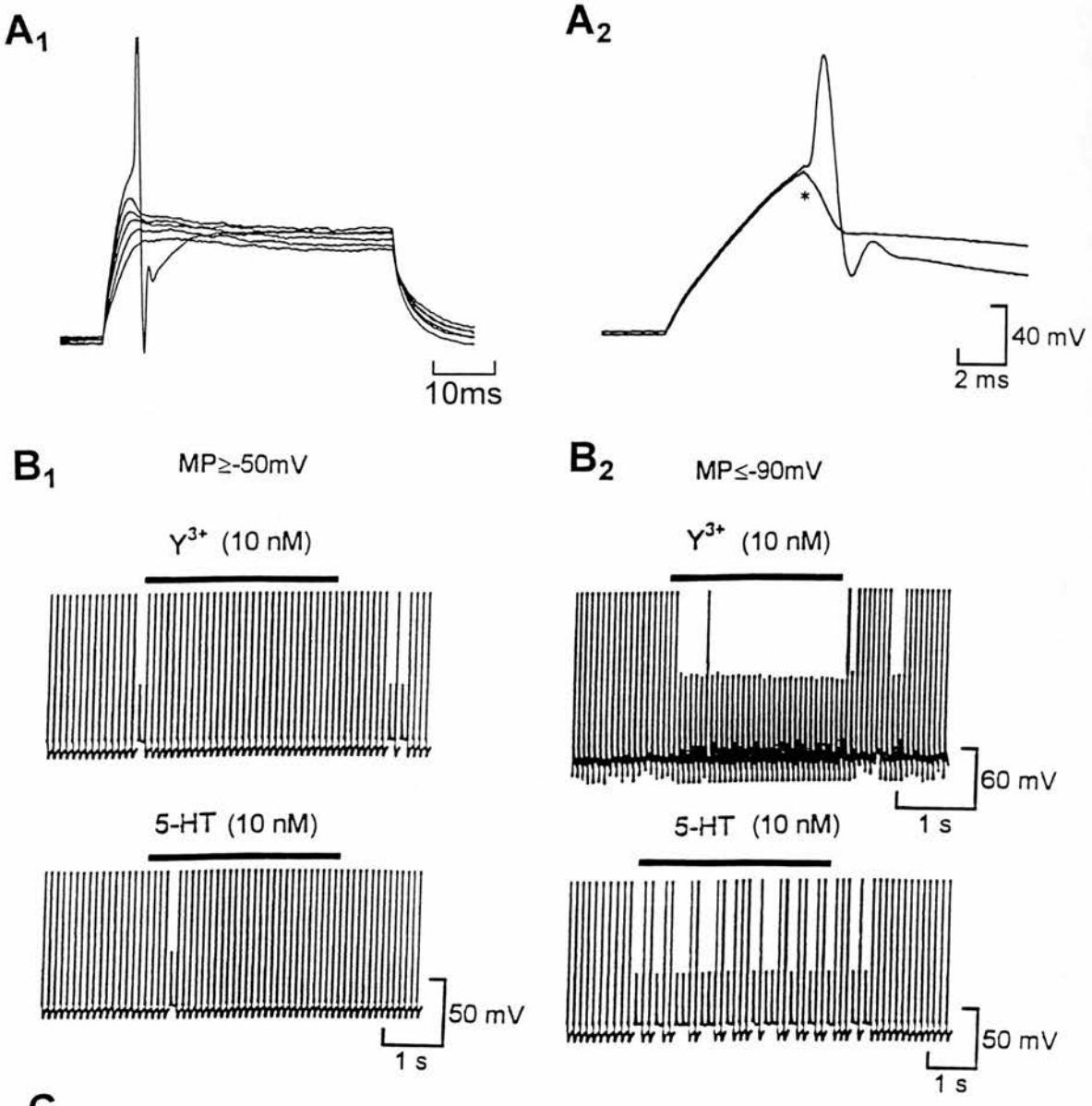

A1
Sweep (5 s)
0
20
40
60
80
0
-50
-100
-150
ICa(pA)
10 nM 1 μM 5 μM 30 μM
A2
Sweep (5 s)
0
20
40
60
80
0
-1000
-2000
-3000
ICa (pA)
10 nM 1 μM 5 μM 30 μM
A3
*
*
0.40 nA
25 ms
B
100
50
0
Current inhibition (%)
n=8
**
n=8
T
HVA

Figure 2.10 Effects of the selective T-type Ca^{2+} channel blockers and 5-HT on R-B neurone firing

A_1, Current clamp recording in a R-B neurone. Using steps of current injection to detect the threshold current that evoked an action potential. A_2, Showing 10 nM Y^{3+} (*), which selectively blocked T-type currents, blocked the action potential evoked at threshold current injection. B_1, Current clamp recording from a R-B neurone showing that injection of repeated current pulses just above the threshold caused reliable firing. At resting membrane potentials more positive than -50 mV, neither Y^{3+} (10 nM) nor 5-HT (10 nM) had any effects on neurone firing. B_2, At more negative resting membrane potentials (-90 mV), both Y^{3+} (10 nM) and 5-HT(10 nM) reversibly reduced the probability of firing in the neurone. C, Summary of the effects of Y^{3+} (10 nM) and 5-HT(10 nM) on the probability of R-B neurone firing in response to repetitive threshold current injection at a resting membrane potential of -90 mV, where the probability was measured as: the number of pulses that evoked a spike/total number of pulses, during control, drug treatment and wash. (**; $p<0.01$ vs. control or wash).


A1
10ms
A2
40 mV
2 ms
B1
MP≥-50mV
Y3+ (10 nM)
5-HT (10 nM)
50 mV
1 s
B2
MP≤-90mV
Y3+ (10 nM)
60 mV
1 s
5-HT (10 nM)
50 mV
1 s
C
Probability of firing
1
0.8
0.6
0.4
0.2
0
n=8
**
n=5
*
control
Y3+
wash1
5-HT
wash2

2.5 Discussion

The larva of the South African amphibian, *Xenopus laevis* is a simple model for studying the spinal mechanisms of sensory modulation. Around the time of hatching, the spinal cord contains only eight classes of differentiated neuron (Roberts and Clarke, 1982). The trunk skin of the tadpole is innervated by the free nerve endings of a single, homogeneous population of mechanosensory afferents, called Rohon-Beard (R-B) neurons (Hughes, 1957). The R-B neurons are highly analogous to human C fibres. Like C fibres, R-B neurons are unmyelinated and possess free nerve endings; use glutamate as a transmitter (Sillar and Roberts, 1988) and substance P as a co-transmitter (Clarke et al., 1984); and have capsaicin receptors (Kuenzi and Dale, 1996). The acutely isolated R-B neurons have a distinctive morphology, maintain similar membrane properties to their *in vivo* counterparts and can easily be recognized *in vitro* (Dale, 1991). The R-B neurons can therefore be used as a highly advantageous model for studying the neuromodulation of sensory pathways.

2.51 Identity of serotonergic receptors

I found that T-type currents were blocked by the 5-HT_{1A} agonist 8-OH-DPAT, and the 5-HT_{1D} agonist, L-694,247. The IC_{50}' s for both agonists were very similar to that reported in mammals (Middlemiss and Fozard, 1983; Beer et al., 1993). The effect of the 5-HT_{1A} agonist was blocked only by the 5-HT_{1A} antagonist, p-MPPI (Kung et al., 1994) whereas the effect of L694,247 was blocked only by the 5-HT_{1D} antagonist, GR127935 (Skingle et al., 1993). Therefore these receptors have a very similar pharmacology to the mammalian 5-HT_{1A} and 5-HT_{1D} receptors and I propose that they are the amphybian equivalents of these receptors.

5-HT_{1A} receptors have been previously described in amphibian spinal cord (Holohean et al., 1992; Tan & Miletic, 1992). The 5-HT_{1A} receptor can inhibit Ca^{2+} entry through HVA channels into afferent terminals of nociceptors in mammalian spinal cord (Del Mar et al., 1994). 5-HT_{1A} receptors have also been reported to be involved in the voltage-dependent G-protein-coupled inhibition of the HVA currents in other CNS neurons (Penington, et al., 1991; Koike et al., 1994; Bayliss et al., 1995; Foehring, 1996). Unlike these previous reports, the inhibition of the HVA currents by 5-HT_{1A} receptors in R-B neurons is not voltage-dependent.

$5HT_{1D}$ receptors have been found mostly in the mammalian brain and in pigeon (Bruinvels et., 1992; Hoyer et al., 1992; for review see Waeber et al., 1990) but not yet in the amphibian nervous system. $5HT_{1D}$ receptors seem to exist in those species where the $5HT_{1B}$ receptor is absent and may play a role in these species equivalent to that of the $5HT_{1B}$ receptor in rat and mouse (see Zifa and Fillion, 1992, for review). Our findings that $5HT_{1D}$ receptors are present in *Xenopus* are the first to suggest that of 5-HT_{1D} receptors exist in the amphibian nervous system and are consistent with previous reports that the $5HT_{1B}$ receptors are absent in frog (see Zifa and Fillion, 1992, for review).

The $5HT_{1D}$ receptor inhibits synaptic transmission (Jones et al., 1995) and acts as an autoreceptor in the mammalian raphe nucleus (Davidson and Stamford, 1995). The actions of 5-HT_{1D} receptor on Ca^{2+} currents and its role in the sensory transmission have not been reported; therefore our results are the first to show that 5-HT_{1D} receptors are also involved in limiting sensory transmission by inhibiting T- type and HVA Ca^{2+} channels. In the *Xenopus* spinal cord, 5-HT_{1D} receptors are also found in a class of glycinergic inhibitory premotor interneurons (Q. Q. Sun and N. Dale, 1997, unpublished results) which contribute to the control of locomotion in *Xenopus*. Therefore the 5-HT_{1D} receptor may play a important role in both the descending supraspinal control of sensory transmission and the regulation of locomotion patterns in *Xenopus* .

2.52 Modulation of the T-type currents

Although some neurotransmitters have been documented to inhibit T-type currents, presumably via G-protein mediated processes (Formenti and Sansone, 1993), serotonin has been reported to enhance rather than inhibit T-type currents in CNS neurons (Berger and Takahashi, 1990; Fraser and MacVicar, 1991). In contrast to this, I found that serotonin reversibly inhibited T-type currents in R-B neurons.

5-HT has been previously reported to mediate presynaptic inhibition of transmitter release from R-B neurons in *Xenopus* (Sillar and Simmer 1994). However T-type channels are not generally thought to be involved in supporting synaptic transmission. The modulation of T-type currents therefore suggests an additional and important locus of modulation: initiation of R-B neuron discharge in response to sensory stimuli. R-B neurons have a very negative resting potential (around -90 mV, Spitzer and Lamborghini, 1976), so the T-type calcium current is unlikely to be in an inactivated state in these neurons at rest. Since the T-type currents are the only voltage-gated inward currents activated in these neurons between -60 and -30 mV, they are may play an important role in triggering action potentials. Small reductions of the T-type calcium current could therefore modulate the responsiveness of Rohon-Beard neuron to sensory stimuli. I found that both selective T-type channel blockers and low doses of 5-HT greatly reduced the probability of R-B neuron firing in response to threshold current injection at a holding membrane potentials of -90 mV but not at more positive membrane potentials of -50 mV, where the T-type channels were in inactivated. This strongly supports our hypothesis that T-type channels play a role in the spike initiation in R-B neurons and that the modulation of T-type channels may raise the R-B firing threshold. I also found that the T-type currents were much more sensitive to serotonin agonists (IC_{50} = 0.1 nM for 5-HT) than HVA currents (IC_{50} = 40 nM for 5-HT). During 5-HT release, the T-type channels are thus likely to be modulated first when the concentrations of 5-HT are low. In our cell-attached recordings, I

found that patches located near the neuronal process but not the nucleus, quasi-macroscopic T-type can be elicited. The most effective locus for the T-type channels to influence spike initiation would be in the peripheral neurites close to the site of mechanotransduction. I therefore propose that both the 5-HT receptors and T-type channels are in the peripheral neurites and that modulation of these channels alters the sensitivity of the sensory pathway.

2.43 Modulation of the N- and P/Q- type HVA Ca^{2+} currents

5-HT inhibits N- and P/Q- type HVA calcium current in neocortical pyramidal neurons (Foehring, 1996), in rat motor neurons (Bayliss, 1995), or N-type in sensory neurons (Del Mar et al., 1994) and other CNS neurons (Pennington et al., 1991; Koike et al., 1994). Consistent with these reports, our results showed that N- and P/Q- type HVA Ca^{2+} current was inhibited by 5-HT in R-B neurons. N-, and P/Q- type calcium channels are involved in supporting the synaptic transmission in CNS and peripheral (Leubeke et al., 1993; Leubeke and Dunlap, 1994; Wall and Dale, 1994; Wheeler et al., 1994; see Dunlap 1997, for review). Therefore the reduction of N- and P-type currents could contribute to the previously reported presynaptic inhibition of transmitter release from R-B neurons (Sillar et al., 1994).

The modulation of N-type and P/Q-type currents in R-B neurons was not accompanied with slowing of activation and was voltage-independent, which is different from most of the examples of G-protein-mediated modulation (for review see Hille, 1994; Wickman and Clapham, 1995), where a kinetic-slowing is always accompanied with the reduction of HVA currents. The difference in voltage-dependence for modulatory components may have different functional implications under various physiological conditions. The voltage-dependent modulation, which can be removed by preceding stimulation, would be phasic in nature; whereas the voltage-independent modulation, would persist under conditions of repetitive firing.

2.54 Distinctive biochemical pathways may underlie the modulation of T-type and HVA currents

In R-B neurons, both HVA and T-type currents were inhibited by 5-HT in a voltage-independent manner, through the same receptors. However, the modulation of T-type channels differed from the HVA channels in that: it had roughly 100 times greater sensitivity to 5-HT and the time course for wash was twice as fast. These differences may reflect the involvement of distinctive biochemical mechanisms. Our results suggest that the reduction of T-type currents occurred through a membrane-delimited pathway, possibly, a direct G protein-channel interaction. In the mammalian CNS, the most common signal transduction pathway for the modulation of N- and P/Q- channels is through a voltage-dependent interaction between the calcium channels and the G protein $\beta\gamma$ subunits (Ikeda, 1996; Waard et al., 1997, see Dunlap, 1997 for review). However, in those cases where the modulation is voltage-independent, it may be mediated by protein kinase C (Diverse-Pierluissi et al., 1995). Further studies to address the participation of various signal transduction pathways, such as whether the G-protein involved are PTX-sensitive and if cAMP/PKA or PKC involved in the modulation will certainly be very useful.

Chapter 3

G-PROTEINS ARE INVOLVED IN 5-HT RECEPTOR MEDIATED MODULATION OF N AND P/Q BUT NOT T-TYPE CA^{2+} CHANNELS

3.1 Summary

5-HT inhibits the N, P/Q and T-type Ca^{2+} currents in sensory neurons of *Xenopus* larvae. The inhibition of HVA currents by 5-HT is mediated by a pertussis toxin-sensitive G-protein that activates a diffusible second messenger. Although modulation of T-type currents is membrane-delimited, it was not affected by GDP-β-S (2 mM), GTP-γ-S (200 μM), GMP-PNP (200 μM), AlF_4^- (100 μM), or pertussis toxin. To investigate the modulation of T current further, I synthesized peptides that were derived from conserved cytoplasmic regions of the rat $5\text{-}HT_{1A}$ and $5\text{-}HT_{1D}$ receptors. Whereas two peptides derived from the third cytoplasmic loop inhibited the HVA currents by activating G-proteins and occluded the modulation of HVA currents by 5-HT, two peptides from the second cytoplasmic loop and the carboxy-tail had no effect. None of the 4 receptor-derived peptides had any effect on the T-type currents. I conclude that 5-HT modulates T-type channels by a membrane-delimited pathway that does not involve G-proteins and is mediated by a functional domain of the receptor that is distinct from that which couples to G-proteins.

3.2 Introduction

Many neurotransmitters and neuropeptides mediate their biological actions by activation of receptors that are coupled to heterotrimeric G-proteins. Ligand binding to the receptor causes interaction with distinct classes of G-proteins and triggers the exchange of GTP for GDP on the α subunits, leading to the dissociation of the Gα from the $\beta\gamma$ complex. The dissociated α-GTP or $\beta\gamma$ subunits are then able to interact with their effector enzymes and ion channels. CNS neurons normally express many different types of receptors that transduce signals through a relatively limited repertoire of herterotrimeric G-proteins. Traditional linear models of signalling that require specific and highly selective coupling of receptor to G-protein to effector proteins have proven to be too simple. For example, a single neurotransmitter can activate and modulate several types of ion channels through a variety of mechanism (Ciranna et al., 1996; Sun and Dale, 1998). Conversely, activation of a single receptor can modulate several types of ion channels through more than one mechanisms (Luebke and Dunlap, 1994; Diversé-Pierluissi, et al., 1993 and 1995; Albillos et al., 1996; Currie and Fox, 1997; Sun & Dale, 1998). Nevertheless, the prevailing orthodoxy is that the superfamily of receptors that includes, for example the muscarinic, adrenergic and serotonergic receptors mediate their major actions on ion channels and other proteins exclusively through G-proteins (but see Hall et al., 1998).

T-type channels are encoded by different genes and have unique kinetic characteristics compared to the HVA channels (Perez-Reyes, et al, 1998). The functions of T-type channels are also distinct from those of the HVA channels (see Huguenard, 1996, for review). Investigations into the modulation of T-type channels by neurotransmitters and G-proteins have yielded contradictory results so far. In rat spinal motoneurons and hippocampal neurons, these currents were enhanced by 5-HT and other transmitters through an unknown mechanism (Berger and Takahashi, 1990; Fraser and MacVicar, 1991), whereas in sensory neurons (Sun and Dale, 1997; Abdulla and Smith, 1997) and rat nucleus basalis

neurons (Margeta-Mitrovic et al., 1997), T-type currents were inhibited by 5-HT or neuropeptides. In sensory neurons, inhibition of T-type currents can alter the neuron firing properties, and could thus be involved in analgesia (Abdulla and Smith, 1997; Sun and Dale, 1997).

In a previous report (Sun and Dale, 1997), I found that the 5-HT_{1A} and 5-HT_{1D} receptors inhibited both the T-type and HVA channels via voltage-independent mechanisms in sensory neurons (Sun and Dale, 1997). We report here that both 5-HT_{1A} and 5-HT_{1D} receptors inhibit the HVA channels through a pertussis toxin-sensitive G-protein and a diffusible second messenger. In a surprising contrast, modulation of the T-type channels, which is membrane-delimited (Sun and Dale, 1997), does not appear to occur through the actions of a G-protein. Indeed by using the peptides derived from the intracellular portions of the 5-HT receptor I have evidence that a functional domain entirely separate from that involved in coupling to the G-protein is likely to mediate the modulation of T-type channels.

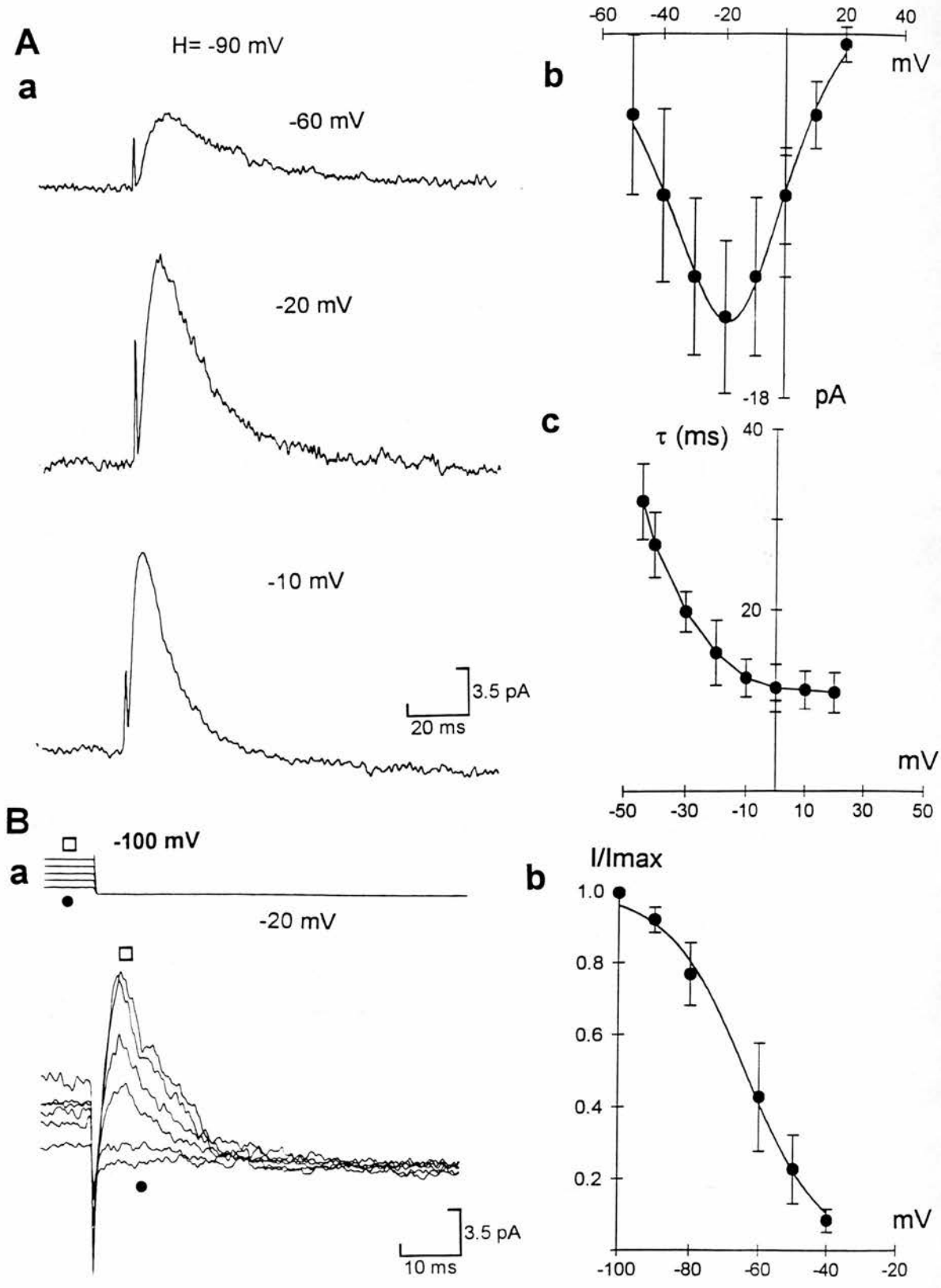
3.3 Results

3.31 Both the HVA channels and the T-type channels can be distinguished in cell-attached patch recordings

In whole-cell recordings, T-type currents only represent a very small amount of total Ca^{2+} current elicited from holding potential of -90 mV (Sun and Dale, 1997). This current density can be produced by some 1000 T-type channels. In cell-attached patch recordings, T-type channels are non-uniformly distributed and were not observed at a holding potential of -90 mV in the majority of patches (~70%, n=120). In the remaining 30% of patches, recorded near the neuronal process but not the nucleus, I observed some large T-type currents. These ranged in amplitude from 3 pA to 20 pA at holding potential of -90 mV, and were therefore produced by some 10 to 80 T-type channels (Fig 3.1 A). Thus the T-type channels are clustered with a non-uniform spatial distribution.

Figure 3.1 Kinetic characters of T-type channel currents in cell-attached patch

A a) T-type currents were elicit by steps of + 10 mV from holding potential of - 90 mV, representative traces were elicited by steps to - 60 mV, -20 mV, and - 10 mV, respectively. b) Current-voltage relation of T-type currents (n=5). Solid line is the best fit of the product of the Goldmann-Hodgkin-Katz equation and Boltzmann equation (cf. Dale, 1995). c) Graph showing time constant of inactivation of T-type currents vs. test voltage in 4 cells. B a) T-type currents were elicited by steps to - 20 mV from holding potential varied from - 100 mV to - 40 mV. b) Steady-State inactivation of T-type currents. Solid line is the best fit of Boltzmann relation, $I = \{1+ \exp[(V+V_{1/2})/k]^{-1}\}^{-1}$, where $V_{1/2}$= -62 ± 6.3 mV, and k=11.6± 3.2 (n=4). Error bars represent SEM in this and all figures unless otherwise stated.


A
H= -90 mV
a
-60 mV
-20 mV
-10 mV
3.5 pA
20 ms
b
-60
-40
-20
20
40
mV
-18
pA
c
τ (ms)
40
20
mV
-50
-30
-10
10
30
50
B
-100 mV
a
-20 mV
3.5 pA
10 ms
b
I/Imax
1.0
0.8
0.6
0.4
0.2
mV
-100
-80
-60
-40
-20

By fitting the current-voltage relation with the Boltzmann equation, I obtained a half-activation voltage for the T-type currents (110 mM $BaCl_2$) of -35 ± 4.5 mV (n=6, Fig 3.1 Aa and b), and activation slope (k) of 9.1 ± 2.4 (n=6, Fig 3.1 Aa and b). The inactivation of T-type currents was very fast with a time constant that ranged from 33.6 ± 3.8 ms (at test potential to -40 mV) to 14 ± 2.6 ms (at + 10 mV, Fig 3.1Aa and c). In some patches which contained more than 50 T-type channels (Fig 3.1 Ba), I tested the steady-state inactivation properties of the T-type, and obtained a half-inactivation voltage of -62 ± 4.3 mV (n=4, Fig 3.1 Bb). These parameters of T-type channels are similar to those of unitary T-type channels recorded under cell-attached patches in mammalian sensory neurons (Fox et al., 1987a, b). However they differ from the voltage-dependence seen in whole-cell recordings (Fox et al., 1987a, b; Sun and Dale, 1997), probably due to the high levels of Ba^{2+} which will screen surface charge and alter channel gating.

By contrast to the T currents, HVA currents had very different characteristics. HVA currents were elicited at more depolarized membrane potentials (> +10 mV) in the cell-attached patches (Fig 3.2 A and Fig 3.3). In *Xenopus* R-B neurons, N- and P/Q- type channels comprised the majority of HVA channels. N currents represent around 70 % of whole-cell currents and P/Q currents represent about 25% (Sun and Dale, 1997). In most patches recorded, the HVA currents resembled the characteristic kinetic pattern of N-type HVA channels (Fig 2.2A and Fig 3.3A). Like N-type channels in mammalian sensory neurons (Fox et al., 1987a, b), *Xenopus* N-channels also appear to be non-uniformly distributed and spatially clustered. HVA currents were only recorded in 35% of the patches, with currents produced by some 10 to 200 channels. In cell-attached patches (110 mM $BaCl_2$), N-channel currents showed strong inactivation at holding potential of -50 mV, but this had time constant that was more than two times more slower than the T-type currents recorded at the same test voltage (τ=35.8 ± 6.2 ms at a test potential of +20 mV, n=5).

Figure 3.2 Modulation of the HVA channel currents by 5-HT and 5HT agonists in cell-attached patches

A) 4 consecutive recordings of HVA channel currents in a cell-attached patch. Bottom: averaged trace from 30 consecutive recordings. The solid line is the best fit of single exponential equation. B) Equivalent traces from the same patch after 5-HT was applied to the cell. C) Summary of the effects of 5-HT, 5-HT_{1A} agonist, 8-OH-DPAT; and 5-HT_{1D} agonist, L-694,247 on the HVA currents recorded in cell-attached patches. D) Summary of the effects of 5-HT on T-type currents recorded in the same batch of neurones. Inset: representative traces of T-type currents (averaged from 20 consecutive recordings) recorded in the cell-attached patches in control, 5-HT and after wash. Error bars represent S.E.M. in this and all figures unless otherwise stated. (n.s. not significant; *: $p<0.05$; ** $p<0.01$ in this and all figures).

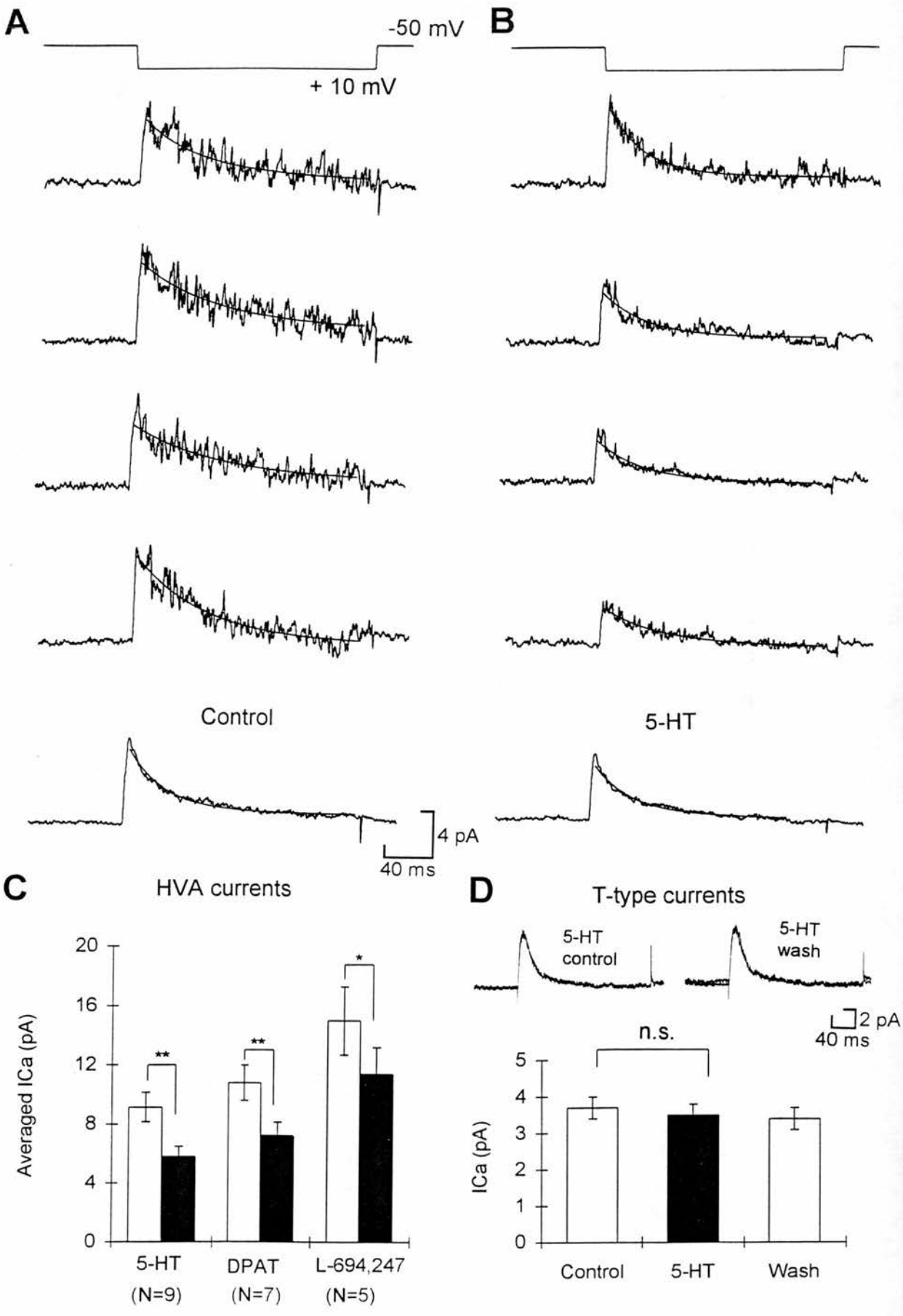
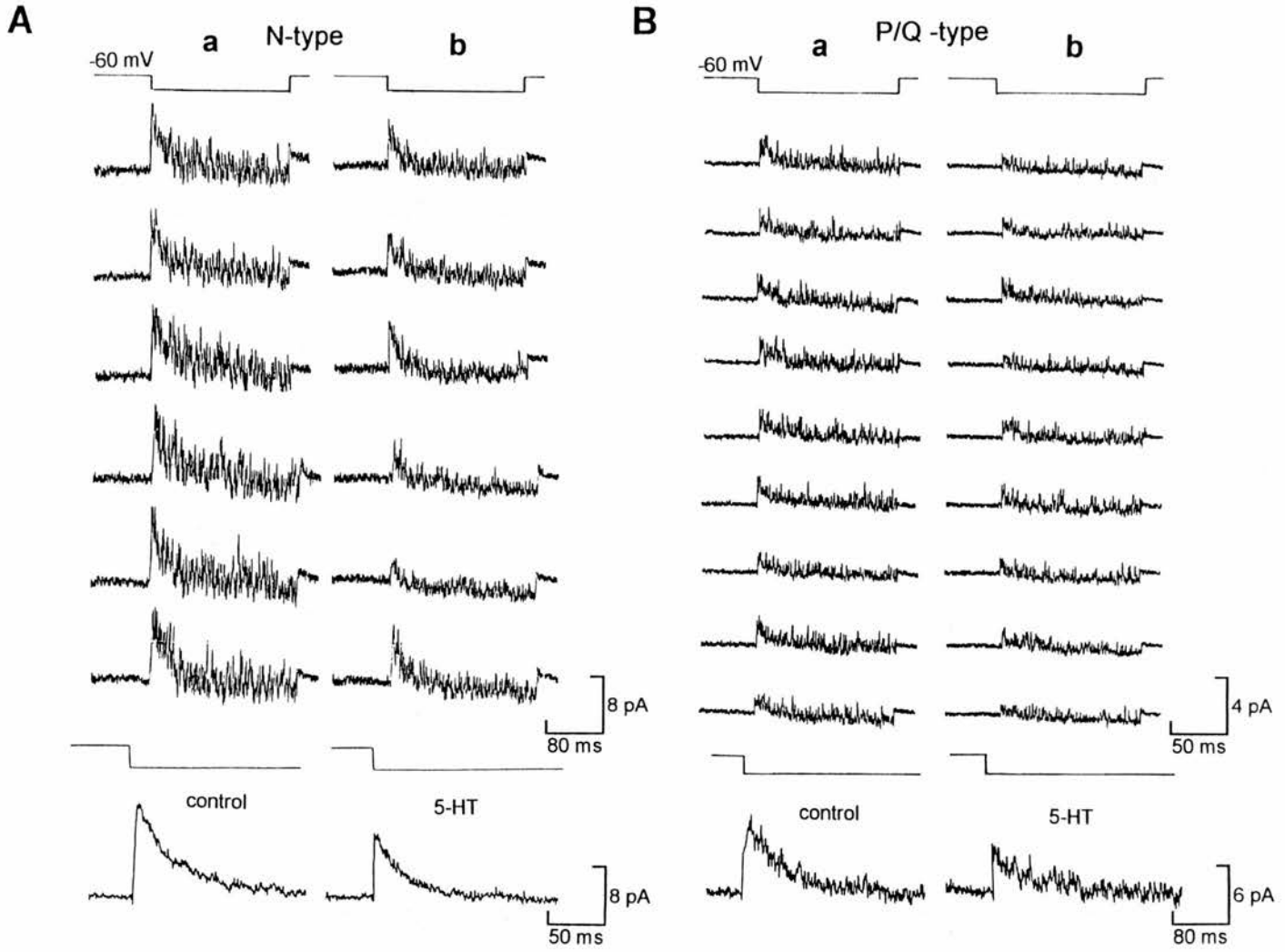

A
-50 mV
+ 10 mV
B
Control
5-HT
4 pA
40 ms
C
HVA currents
Averaged ICa (pA)
20
16
12
8
4
0
**
**
*
5-HT
(N=9)
DPAT
(N=7)
L-694,247
(N=5)
D
T-type currents
5-HT
control
5-HT
wash
2 pA
40 ms
n.s.
ICa (pA)
5
4
3
2
1
0
Control
5-HT
Wash

Figure 3.3 Modulation of N- and P/Q -type channels via diffusible second messengers

A) Representative consecutive records of N-type Ca^{2+} channel currents (ω-Agatoxin-IVA 200 nM in pipette) in control (a) and 5-HT (b). Bottom: averaged trace from 50 consecutive sweeps. B) Representative records of P/Q-type Ca^{2+} channel currents (ω-Conotoxin-GVIA 1 μM in pipette) in control (a) and 5-HT (b). Bottom: averaged trace from 30 consecutive sweeps.


A
a
N-type
b
-60 mV
8 pA
80 ms
control
5-HT
8 pA
50 ms
B
a
P/Q -type
b
-60 mV
4 pA
50 ms
control
5-HT
6 pA
80 ms

3.32 Modulation of HVA channels but not T-type channels involves a freely diffusible second messenger

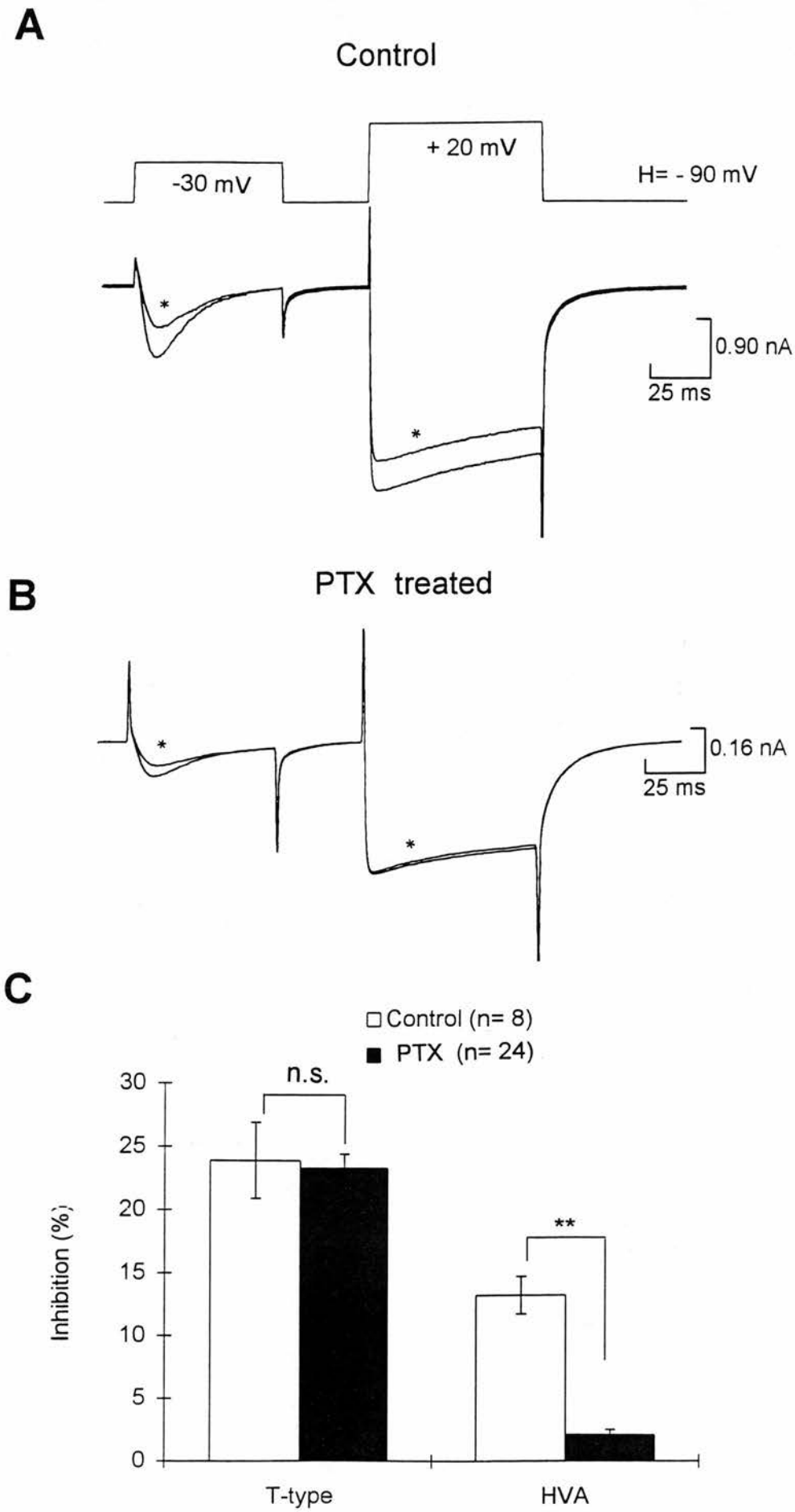
To confirm our earlier findings (Sun and Dale, 1997), I studied whether 5-HT could modulate the T-type currents recorded in the cell-attached patch mode. Once again I found that 5-HT had no effect on the T channels (Fig 3.2D, n=6), showing that modulation of whole-cell T-type currents by 5-HT is indeed membrane-delimited. In contrast to the T-type currents, the HVA currents recorded in cell-attached patches were inhibited by 5-HT applied outside the pipette (Fig 3.2A , B and C; $p<0.01$, n=9). The selective 5-HT_{1A} agonist 8-OH-DPAT (Fig 3.2C, $p<0.01$, n=7) and 5-HT_{1D} agonist L-694,247 (Fig 3.2C, $p<0.05$, n=5) also caused inhibition. The inhibition of HVA currents by both 5-HT_{1A} and 5-HT_{1D} receptors must therefore be mediated through a freely diffusible second messenger. The inhibition of HVA currents by 5-HT was 35.6 ± 4.2 % (n=9), which was much higher than the inhibition in whole-cell recordings (16%, Sun and Dale, 1997). This is probably due to greater preservation of the cell structure and content in the cell-attached form of recordings.

I next tested whether both types of HVA channels were individually modulated via a diffusible second messengers. Patches which contained L-type channel activity (readily distinguished by unitary conductance and voltage-dependence of activation and inactivation) were excluded from analysis. Pure N- or P/Q channel recordings were obtained by including in the patch pipette either ω-conotoxin-GVIA (1 μM) to block N- channels or ω-agatoxin-IVA (200 nM) to block P/Q channels. Both N-type channels (Fig 3.3A , 3 neurons) and P/Q channels (Fig 3.3B, 2 neurons) were inhibited by 5-HT. Our results therefore suggest that N- and P/Q channels are inhibited via a diffusible second messenger that can be activated by both 5-HT_{1A} and 5-HT_{1D} receptors.

3.33 Modulation of HVA but not T-type currents involves a pertussis toxin-sensitive G-protein

Figure 3.4 Effects of overnight pre-incubation of pertussis toxin (PTX) on the inhibition of Ca^{2+} currents by 5-HT

A) 5-HT produced reversible inhibition on both T-type and HVA currents in a neurone which had been incubated overnight but without addition of PTX. B) In a neurone that was incubated with PTX (1 ng/ml) for 12 h, 5-HT had very little effect on the HVA currents but still inhibited the T-type currents. C) Summary of the effects of 5-HT on both T-type and HVA currents in the control and PTX-treated neurones.


A
Control
+ 20 mV
-30 mV
H= - 90 mV
0.90 nA
25 ms
B
PTX treated
0.16 nA
25 ms
C
Control (n= 8)
PTX (n= 24)
n.s.
**
Inhibition (%)
30
25
20
15
10
5
0
T-type
HVA

I examined the effects of preincubation of pertussis toxin (PTX) on the modulation of T-type and HVA currents by 5-HT. PTX (500 ng-1 μg/ml) was added to dishes of neurons and left for around 12 hours before patch-clamp recordings were commenced. Control dishes of neurons dissociated from the same batch of spinal cords were left for the same period but without addition of PTX. PTX greatly reduced the inhibition of the HVA currents by 5-HT (Fig 3.4), but had no effect on the inhibition of the T-type currents by 5-HT (Fig 3.4). This suggests that the inhibition of HVA currents is mediated by a PTX-sensitive G-protein, whereas modulation of T-type currents in the same neuron is insensitive to PTX.

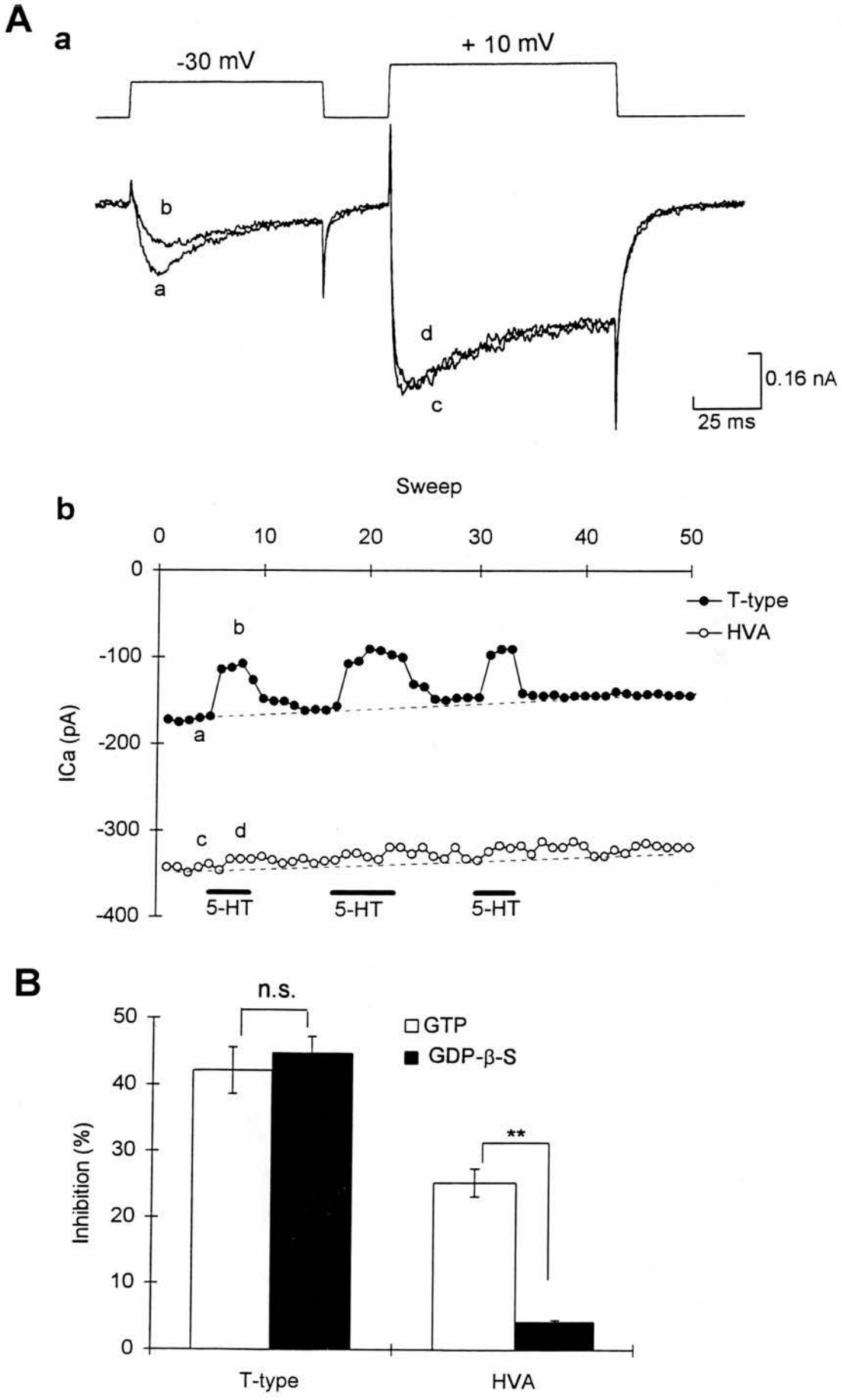
3.34 Non-hydrolyzable analogues of GTP and GDP modify modulation of HVA but not T-type currents

To test further the possible involvement of G-proteins on the modulation of Ca^{2+} currents by 5-HT, 500 μM to 2 mM GDP-β-S was added to the pipette to replace the 1 mM GTP included in the control. A twin pulse protocol from holding potential of -90 mV was used to elicit both the T-type and the HVA currents (Fig 3.5Aa). Unlike in mammalian DRG neurons, where intracellular addition of GDP-β-S caused an enhancement of the transient Ca^{2+} currents (Dolphin and Scott, 1987), I found that GDP-β-S had very little effect on either the HVA currents or the T-type currents (n=20), suggesting that there was little or no tonic modulation of these currents by G-proteins in R-B neurons.

In all recordings in which GDP-β-S was added into the pipette (n=14, Fig 3.5A and B), 5-HT (1-10 μM) still reversibly inhibited the T-type currents. The amount of inhibition evoked by 5-HT in these neurons was similar to those recordings in the same dish in which GTP (1 mM) instead of GDP-β-S (1 mM) loaded into patch pipette (Fig 3.5C). However, GDP-β-S did prevent the inhibition of HVA currents (n=14, Fig 3.5A , B and C). The amount of inhibition produced by 5-HT in those neurons loaded with GDP-β-S was much smaller than those neurons loaded with 1 mM GTP ($p<0.01$, GDP-β-S vs. control, n=14; Fig 3.5C).

Figure 3.5 Intracellular dialysis of GDP-β-S diminished modulation of HVA currents but T-type Ca^{2+} currents

A) In a cell loaded with GDP-β-S (2 mM), 5-HT produced reversible inhibition on T-type currents but had very little effect on the HVA currents; (a) representative traces of the Ca^{2+} currents b) graph showing amplitude of current vs. time in the same cell. Symbols in graph correspond to those on the current traces. B) Summary of the effects of 5-HT on T-type currents and HVA currents in cells loaded with either GTP (control) or GDP-β-S.


A
a
-30 mV
+ 10 mV
b
a
d
c
0.16 nA
25 ms
b
Sweep
0
10
20
30
40
50
0
-100
-200
-300
-400
ICa (pA)
T-type
HVA
b
a
c
d
5-HT
5-HT
5-HT
B
n.s.
GTP
GDP-β-S
**
Inhibition (%)
50
40
30
20
10
0
T-type
HVA

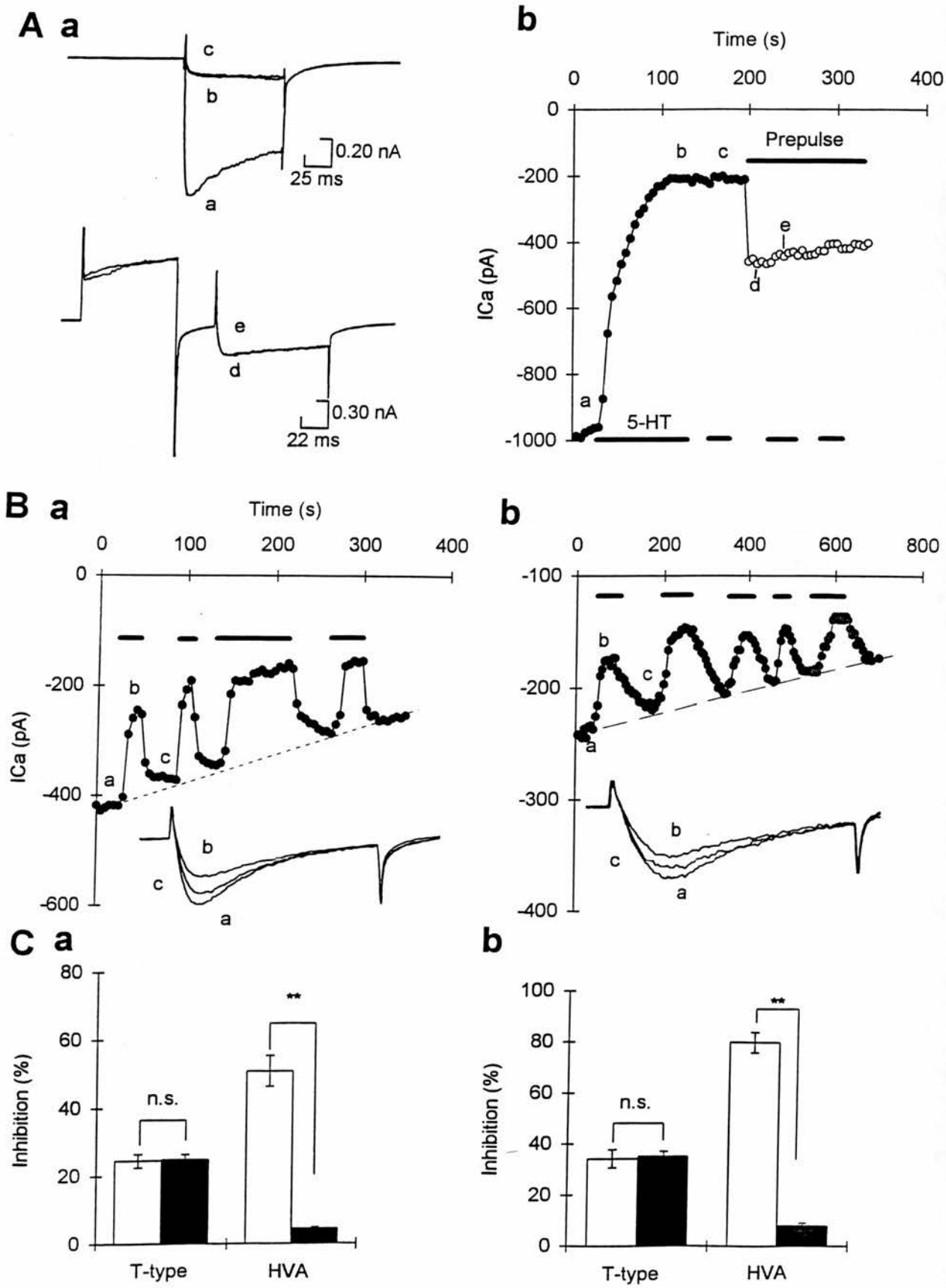
GMP-PNP is a nonhydrolyzable analogue of GTP, that was first used to demonstrate that GTP regulates adenylyl cyclase (Londos et al., 1974). In pipettes loaded with GMP-PNP (200 μM), there was very little change in the T-type current (Fig 3.6Ba, n=8) and 5-HT still produced reversible inhibition, even 10 minutes after the start of recordings (Fig 3.6Ba). The amount of T currents inhibition produced by 5-HT (1 μM) remained similar to that in neurons with control pipette solution recorded in the same dish (GTP 1 mM, n=8, $p>0.1$; Fig 3.6Ba and Ca).

In contrast, GMP-PNP (200 μM) greatly enhanced the inhibition of HVA currents by 5-HT. However the inhibition in most cells was not reversible upon wash (n=8, Fig 3.6A), whereas in a few other cells (n=4), there was a partial reversal. Subsequent applications of 5-HT gave virtually no further inhibition (Fig 3.6A and Ca). Unlike cells which had been loaded with control pipette solution (GTP 1 mM) and where inhibition is not associated with slowing and shifting of voltage-dependence (Sun and Dale, 1997), the inhibition of the HVA currents in the presence of GMP-PNP was associated with slowing of activation that could be relieved partially by a strong-depolarizing prepulse to +120 mV (Fig 3.6A). After loading with GMP-PNP, the onset of the inhibition of HVA currents by 5-HT was rather slow, ranging from 100 s to 200 s (Fig 3.6Ab). Since the inhibition of HVA currents by 5-HT was not only enhanced but was also accompanied by a kinetic change and voltage-dependence, this suggests that new modulatory components were activated in the presence of GMP-PNP (cf. Sun and Dale, 1998). To observe the effects of GMP-PNP on the voltage-independent suppression of HVA currents in R-B neurons, I eliminated the voltage-dependent interaction by applying a prepulse to +120 mV, and I found that 5-HT still had no further effect (Fig 3.6A, n=3).

GTP-γ-S is another nonhydrolyzable analogue of GTP which has similar effects to GMP-PNP on G-proteins but with a higher affinity for G-proteins (Olate and Allende, 1991). Like GMP-PNP, GTP-γ-S had very little effect on T-type currents by itself (Fig 3.6Bb). The mean peak current in neurons loaded with

Figure 3.6 Effects of intracellular dialysis of GMP-PNP or GTP-γ-S on the modulation of whole-cell Ca^{2+} currents by 5-HT

A) In a cell loaded with GMP-PNP (200 μM), 5-HT produced irreversible inhibition of HVA currents which was accompanied by slowing of activation. Further application of 5-HT did not have any additional effect; even in presence of prepulse to remove the voltage-dependent inhibition. a) HVA currents elicited by test potential to + 10 mV from holding potential of - 90 mV, without (top traces) or with prepulse to + 120 mV (bottom traces). b) Time series recordings in the same cell, symbols corresponding to current traces in a. Filled circle represent recordings made before applying prepulse and open circles represent recordings made during prepulse application, symbols correspond to current traces. B) Time series measurements and T-type currents in the same cell showing that in a cell loaded with GMP-PNP (a, 200 μM) or GTP-γ-S (b, 200 μM), 5-HT reversibly inhibited the T-type currents. Insets: T-type currents elicited by test potential of -30 mV from holding potential of - 90 mV in control (trace a) 5-HT (trace b) and after wash (trace c). C) The first application of 5-HT caused increased inhibition of the HVA but not the T-type currents (shown by white bar) in neurones loaded with GMP-PNP (Ca) or 200 μM GTP-γ-S (Cb). The filled bars showing the effects of subsequent addition of 5-HT (5 min after the first addition) on the T-type and the HVA currents.


A a
c
b
a
0.20 nA
25 ms
e
d
0.30 nA
22 ms
b
Time (s)
0
100
200
300
400
0
-200
-400
-600
-800
-1000
ICa (pA)
Prepulse
b
c
e
d
a
5-HT
B a
Time (s)
0
100
200
300
400
0
-200
-400
-600
ICa (pA)
a
b
c
b
0
200
400
600
800
-100
-200
-300
-400
a
b
c
C a
80
60
40
20
0
Inhibition (%)
n.s.
**
T-type
HVA
b
100
80
60
40
20
0
Inhibition (%)
n.s.
**
T-type
HVA

GTP-γ-S (100-200 μM at -30 mV test potential) was 228 ±18.8 pA (n=29), which was not significantly different from the mean T-type currents in control recordings (196±16.8 pA, n=30 p>0.1). This is in contrast to the effects of photo-release of GTP-γ-S on T-type currents in cultured rat dorsal-root ganglion neurons (Scott et al., 1989) and suggests that activation of G-proteins by intracellular dialysis of GTP-γ-S or GMP-PNP had no effects on the T-type currents in R-B neurons.

After allowing the GTP-γ-S (200 μM) to diffuse into the cytoplasm (>5 min), 5-HT still produced reversible inhibition of around 24.5±2% (n=7) of the T-type currents. This is similar to that recorded under control pipette solution (24.9±1%, GTP 1 mM, n=8; Fig 3.6Bb and Cb). In contrast to its effects on the T-currents, GTP-γ-S (200 μM) caused 5-HT to produce much stronger inhibition on the HVA currents (Fig 3.6Cb). Like the effects of 5-HT in cells loaded with GMP-PNP, the effects of 5-HT in cell loaded with GTP-γ-S were not reversible and subsequent addition of 5-HT evoked no further inhibition (Fig 3.6Cb).

The effects of 5-HT on the T-type and HVA currents were also examined in neurons loaded with aluminum fluoride (AlF_4^-), which can permanently stimulate the GTP hydrolysis. Whereas AlF_4^- greatly enhanced the effects of 5-HT on the HVA currents in a partially reversible manner (38±4.2% in AlF_4^-, n= 6, vs. 18.2±4.2% in control, n=20, P<0.01). The modulation of T-type currents by 5-HT remained totally reversible and was unaffected by loading AlF_4^- (35±6.4% in ALF_4^-, n=6, vs. 32± 4.8% in control, n=6).

The very different responses of HVA currents and T-type currents to 5-HT in cells loaded with GDP-β-S, GMP-PNP, GTP-γ-S or AlF_4^- suggest that although G-proteins are involved in the modulation of HVA currents they may not be involved in the modulation of T-type currents.

3.35 Receptor-derived peptides activate G-proteins and modulate HVA but not T-type currents

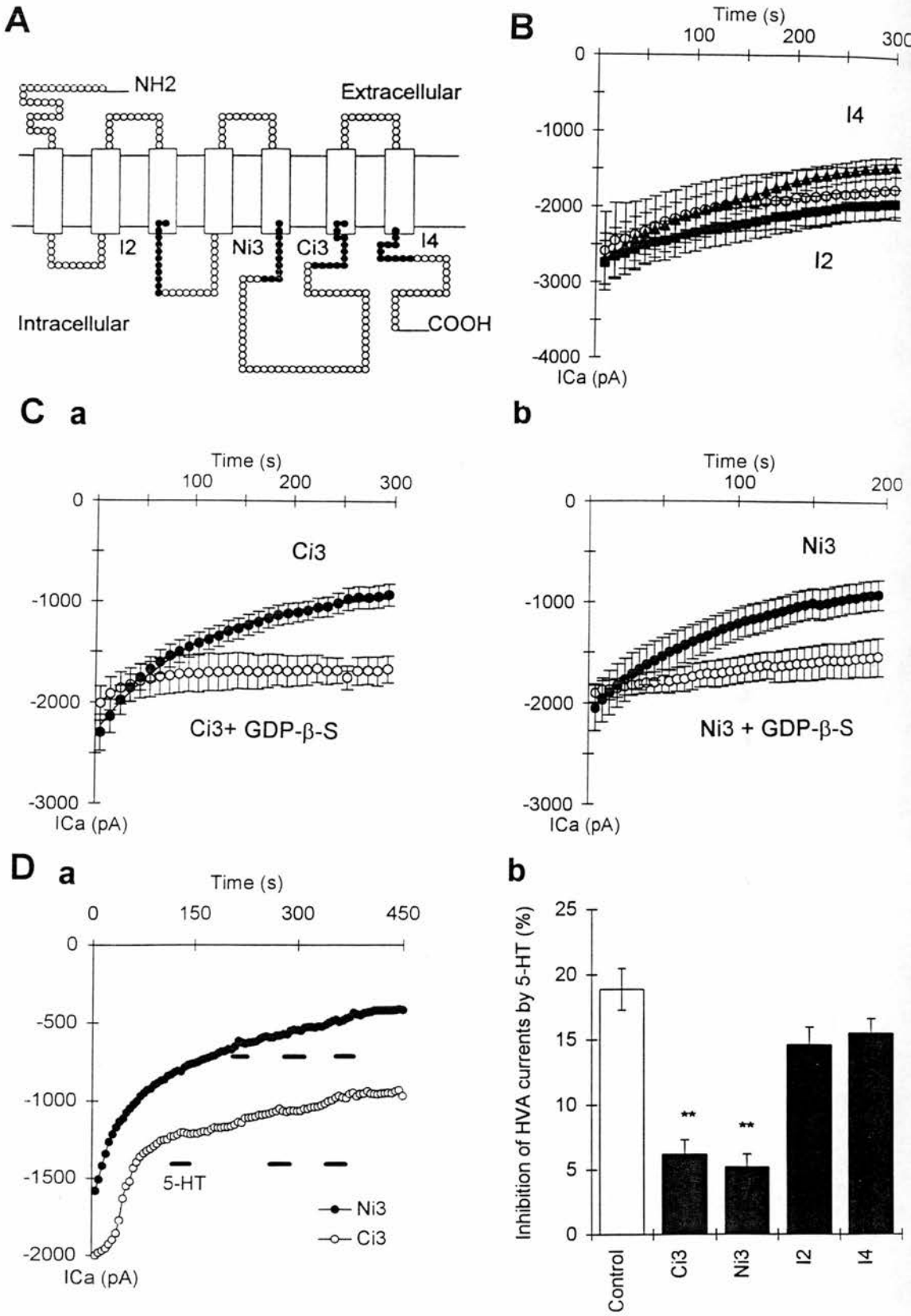
Since GDP-β-S, GMP-PNP and GTP-γ-S all act competitively at the GTP binding site of G-proteins, the lack of effects of these substances on the modulation of T-type currents could still be explained by involvement of a novel G-protein with a very much higher affinity for GTP and GDP than for the non-hydrolyzable GTP and GDP analogues. G-protein activation involves a conformational change of receptor that enables the G-protein to interact with previously inaccessible regions on the receptor protein (cf. Wess 1997). Biochemical studies from several laboratories suggest that peptides corresponding to the second intracellular loop (hereafter referred to as I2), and N and C terminal regions of the third intracellular loop (hereafter referred to as Ni3 and Ci3) can mimic or inhibit the receptor/G-protein interaction (cf. Savarese and Fraser, 1992; Strader et al., 1994; Zhu et al., 1997, Wess, 1997). I therefore synthesized four highly conserved segments of 5-HT_{1A} and 5-HT_{1D} receptor from regions which are generally thought to be involved in receptor G-protein interaction with other types of receptors (Fig 3.7A). These peptides were applied via patch pipette to see whether they could induce inhibition of the T-type and HVA channels. I used four peptides, the first peptide was derived from the carboxy- end of the third cytoplasmic loop and had an amino acids sequence of **RKRISAARERKATK** (Ci3 peptide); the second was from amino end of the third cytoplasmic loop with an amino acid sequence of **LYGRIYVAARSRI** (Ni3 peptide); the third was from the second cytoplasmic loop (**IALDRYWAITD**: I2 peptide) and the fourth was from cytoplasmic carboxy- tail (**DFRQAFQRVV**: I4 peptide).

Effects of the peptides on HVA currents and T-type currents

I first observed whether addition of the four 5-HT receptor-derived peptides via the patch pipette could alter the HVA and T-type currents directly. I found that addition of peptides from the third cytoplasmic loop (Ci3 and Ni3 peptides), but not the other regions (I2 and I4), caused inhibition of the HVA currents manifested as abnormally fast run-down of the HVA currents (Fig 3.7B and C). Run-down of HVA currents can also occur in control pipette solutions and is

Figure 3.7 Effects of 5-HT receptor-derived peptides on the HVA currents and their modulation by 5-HT

A) A topographical model of the 5-HT_1 receptors to show the location of the four synthesized cytoplasmic peptides (filled circles). B) Time series measurements of HVA currents showing the run-down of the HVA currents in cells loaded with control recording solution (open circles, n=16), I4 (filled triangles, n=9) and I2 peptide (filled squares, n=6). The solid line is the best fit of single exponential curve. Ca) Time series measurements of HVA currents in cells loaded with Ci3 peptide alone (filled circles, n=8), and in cells loaded with Ci3 peptide plus 1 mM GDP-β-S (open circles, n=6). Cb) Time series measurements of HVA currents in cells loaded with Ni3 peptide alone (filled circles, n=10), and in cells loaded with Ni3 peptide plus 1 mM GDP-β-S (open circles, n=6). D a) Example of time series measurements in cells loaded with Ci3 (open circles) and Ni3 peptides (filled circles), showing bath administration of 5-HT (1μM) caused virtually no further inhibition. Db) Summary of the inhibition of HVA currents produced by 5-HT in cells loaded with the four synthetic peptides (**: $P<0.01$ vs. control).

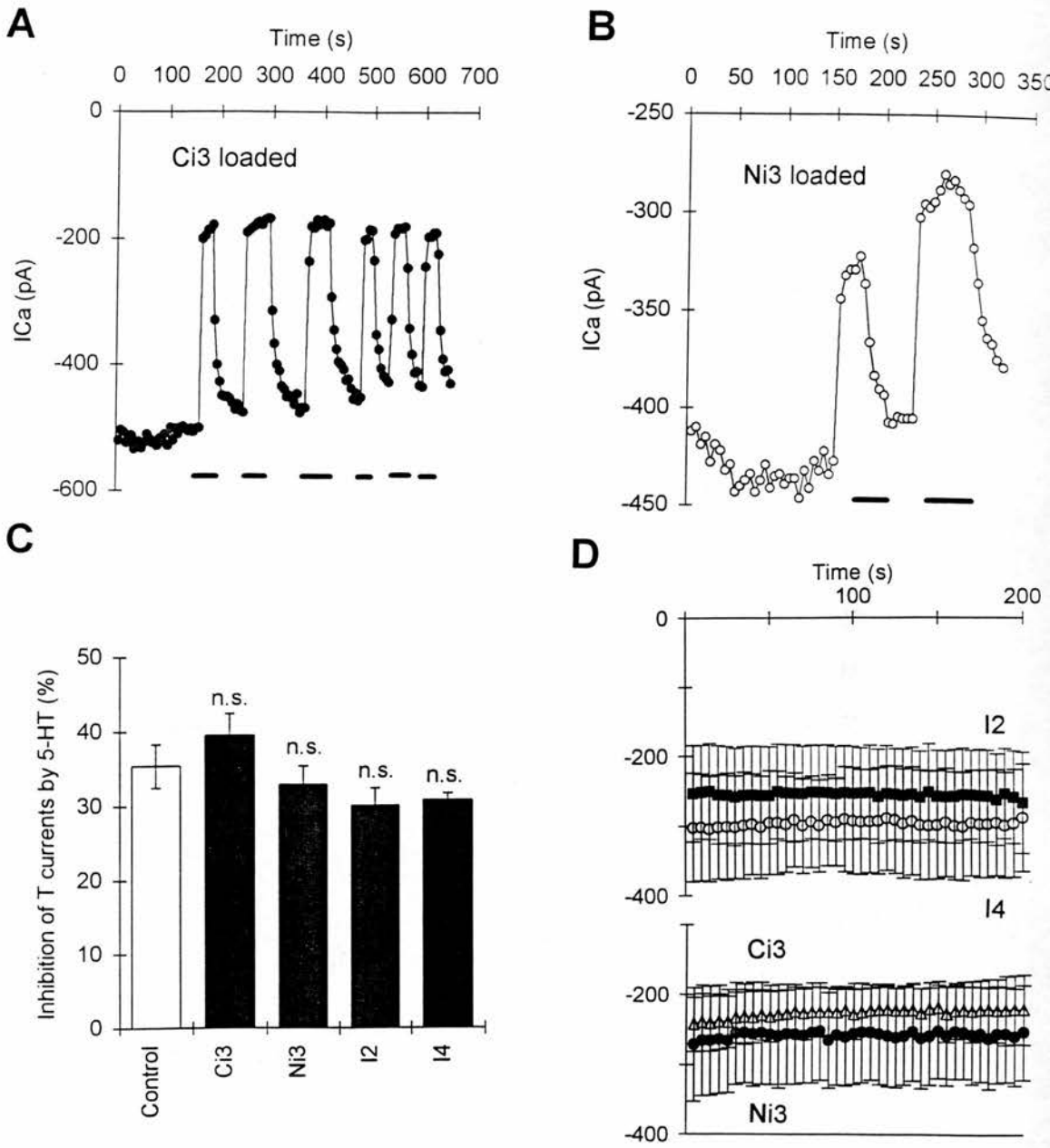

A
NH2
Extracellular
I2
Ni3
Ci3
I4
Intracellular
COOH
B
Time (s)
100
200
300
0
-1000
-2000
-3000
-4000
I4
I2
ICa (pA)
C a
Time (s)
100
200
300
0
-1000
-2000
-3000
Ci3
Ci3+ GDP-β-S
ICa (pA)
b
Time (s)
100
200
0
-1000
-2000
-3000
Ni3
Ni3 + GDP-β-S
ICa (pA)
D a
Time (s)
0
150
300
450
0
-500
-1000
-1500
-2000
5-HT
Ni3
Ci3
ICa (pA)
b
Inhibition of HVA currents by 5-HT (%)
25
20
15
10
5
0
**
**
Control
Ci3
Ni3
I2
I4

exacerbated if re-sealing and blocking of the pipette tip occurs. I therefore excluded recordings which were accompanied by changes in electrode access resistance. The amount and rate of run-down were compared between neurons loaded with control pipette solutions (with 1 mM GTP) and neurons loaded with the synthetic peptides. Both Ci3 and Ni3 peptides, but not the I2 peptide and the I4 peptide (Fig 3.7B and C), caused much bigger amount of run-down of the HVA currents as measured at 5 min after the start of recording (Fig 3.7Ca and b vs. B). The time course of the run-down could be fitted by a single exponential equation (Fig 3.7B and C). The mean time constant of run-down for cells injected with Ci3 and Ni3 peptides was significantly shorter (77.5±14.5 s in Ci3 and Ni3, n=16) than the control (184.5±47 s, n=11, $p<0.05$). However, the time constant for run down' of HVA currrents in neurons injected with the other two 5-HT receptor-derived peptides (I2 and I4 peptides) remained similar to control (197±27 s for I4, n=9, and 224±21 s for I2, n=6). To test whether the abnormal run-down of the HVA currents was mediated via G-proteins, we measured the run-down in cells loaded with the Ni3 (or the Ci3) peptides with GDP-β-S (1 mM) instead of GTP. GDP-β-S (1 mM) significantly reduced the run-down of HVA currents caused by both peptides (Fig 3.7C), and also slowed the time constant of run-down (180±41.5 s, n=12, $p<0.05$ vs. peptides only). These results suggests that both peptides activated G-proteins and mimicked the effects of 5-HT. However, unlike the effects of 5-HT, whose effects on the HVA currents in R B neurons were mediated via PTX-sensitive G-proteins, the effects of the Ni3 and Ci3 peptides on the HVA currents was not changed by preincubation with PTX overnight. The run-down of HVA currents at 5 min in neurons treated with PTX (>12 hr, 1 μg/ml) remained similar (64.9±5.2%; n=6) to that without PTX incubation (60.3±7.3%, n=8; $p>0.5$). This treatment did however, significantly reduced the effects of 5-HT on HVA currents in neurons loaded with control pipette solution (5.8±2.6% inhibition in PTX, n=6; and 16.7±3.5% inhibition in control, n=8, $p<0.05$). These receptor-derived peptides must therefore activate several types of G-proteins.

In contrast to the effects of Ci3 and Ni3 peptides on the HVA currents, none of the four synthetic peptides had any direct effect on the T-type currents (Fig 3.8D)

Figure 3.8 Effects 5-HT receptor-derived peptides on the T-type currents and their modulation by 5-HT

A) Time series measurements of T-type currents in a neurone loaded with Ci3 peptide. There was no run-down during the time series recordings and 5-HT (1 μM) still reversibly inhibited the T-type currents. B) Time series measurements of T-type currents in a neurone loaded with Ni3 peptide. There is very little run-down during the time series recordings, 5-HT (1 μM) also caused reversible inhibition on T-type currents. C) Summary of the inhibition of T-type currents by 5-HT in cells loaded with different synthetic peptides (n.s. vs. control). D) The mean time series measurements in cells loaded with different synthetic peptides (open circles, I4; filled circles, Ni3; open triangles, Ci3; filled triangles, I2). No run-down of the T current is apparent with any peptide.


A
Time (s)
0 100 200 300 400 500 600 700
0
-200
-400
-600
ICa (pA)
Ci3 loaded
B
Time (s)
0 50 100 150 200 250 300 350
-250
-300
-350
-400
-450
ICa (pA)
Ni3 loaded
C
Inhibition of T currents by 5-HT (%)
50
40
30
20
10
0
n.s.
n.s.
n.s.
n.s.
Control
Ci3
Ni3
I2
I4
D
Time (s)
100
200
0
-200
-400
I2
I4
Ci3
-200
-400
Ni3

which also did not undergo run-down during control recordings (not shown in figures). These observations together with the observations of the effects of non-hydrolyzable G-protein activators, suggest that T-type channels in R-B neurons seem to be unaffected by G-protein activation.

Effects of synthetic peptides on modulation of HVA currents and T-type currents

I next examined whether the synthetic 5-HT receptor peptides could alter the modulation of T-type and HVA currents by 5-HT. The effects of 5-HT on the T-type currents was not altered by any of these peptides (Fig 3.8). However the effect of 5-HT on the HVA currents was almost totally abolished by the Ci3 (n=8) and Ni3 (n=10) peptides derived from the third cytoplasmic loop of 5-HT receptors (Fig 3.7Da and b), but not by peptides from the other cytoplasmic regions of 5-HT receptor (I2, n=6; I4, n=9; Fig 3.7Db). Since fragments of the 3rd cytoplasmic loop mimicked and occluded the effects of 5-HT on the HVA channels but had no effect on T-type channels or their modulation by 5-HT, I suggest that receptor domains distinct from those involved in activation of G-proteins are required for modulation of T channels by 5-HT.

3.4 Discussion

3.41 GTP-insensitive T-type channel modulation

I previously found that T-type currents in *Xenopus* R-B neurons were inhibited by 5-HT_{1A} and 5-HT_{1D} receptors via a membrane-delimited pathway (Sun and Dale, 1997). Membrane-delimited inhibition of HVA channels occurs through a direct interaction between Gβγ subunits and the channels themselves (Herlitze et al., 1996; Ikeda et al., 1996; De Waard et al., 1997). Surprisingly, however, activation of G-proteins did not appear to be required for the modulation of T-type currents by 5-HT: in neurons loaded with GDP-β-S , GTP-γ-S, GMP-PNP, or AlF_4^-, the inhibition of T-type currents by 5-HT was not diminished or enhanced. This is not likely to be due to the lack of access of these GTP-

analogues, because these agents were effective in altering inhibition of HVA currents in the same cell. However, these GTP analogues act as competitive ligands at the GTP binding site on the Gα subunit and the inability of these agents to block modulation does not rule out the involvement of a novel G-protein with a very high affinity for GTP which could be activated at very low concentrations of GTP (cf. Sprang, 1997). Since we have also demonstrated strongly that the inhibition can be blocked by 5-HT_{1A} and 5-HT_{1D} receptor antagonists (Sun and Dale, 1997), this also rules out a direct effect of 5-HT on the T-type channels themselves. Thus our evidence suggests that the modulation of the T channels occurs through an unknown GTP-insensitive pathway. In another report, an opioid-like peptide nociceptin (orphanin FQ) has very similar effects to 5-HT on HVA and T-type currents (Abdulla and Smith, 1997). Nociceptin caused inhibition of HVA currents via G-protein pathways but inhibition of the T-type currents was not altered by non-hydrolyzable analogues of GTP.

3.42 Receptor-derived peptides and receptor-G protein coupling

Biochemical experiments using synthetic peptides have been increasingly used to study the receptor activation and selectivity of G-protein recognition, and the results generally agree well with studies that use chimeric or mutated receptors cf. Wess, 1997). The majority of such studies indicate that the selectivity of G-protein recognition is primarily determined by amino acids located in the I2 loop, and the amino- and carboxy- ends of the third cytoplasmic loop (Ci3 and Ni3 , cf. Hedin et al, 1993; Wess, 1997). Several laboratories have shown that peptides corresponding to the I2, Ni3 and Ci3 region (in some cases also the membrane-proximal portion of the carboxy-terminal I4 region) can mimic or inhibit receptor/G-protein interaction in a variety of receptors (cf. Savarese and Fraser, 1992; Strader et al, 1994; Zhu et al, 1997, Wess, 1997). Attempts have also been made to determine the critical amino acid sequences that determine specificity of 5-HT receptor G-protein coupling. Current evidence suggests important roles for the second loop (Varrault 1994; Lembo et al, 1997), the carboxyl end of the third intracellular loop (Varrault 1994; Oksenberg et al, 1995). However, the role

of individual amino acid residues in determining the receptor-G-protein activation is not clear.

Our findings that the Ni3 and Ci3 peptides inhibit HVA currents are therefore consistent with these general ideas. However our peptides, derived from the Ni3 and Ci3 regions of 5-HT_1 receptors, activated a variety of G-proteins in a non specific manner. Thus, if there is specificity in the interaction between receptor and G-proteins, it must reside in some other part of the receptor. Although some evidence suggests that the I2 loop is important to determine the signalling specificity of 5-HT_{1A} receptor (Lembo et al., 1997), we did not find that the I2 peptide alone had any direct effect on HVA currents or on the inhibition of HVA currents by 5-HT. However peptides derived from different regions of the receptor may need to act in a cooperatively fashion to allow specificity of signalling.

By applying these peptides via patch pipette, I have demonstrated that the activation of G-protein by these peptides caused substantial voltage-independent inhibition of the HVA currents (in some cells more than 80% inhibition of total HVA currents). Nevertheless these peptides still had no effects on the T-type current recorded in the same neurons. As this is a way of manipulating G-protein activation that is mechanistically completely distinct from the use of GTP analogues, this strongly suggests that G-proteins are not involved in modulating the T-type channels in R-B neurons. Furthermore I propose that a functional domain of the receptor that is distinct from that involved in activating G-proteins may cause inhibition of T channel either directly or through an unknown intermediate.

The effects of 5-HT receptors (except for 5-HT_3 receptor), and other G-protein-coupled receptors activation are thought to be mediated exclusively via activation of G-proteins. This was challenged by recent findings of agonist-promoted association of the β(2)-adrenergic receptor with the Na^+/H^+ exchanger regulatory factor (Hall et al, 1998). This regulatory protein binds to the β(2)-adrenergic receptor and interacts specifically with the last few residues of the carboxyl-

receptor and interacts specifically with the last few residues of the carboxyl-terminal cytoplasmic domain of the receptor (Hall et al., 1998).

3.43 The enhancement effects of non-hydrolyzable GTP analogs on the inhibition of HVA currents

Intracellular loading with GTP-γ-S, GMP-PNP, or AlF_4^- did not produce much change on the amplitude of HVA currents by itself, but did produce a great enhancement of the effects of 5-HT on the HVA currents. Furthermore, the inhibition produced by 5-HT in cells loaded with non-hydrolyzable GTP analogs was also accompanied by slowing of activation kinetics, and could be partially relieved by strong depolarizing prepulses. This is very different from the effects of 5-HT on in RB neurons loaded with GTP, where the inhibition of N and P/Q currents is pure voltage-independent (Sun & Dale, 1997). Although GTP-γ-S mediated direct and voltage-dependent inhibition of the HVA currents have been reported (Elmslie et al., 1990; Page et al., 1998), our results differ from this report in that the effects of GTP-γ-S were only produced in presence of extracellular 5-HT. The additional modulation seen when G-proteins were irreversibly activated, may result from higher local concentrations of free Gα and Gβγ subunits, which could drift in the plane of membrane further and act directly on more distant HVA channels, that would normally be out of reach under control conditions (cf. Ikeda, 1996; De Waard, et al., 1997; Herlitze, et al., 1996).

3.5 Experimental Procedures

Whole-cell patch clamp recordings

Spinal neurons were acutely isolated from *Xenopus* larvae (stage 40-42, Nieuwkoop and Faber, 1956) by methods based on those described by Dale (1991). Conventional whole-cell recordings and cell-attached recordings were made in the primary sensory neurons (Rohon-Beard, R-B neurons). Owing to their unique morphological characteristics, Rohon-Beard neurons were readily identifiable under phase contrast microscopy, based on the criteria of Dale (1991): a large spherical soma, a large nucleus and dark nucleolus. Electrodes were fabricated using a Sutter Instrument P97 puller from capillary glass obtained from World Precision Instruments (TW 150F) and Clark Electromedical Instruments (GC150F-10) and coated with Sylgard® and fire polished. A List L/M-PC amplifier together with a DT2831 interface (Data Translation) was used to record and digitize the voltage and current records. Data were acquired to the hard disk of an IBM-compatible PC , while an optical disk was used for long-term storage of experimental records. The sampling and analysis software were written by Dale (1995). The whole-cell recordings had access resistance ranging from 4 to 12 MΩ. Between 70 and 85% of this access resistance was compensated for electronically. For recording of whole-cell Ca^{2+} currents, external solutions were composed of (in mM) 57.5 Na^{+} , 57.5 TEA, 2.4 HCO_3^{-}, 3 K^{+}, 10 Ca^{2+}, 1 Mg^{2+}, 10 HEPES, 1 4 -aminopyridine(4-AP), and TTX (140 nM), pH 7.4, adjusted to 260 mosmol l^{-1}. The pipette solution contained (in mM) 100 Cs^{+}, 1 Ca^{2+}, 6 Mg^{2+}, 20 HEPES, 5 ATP and 10 EGTA, 1 GTP, pH 7.4, adjusted to 240 mosmol l^{-1}. Leak subtraction was performed on whole cell recordings by either of two methods. For one method, the current of interest was blocked (Y^{3+} 30 μM or Cd^{2+} 120 μM) and the remaining leak currents subtracted from the equivalent experimental records from the same cell. In the other method, a scaled negative version of the experimental pulse protocol was given to the same cell. This was subsequently scaled up and added to the experimental records. In both cases, the leak currents were obtained immediately before or after each set of experimental records. Drugs were applied through a multibarrelled microperfusion pipette that was positioned within 1 mm of the cell. All experiments were performed at room temperature, 18-22°C.

Cell-attached patch-clamp recordings

Unitary channel recordings (cell-attached mode) were made by methods described by Fox et al (1987a, b) and Delcour and Tsien (1993): the membrane potential outside the bath was zeroed with the following external solution: 110 mM K-aspartate , 10 mM EGTA, 10 mM HEPES, 1 mM $MgCl_2$, 10 mM glucose (pH 7.4 adjusted with KOH). The pipette solution contained 110 mM $BaCl_2$, 10 mM TEA, 5 mM 4-AP and 10 mM HEPES, pH adjusted to 7.5 with TEA-OH. Leak subtractions were made by subtracting the fitting of traces without channel openings, or given to the same cell a scaled negative version of the experimental pulse protocol, this was subsequently scaled up and added to the experimental records.

Chemicals used

5-hydroxytryptamine (5-HT, RBI), 2- [5- [3- (4-Methylsulphonylamino) benzyl-1,4-oxadiazol-5-yl]-1-H-indole-3-yl]ethylamine (L-694,247, Tocris Cookson, UK). tetrodotoxin (TTX, Sigma), ω-Agatoxin IVA (agatoxin, Alomone labs and Sigma), ω-Conotoxin-GVIA (ω-CgTx, Bachem Califonia), Nifedipine (Sigma), tetraethylammonium chloride (TEA, Aldrich), Yttrium nitrate (Y^{3+}, Sigma). 5' -Guanylyl-imidodiphosphate tetralithium (GMP-PNP, Sigma), Pertussis toxin (PTX, Sigma), Guanosine-5' -Tetraphosphate (GTP, Sigma), Guanosine-5' -o-(2-thiodi-phosphate) (GDP-β-S, Sigma), Guanosine-5' -O-(2-thiodi-phosphate) (GTP-γ-S, Sigma). 5-HT_{1A} and 5-HT_{1D} receptor peptides were derived from the conserved regions of the rat 5-HT_{1A} and 5-HT_{1D} receptors. Peptides were synthesized by the FMOC-polyamide method of Atherton, et al. (1988) and purified by reverse phase chromatography on a C18 column equilibrated with 0.1% (v/v) trifluoracetic acid. Peptides were eluted by increasing concentrations of acetonitrile and the sequence confirmed using an Applied Biosystems Procise Protein Sequencer.

Chapter 4

DIFFERENTIAL INHIBITION OF N AND P/Q CA^{2+} CURRENTS BY 5-HT_{1A} AND 5-HT_{1D} RECEPTORS IN SPINAL NEURONES OF *XENOPUS* LARVAE

4.1 Summary

1. In whole-cell patch clamp recordings made from non-sensory neurons acutely isolated from the spinal cord of *Xenopus* (stage 40-42) larvae, two forms of inhibition of the HVA Ca^{2+} currents were produced by 5-HT. One was voltage-dependent and was associated with both slowing of the activation kinetics and a shifting of the voltage-dependence of the HVA currents. This inhibition was relieved by strong depolarizing prepulses. A second form of inhibition was neither associated with slowing of the activation kinetics nor relieved by depolarizing prepulses and was thus voltage-independent.

2. In all neurons examined, 5-HT (1 μM) reversibly reduced 34 ± 1.6 % (n=102) the HVA Ca^{2+} currents. In about 40% of neurons, the inhibition was totally voltage-independent. In another 5% of neurons, the inhibition was totally voltage-dependent. In the remaining neurons, inhibition was only partially (by around 40%) relieved by a large depolarizing prepulse, suggesting that in these neurons, the inhibition consisted of voltage-dependent and -independent components.

3. By using the selective channel blockers, I found that 5-HT acts on both N- and P/Q- type channels. However, whereas the inhibition of P/Q-type currents was only voltage-independent, the inhibition of the N-type currents had both voltage-dependent and -independent components.

4. The effects of 5-HT on HVA Ca^{2+} currents were mediated by $5\text{-}HT_{1A}$ and $5\text{-}HT_{1D}$ receptors. The $5\text{-}HT_{1A}$ receptors not only preferentially cause voltage-independent inhibition, but do so by acting mainly on the ω-agatoxin-IVA sensitive Ca^{2+} channels. In contrast, the $5\text{-}HT_{1D}$ receptor produced both voltage-dependent and -independent inhibition and was preferentially coupled to ω-conotoxin-GVIA sensitive channels. This complexity of modulation may allow fine tuning of transmitter release and calcium signaling in the spinal circuitry of *Xenopus* larvae.

4.2 Introduction

Calcium influx via voltage-gated Ca^{2+} channels controls a variety of physiological functions such as neurotransmitter release, membrane excitability, and gene expression. The diversity of Ca^{2+} channels types and their differential localization is of fundamental importance in control of Ca2+ signaling. In the synaptic terminals, N- and P/Q type HVA Ca^{2+} channels play a critical role in triggering neurotransmitter release (Luebke, Dunlap & Terner, 1993; Turner, Adams & Dunlap, 1993; Wheeler, Randall & Tsien, 1994). Modulation of these Ca^{2+} channels by neurotransmitters, such as GABA, glutamate, 5-HT and noradrenaline is a widespread mechanism that contributes to presynaptic inhibition (see review by Dolphin, 1998).

Although several mechanisms may be involved, perhaps the commonest form of modulation thus far described is the G-protein-mediated, voltage-dependent inhibition. This has the following characteristics: the inhibition is always partial; it is accompanied by a slowing of current activation kinetics at moderately depolarized membrane potentials; channel activation is shifted to more depolarized voltages; a strong depolarizing prepulse restores normal fast activation kinetics, and increases the current amplitude (see review by Hille, 1994; Dolphin, 1998). The likely signal transduction pathway for such inhibition may involves a direct interaction between Ca^{2+} channel $\alpha 1$ subunits and the G protein $\beta\gamma$ subunits (Ikeda, 1996; Waard, Liu, Walker, Scott, Gurnett & Campbell, 1997; Herlitze, Garcia, Mackie, Hille, Scheuer & Catterall, 1996). This membrane-delimited pathway suggests close colocalization of G-protein-coupled receptors and their target Ca^{2+} channels.

However, other forms of modulation of Ca^{2+} channels also occur. In particular, voltage-independent inhibition can be produced either separately or in

conjunction with the well-studied voltage-dependent inhibition. In chick embryonic sensory neurons, N-type channels are inhibited by noradrenaline through both voltage-dependent and -independent mechanisms (Luebke & Dunlap, 1994; Diversé-Pierluissi, Goldsmith, & Dunlap, 1995). Voltage-dependent and -independent inhibition produced by one neurotransmitter has also been reported for several subtypes of HVA channels in various preparations (Ciranna, Feltz, & Schlichter, 1996; Albillos, Carbone, Gandia, Garcia, & Pollo, 1996, Currie & Fox, 1997; Sun & Dale,1997). Voltage-independent inhibition is characterized by the presence of similar inhibition throughout all test potentials and an incomplete ability of depolarizing prepulses to remove the inhibition (cf. Luebke & Dunlap, 1994; Sun & Dale, 1997). It remains unclear whether these two forms of modulation represent different signaling pathways or two facets of the same inhibitory mechanism.

As transmitter release depends on the square of the local Ca^{2+} concentration, reduction of Ca^{2+} entry through either the P/Q or N channels could produce a disproportionately large reduction of synaptic strength (Wheeler, et al, 1994; Luebke, et al, 1993; Turner, et al, 1993). The different types of inhibition of Ca^{2+} channels could have distinct effects on transmitter release. For example, voltage-dependent modulation may be relieved by firing of action potentials and may thus be transient in nature (cf. Brody, Patil, Mulle, Snutch & Yue, 1997). By contrast, voltage-independent inhibition cannot be relieved by spiking and is therefore tonic (cf. Luebke & Dunlap, 1994). These differences are likely to have functional consequences for transmitter release during patterned presynaptic activity.

As many as 7 classes of 5-HT receptors have been defined. Most of the 5-HT receptor subtypes are G-protein-coupled receptors and can activate multiple downstream signal transduction pathways (Hoyer, Clarke, Fozard, Hartig, Martin, Mylecharane, Saxena, & Humphrey, 1994). Surprisingly, it is mainly the 5-HT_{1A} receptor subtype that has been shown to be involved in the suppression

Ca^{2+} channels. For example, 5-HT_{1A} receptors inhibit N-type, or P/Q-type Ca^{2+} channels in mammalian motor neurons (Bayliss, et al., 1995) and other CNS neurons via a voltage-dependent membrane-delimited G-protein pathway (Pennington, Kelly, & Fox, 1991; Koike, Saito, & Matsuki, 1994; Foehring, 1996). The 5-HT_{1A} receptor has also been found to mediate voltage-independent suppression of HVA Ca^{2+} currents (Ciranna et al., 1996; Sun & Dale, 1997), but these accounts are sparse compared to the vast literature on the voltage-dependent inhibition. Wider knowledge as to whether other 5-HT receptor subtypes also inhibit Ca^{2+} channels and the mechanisms that could be involved is largely lacking.

In the present study of non-sensory spinal neurons of *Xenopus*, I have found that 5-HT mediates, via the 5-HT_{1A} and 5-HT_{1D} receptor subtypes, a mixture of voltage-dependent and -independent inhibition of the HVA currents. However the two types of channel comprising the HVA current were differentially modulated: while the P/Q channels received only voltage-independent inhibition, the N channels were inhibited by both mechanisms. In addition, the 5-HT_{1A} receptors preferentially inhibited the P/Q channels whereas the 5-HT_{1D} receptor preferentially acted on the N channels.

4.3 Methods

4.31 Preparation of the acutely isolated spinal neurons

Acutely isolated spinal neurons were prepared by methods based on those described by Dale (1991). In accordance with the UK Animals (Scientific Procedures) Act 1986, stage 40-42 *Xenopus* larvae (Nieuwkop & Faber, 1956) were anaesthetized in a solution of MS222 (0.5 mg/ml, tricaine, Sigma); pinned to a rotatable Sylgard® table in HEPES saline with the following composition (in mM): 117.4 Na^+, 3 K^+, 1 Mg^{2+}, 2 Ca^{2+}, 2 NO_3^-, 2.4 HCO_3^-, 124 Cl^-, 10 HEPES, and 10 Glucose at a pH 7.4. Their spinal cords were carefully removed,

transferred to a dish containing 0.1-0.3 mg/ml DNase in HEPES saline and incubated at room temperature for 3 minutes. After this, the spinal cords were placed in a dish containing 8 mg/ml pronase (Sigma) in a low chloride trituration saline, composed of (in mM): 117.4 Na^+, 115 $MeSO_3^-$, 3 K^+, 1 Mg^{2+}, 2 Ca^{2+}, 2 NO_3^-, 2.4 HCO_3^-, 9 Cl^-, 10 HEPES, and 10 Glucose at pH 7.4, and incubated at room temperature for 2 minutes. They were then transferred to a dish of dissociation saline, composed of (in mM): 115 Na^+, 115 $MeSO_3^-$, 3 K^+, 2 EDTA, 3 Cl^-, 10 HEPES, 10 Glucose, and 10 piperazine-N, N' -bis (2-ethanesulphonic acid, PIPES) at pH 7.0 for 1 minute, and then to a dish of saline , containing (in mM): , 115 Na^+, 115 $MeSO_3^-$, 3 K^+, 0.1 Mg^{2+}, 0.1 Ca^{2+}, 3 Cl^-, 20 Glucose, and 10 PIPES, at pH 7.0 for 3 minutes. The spinal cords were then gently triturated in trituration saline containing 3 mg/ml DNase in a microfuge tube until the cords had dissociated. The cells were finally transferred to 35 mm poly-D-lysine-coated dishes in HEPES saline and allowed to settle and stick to the substrate for at least 1 hour before recording.

4.32 Whole-cell patch clamp recordings

Conventional whole-cell patch clamp recordings were made. Electrodes were fabricated from capillary glass obtained from World Precision Instruments (TK 150F) using a Sutter Instrument P97 puller. For the recording of tail currents, the electrodes were coated with Sylgard® and fire polished. A List L/M-PC amplifier together with a DT2831 interface (Data Translation) was used to record and digitize the voltage and current records. Data were acquired to the hard disk of an IBM-compatible PC, while an optical disk was used for long-term storage of experimental records. The sampling and analysis software were written by Dale (1995). The whole-cell recordings had access resistance ranging from 4 to 12 MΩ. Between 70 and 85% of this access resistance was compensated for electronically. For recording Ca^{2+} currents, external solutions were composed of (in mM) 57.5 Na^+, 57.5 TEA, 2.4 HCO_3^- , 3 K^+, 10 Ca^{2+}, 1 Mg^{2+}, 10 HEPES, 1 4 - aminopyridine(4-AP) and TTX (140 nM), pH 7.4, osmolarity adjusted to 260 mosmol l^{-1}. The pipette solution contained (in mM) 100 Cs^+, 1 Ca^{2+}, 6 Mg^{2+}, 20 HEPES, 5 ATP, 1 GTP (in some earlier experiments, GTP was omitted from the

pipette but this had no effect on the actions of 5-HT) and 10 EGTA, pH 7.4, osmolarity adjusted to 240 mosmol l^{-1}. Leak subtraction was performed on whole cell recordings by the following method: a scaled negative version of the experimental pulse protocol was given to the same cell. The leak currents were obtained immediately before or after each set of experimental records and subsequently scaled up and added to the experimental records. Drugs were applied through a multibarrelled microperfusion pipette that was positioned within 1 mm of the cell. All experiments were performed at room temperature, 18-22°C. Experiments using nifedipine were carried out in the dark.

The identification of the acutely isolated stage 40-42 *Xenopus* spinal neurons was similar to that described by Dale (1991) for stage 37/38, The more mature neurons had smaller diameters and lower cell capacitance (3.2±0.5 pF). Recordings were not made from the large and readily identifiable primary sensory Rohon-Beard neurons. Instead, 90% of recordings were made from unipolar cells, which in the embryo have a 75% chance of being glycinergic reciprocal inhibitory interneurones from the locomotion central pattern generator CPG (Dale, 1991). 10% of the neurons recorded were multipolar neurons, which in the embryo are also CPG neurons: mostly motorneurones and excitatory interneurones. Although most dissociations were made from stage 42 *Xenopus*, some were made from stage 41 and 40. The results from neurons of these earlier larvae were very similar to those of stage 42 neurons.

4.33 Chemicals used

Serotonin agonists and antagonists: 5-hydroxytryptamine (5-HT, RBI), N- [4-methoxy-3- (4-methyl-1-piperazinyl) phenyl] -2' -methyl-4' - (5-methyl-1, 2, 4-oxadiozol-3-yl) [1, 1-biphenyl] -4-carboxamide (GR127935, GLAXO Wellcome Research and Development Ltd.), R (+) -8-OH-DPAT (DPAT, RBI), ketanserin tartrate(RBI), 2- [5- [3- (4-Methylsulphonylamino) benzyl-1, 4-oxadiazol-5-yl] -1 H-indole-3-yl]ethylamine (L-694,247, Tocris Cookson), 1- (2-Methoxyphenyl)-4-[4-(2-phthalimido) butyl]piperazine (NAN-190, RBI), 4- [Iodo-N- [2- [4-(methoxyphenyl) -1-piperazinyl]ethyl] -N-2-pyridinyl-benzamide (*p*-MPPI,

RBI), N-methyl-clozapine (RBI). L-694,247, clozapine, and NAN-190 were initially dissolved in a few drops of dimethyl sulphoxide and stored in Freezer.

***Ion channel blockers*:** ω-Agatoxin-IVA and ω-Agatoxin-TK (agatoxin, Alomone labs), ω-Conotoxin GVIA (ω-CgTx, Bachem Califonia), ω-Conotoxin MVIIC (Alomone labs), Nifedipine (Sigma), tetraethylammonium chloride (TEA, Aldrich), Tetrodotoxin (TTX, Sigma).

4.34 Fitting

The Levenberg-Marquardt algorithm was used to fit Boltzmann equations. This gave the best fitting parameters and their standard errors. For all other fitting procedures, the simplex algorithm was used.

4.35 Statistics

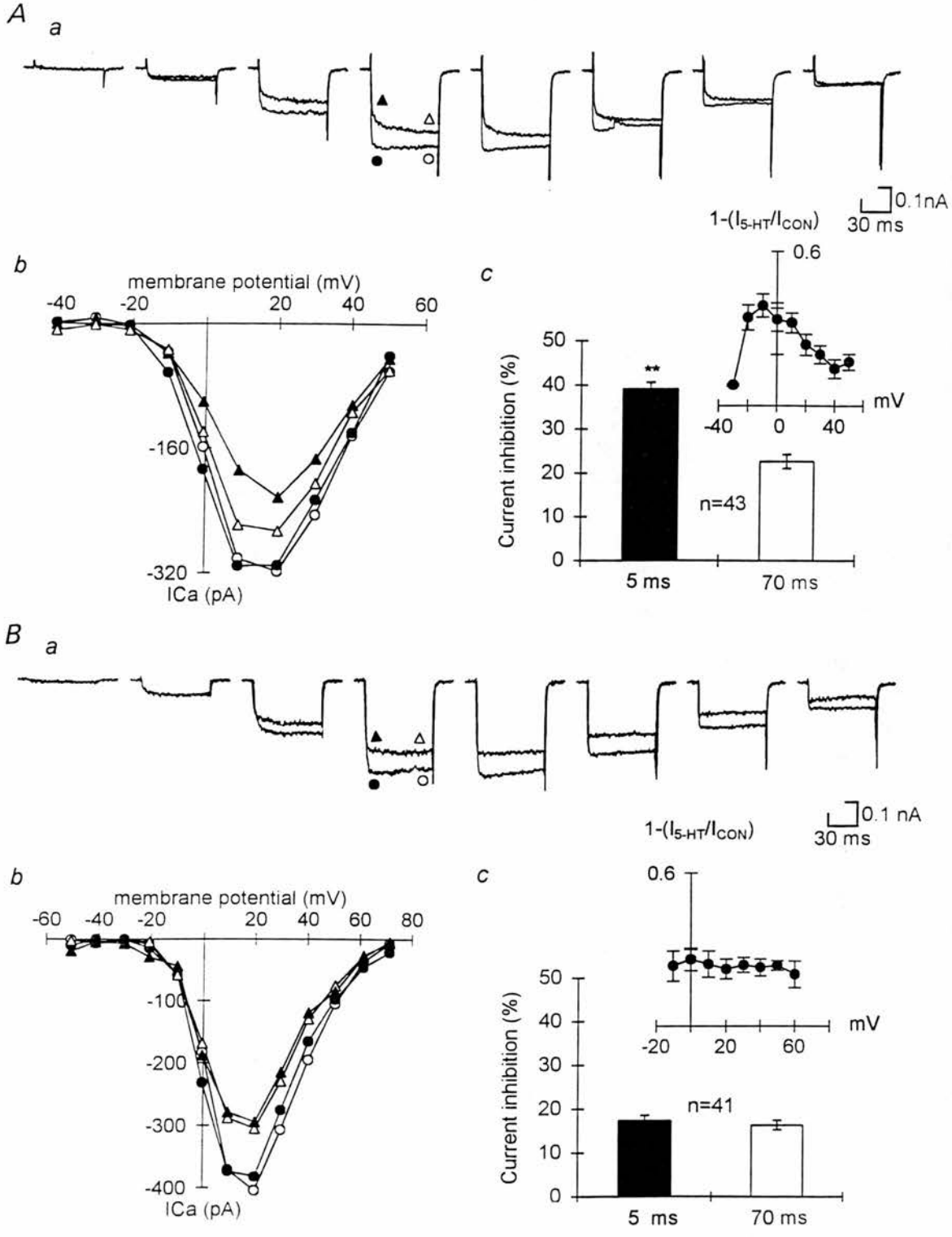
All data presented as means ± S.E.M. unless otherwise stated. Analysis by Student' s t-test was performed for paired and unpaired observations. Differences in frequency of occurrence were assessed by using 2×2 contingency table and χ^2 parameter. P values of less than 0.05 were considered statistically significant.

4.4 Results

In 78% (102/130) of the acutely isolated *Xenopus* spinal neurons, 5-HT reversibly reduced the HVA Ca^{2+} currents with a mean inhibition of 34 ±1.6% (range 20-90% n=102, at a membrane potential of +10 mV). Of neurons that responded to 5-HT, 90% (90/102) were unipolar, which at stage 37/38 are highly

Figure 4.1 Inhibition of the HVA Ca^{2+} currents by 5-HT can be through ' kinetic-slowing or steady-state mechanisms

A a) HVA Ca^{2+} currents elicited by steps of +10 mV from holding potential of -50 mV in control (circle) and 1 μM 5-HT (triangle), showing a ' kinetic-slowing' of activation. b) Effect of 5-HT on current-voltage relation of the HVA Ca^{2+} currents in the same cell, symbols correspond to measurements in (a), showing that the inhibition 5 ms after start of the test pulse (filled triangle) is greater than at 70 ms (open triangle). c) The mean inhibition of HVA Ca^{2+} currents measured at 5 ms is significantly greater than at 70 ms in 43 neurones that showed similar ' kinetic-slowing' (**: $p<0.01$ vs. 70 ms). The inset shows the variation of inhibition (measured at 5 ms of test pulse) with membrane potential. B a) HVA Ca^{2+} currents were elicited by steps of +10 mV from a holding potential of -50 mV in control (circle) and 1 μM 5-HT (triangle). The reduction of HVA currents was not accompanied by ' kinetic-slowing' of activation. b) Effect of 5-HT on current-voltage relation of the HVA Ca^{2+} currents, symbols correspond to measurements in (a). c)The mean inhibition of HVA Ca^{2+} currents measure at 5 ms and 70 ms of the test pulse is not significantly different (n=41). The inhibition of Ca^{2+} currents (measured at 5 ms of test pulse) was similar at different membrane potentials in these neurones (inset); error bars represent standard errors in this and all figures unless otherwise stated.


A
a
0.1nA
30 ms
1-(I5-HT/ICON)
b
membrane potential (mV)
-40
-20
20
40
60
-160
-320
ICa (pA)
c
0.6
mV
-40
0
40
50
40
30
20
10
0
Current inhibition (%)
**
n=43
5 ms
70 ms
B
a
0.1 nA
30 ms
1-(I5-HT/ICON)
b
membrane potential (mV)
-60
-40
-20
20
40
60
80
-100
-200
-300
-400
ICa (pA)
c
0.6
mV
-20
20
60
50
40
30
20
10
0
Current inhibition (%)
n=41
5 ms
70 ms

likely to be glycingergic commissural interneurons (Dale, 1991), the remaining neurons were multipolar.

From a negative holding potential (-90 mV), T-type (LVA) Ca^{2+} currents were elicited by test potentials of -40 mV with an average current density of 24.3 ±0.6 pA/pF (n=10). However, 5-HT had no effect on the T-type Ca^{2+} currents. This contrasts with *Xenopus* sensory Rohon-Beard neurons, where 5-HT inhibits the T-type Ca^{2+} currents via a membrane-delimited pathway. (Sun & Dale, 1997).

In 60% (61/102) of the neurons that responded to 5-HT, the time course of activation was clearly biphasic. The inhibition measured at 5 ms after the start of the test pulse was much stronger than that measured at 70 ms (at a test potential of +10 mV, Fig 4.1Ab & c). I define this type of inhibition as involving 'kinetic-slowing' (cf. Luebke & Dunlap, 1994; Hille, 1994; Dolphin, 1998). The mean inhibition varied with membrane potential and had a bell-shaped relation when plotted against membrane voltage (Fig 4.1 Ac inset). This suggests that the inhibition of HVA currents was at least partly voltage-dependent in these neurons.

In the remaining neurons (40%, 41/102), the inhibition of Ca^{2+} currents did not show the 'kinetic-slowing'. Instead, the inhibition was achieved through a 'steady-state' mechanism (Luebke & Dunlap, 1994). In these neurons, the inhibition did not subside during a test pulse (Fig 4.1Ba), and the amount of inhibition was similar at all test potentials (Fig 4.1 Bc inset). This suggests that the 'steady-state' inhibition was not voltage-dependent.

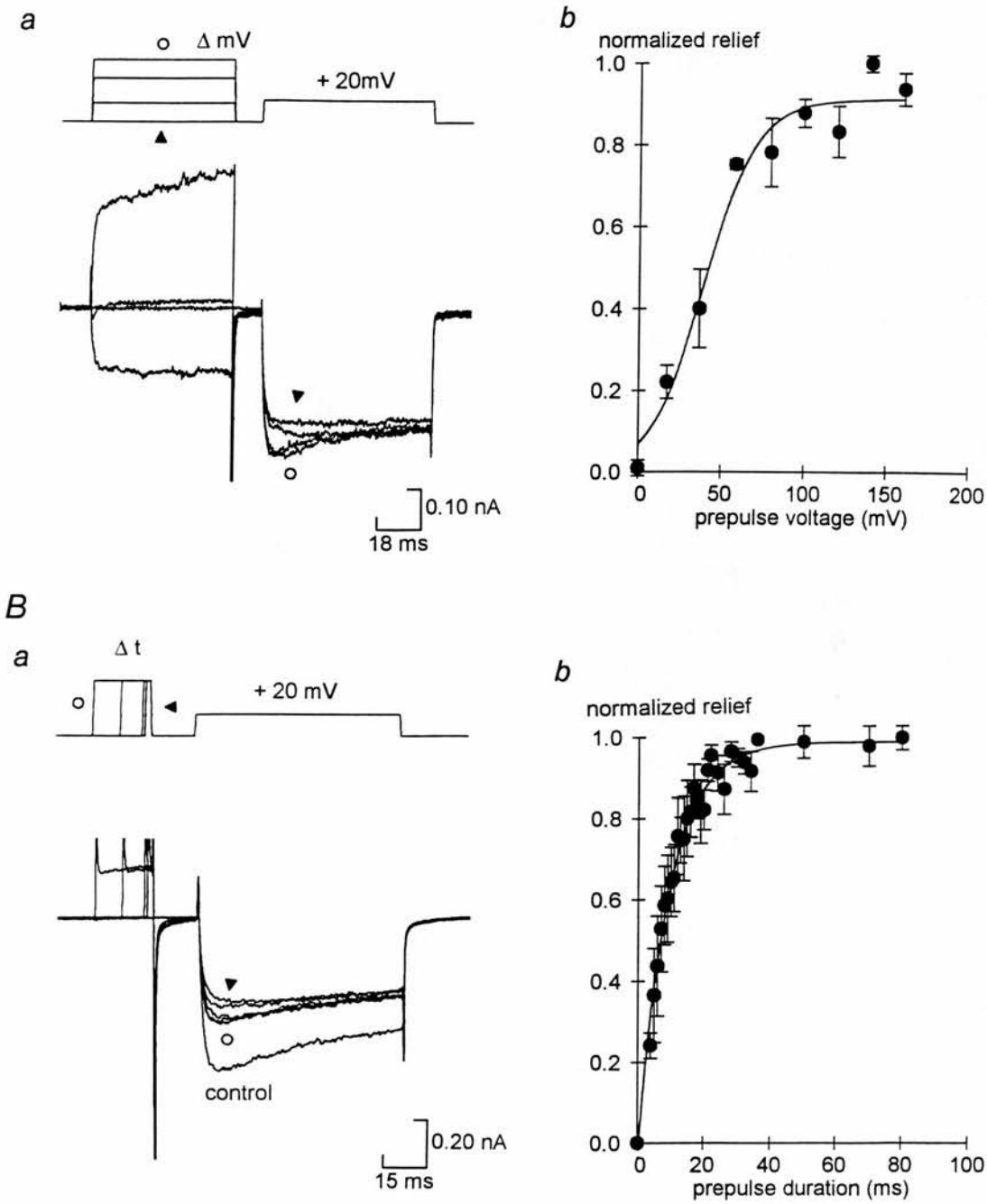
4.41 Relief of voltage-dependent inhibition by strong depolarizing prepulse

In many cases, the voltage-dependent inhibition of Ca^{2+} currents can be almost completely removed by a very positive prepulse applied immediately before the test pulse (Bean, 1989; Penington et al., 1991; Ikeda, 1996; Herlitz et al., 1996; Waard et al., 1996; see Hille, 1994 & Dolphin, 1998 for review); but in other cases, there is only a partial relief of the ' voltage-dependent inhibition' by large depolarizing prepulses (Luebke & Dunlap, 1994; Diversé-Pierluissi, et al., 1995; Bayliss, et al., 1995; Ciranna, et al., 1996; Albillos, et al., 1996; Currie & Fox, 1997).

To determine the optimal voltage and width of prepulse to use in *Xenopus* spinal neurons, I examined the effects of step amplitude and duration on the relief of the inhibition of HVA currents by 5-HT. By using a long (> 60 ms) prepulse step immediately before test pulse to + 20 mV (allowing tail currents of prepulse to decay completely), I found that prepulse steps to + 50 mV produced 60% of maximum relief, whereas depolarizing to around + 100 mV almost produced maximum relief (Fig 4.2 A). The relation between relative relief of inhibition and prepulse voltage was fitted with the Boltzmann equation $I/I_{max}= \{1+\exp[(V+V_{1/2})/k]\}^{-1}$, where $V_{1/2}$= -40.5 ±10.6 mV, k= 13.7 ± 4.5 (n=6). I next tested the effect of varying the duration of the prepulse (to + 120 mV) on the relief of inhibition by 5-HT. Increasing the prepulse duration from 1 to 20 ms dramatically increased the relief of inhibition by 5-HT. The graph of the normalized relief versus prepulse duration was fitted with a single exponential equation. This gave a mean time constant for relief of block of 9.1± 1.1 ms. A prepulse of 30 ms gave > 90% of maximum relief.

Figure 4.2 Time and voltage-dependence of relief by depolarizing prepulse of inhibition of Ca^{2+} currents by 5-HT

A, The voltage-dependence of relief of inhibition by prepulses. a) Voltage-protocols used (top) and the resulting relief of inhibition by 10 μM 5-HT at different prepulse voltages. b) The voltage-dependence of relief of inhibition was fitted with Boltzmann relation. B, Effect of prepulse duration on relief of voltage-dependent inhibition. a) Voltage-protocols used (top); HVA Ca^{2+} currents in 10 μM 5-HT. b) The time-dependent relief of inhibition was fitted with single exponential equation: $I/Imax = 1 - \exp(-x/\tau)$, where $\tau = 9.2 \pm 1.1$ ms (n=5).


A
a
Δ mV
+ 20mV
0.10 nA
18 ms
b
normalized relief
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
prepulse voltage (mV)
B
a
Δ t
+ 20 mV
control
0.20 nA
15 ms
b
normalized relief
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
prepulse duration (ms)

I next examined the proportion of relief produced by depolarizing prepulses to +120 mV (duration 60 ms, n=40). In 60% (24/40) of neurons (group A), the inhibition of HVA currents produced by 5-HT (10 μM) was accompanied by a 'kinetic-slowing' with mean inhibition of 44.2 ±1.3 % at a membrane potential of +20 mV. When the conditioning prepulse was applied, the 'kinetic slowing' was removed; however, a large amount of 'steady-state' inhibition (27.6 ±1.5%, n=24) remained (Fig 4.3 A left). This means that only 39% of the inhibition was voltage-dependent while the remainder was not relieved and was presumably voltage-independent. In the other 40% (16/40) of neurons examined (group B), inhibition of HVA Ca^{2+} currents by 5-HT was not accompanied by 'kinetic-slowing'. The amount of inhibition in these neurons was significantly less than those with a 'kinetic slowing' (mean 23.3 ± 2.4%, $p<0.01$ vs. group A). Furthermore, the prepulse did not even partially relieve the inhibition by 5-HT (mean inhibition following prepulses 26.1 ± 1.5%). The inhibition of HVA Ca^{2+} currents in these neurons thus occurred only via voltage-independent mechanisms. The amount of total inhibition in group B was similar to the 'steady-state' inhibition that was not relieved by the prepulse in group A (Fig 4.2 B), raising the possibility that the steady state inhibition in the two groups was the same phenomenon.

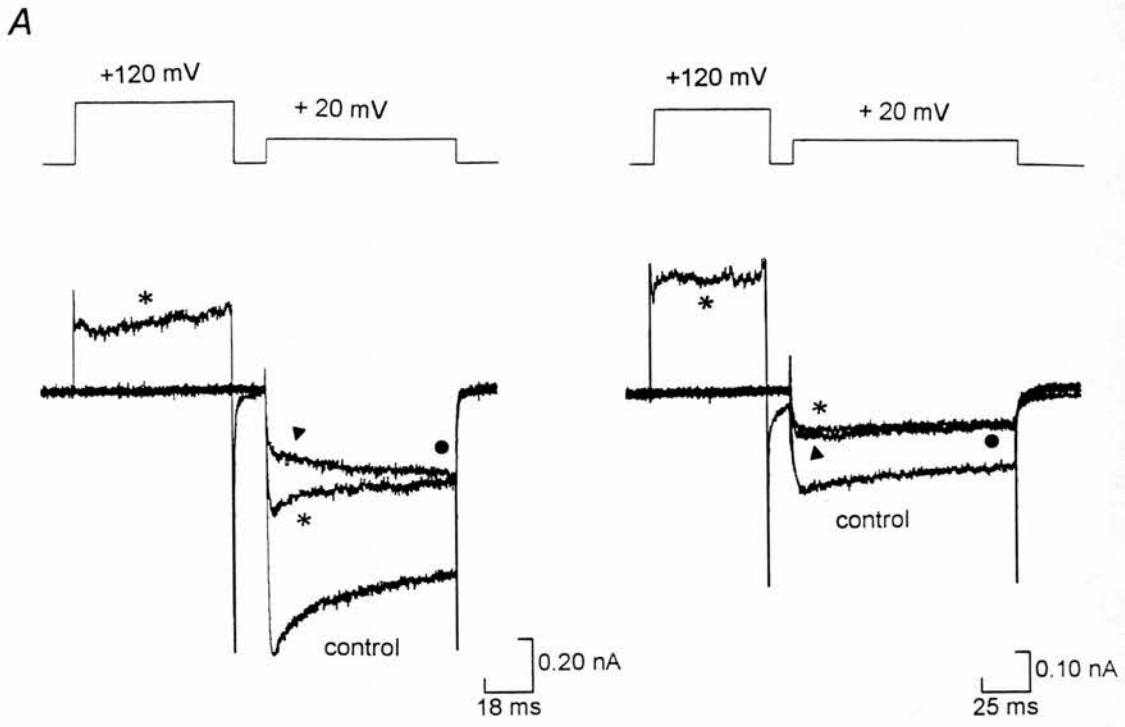
Since the voltage-dependent and independent inhibition could be dissociated and were seen either separately or in combination, I suggest that these two forms of inhibition are mechanistically separate. The steady-state inhibition is thus unlikely to be an irreversible part of the voltage-dependent inhibition.

4.42 Two different mechanisms of modulation involved

I next examined the effects of 5-HT and specific agonists on tail currents elicited by series of test pulses. When inhibition of HVA currents was evolved by either 5-HT or L-694,247, a 5-HT_{1D} agonist, the mid point of the voltage-dependent activation of the Ca^{2+} conductance was shifted to more positive

Figure 4.3 The inhibition of HVA Ca^{2+} currents by 5-HT was mediated by voltage-dependent and -independent mechanisms

A Left) In neurones which showed both ' kinetic slowing' (see triangle) and ' steady-state' inhibition (see filled circle), a prepulse to +120 mV (asterisk) only relieved the ' kinetic slowing' but not the ' steady state' inhibition. Right) In another neurone inhibition of HVA currents was not accompanied by ' kinetic-slowing' (compare current at triangle with previous record), and a prepulse to + 120 mV (asterisk) did not relieve the inhibition. B, Summary of relief of voltage-dependent inhibition of HVA Ca^{2+} currents by prepulse. In those neurones (n=24), that showed ' kinetic-slowing', around 40% of the inhibition was relieved by a prepulse to +120 mV; whereas in other neurones (n=16), the prepulse did not reduce the inhibition. (**: $p<0.01$).


A
+120 mV
+ 20 mV
control
0.20 nA
18 ms
+120 mV
+ 20 mV
control
0.10 nA
25 ms

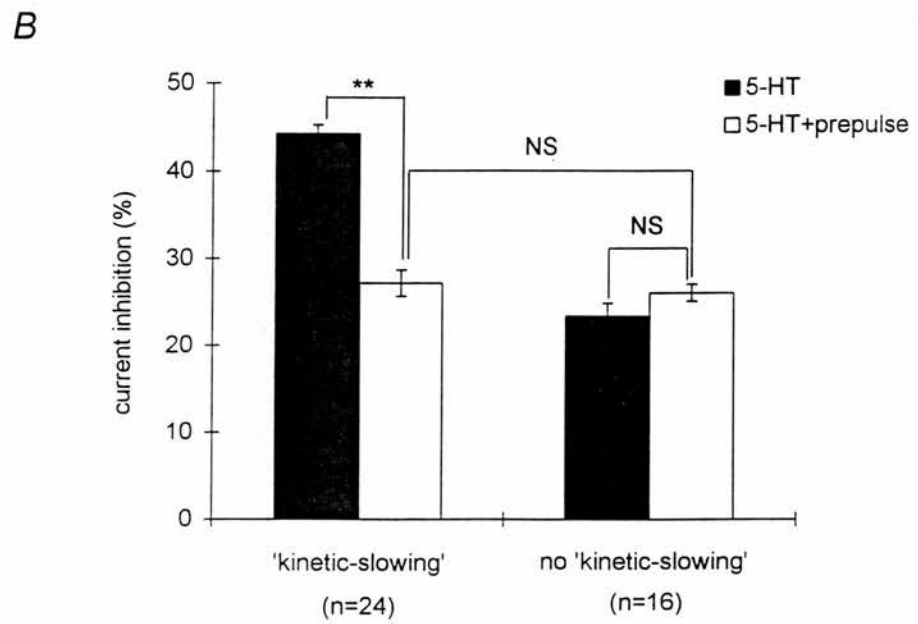

B
5-HT
5-HT+prepulse
current inhibition (%)
50
40
30
20
10
0
**
NS
NS
'kinetic-slowing'
(n=24)
no 'kinetic-slowing'
(n=16)

Figure 4.4 Voltage-dependent inhibition of HVA Ca^{2+} currents

A Top) HVA Ca^{2+} currents elicited by steps of +10 mV from holding potential of -50 mV in control (a) and 1 μM 5-HT_{1D} agonist, L-694,247(b) showing a ' kinetic-slowing' of activation. Bottom) HVA currents elicited in the same neurone by same steps in L-694,247 with (c) and without (b) prepulse (asterisk showing currents elicited by prepulse). B) An expanded recording from the same neurone showing that the suppression of HVA tail currents (solid arrow) by L-694,247 was relieved by prepulse (open arrow). C) The tail currents elicited by voltage-steps were fitted with Boltzmann relation, $I = A\{1+ \exp[(V+V_{1/2})/k]^{-1}\}$, where $V_{1/2}$= -8.2 mV , k= 8.1 for control (open circle). 1 μM L-694,247 (filled circle) reduced the slope of activation (k= 11.5) and shifted the half activation voltage to more positive value ($V_{1/2}$= -15.6 mV), however, this slowing and shifting of activation was totally reversed by prepulse ($V_{1/2}$= -7.6 mV , k= 7.9, filled triangle).

Voltage-dependent inhibition

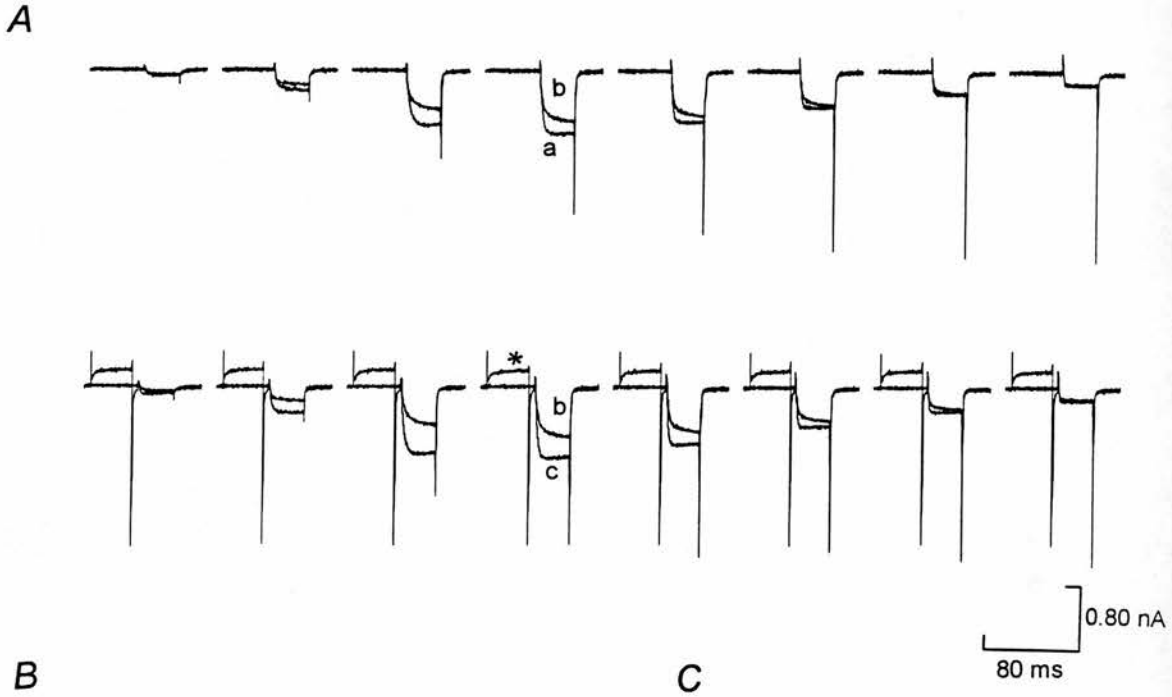
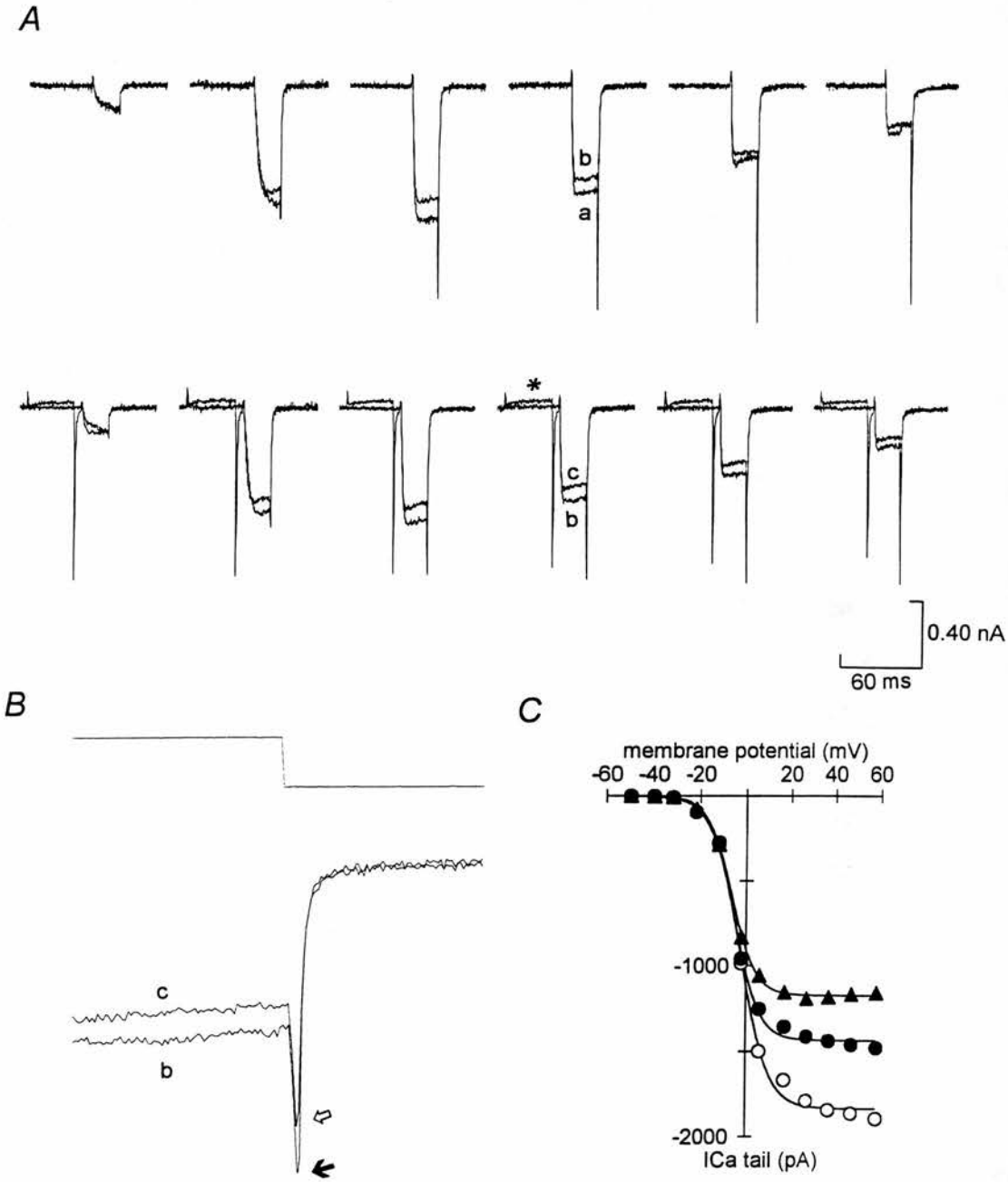


Figure 4.5 Voltage-independent inhibition of HVA Ca^{2+} currents

A Top) HVA Ca^{2+} currents elicited by steps of +10 mV from holding potential of -50 mV in control (a) and 1 μM 5-HT_{1A} agonist, 8-OH -DPAT (b) The was no ' kinetic-slowing' . Bottom) HVA currents elicited in the same neurone by same steps in 8-OH-DPAT with (c) and without (b) a prepulse (asterisk showing currents elicited by prepulse). B) An expanded recording showing the suppression of HVA tail currents (solid arrow) by 8-OH-DPAT was not relieved by prepulse (open arrow). C) The tail currents elicited by voltage-steps were fitted with Boltzmann relation, $I = A\{1+ \exp[(V+V_{1/2})/k]^{-1}\}$, , where $V_{1/2}$= 2.6 mV , k= 6.1 for control (open circle). 1 μM 8-OH-DPAT (filled circle) did not reduce the slope of activation (k= 5.6) and also did not change the half activation voltage ($V_{1/2}$= 4.1 mV). The prepulse reduced the tail currents further (filled triangle), but had no effect on kinetic parameters ($V_{1/2}$= 4.9 mV , k= 5.4).

Voltage-independent inhibition



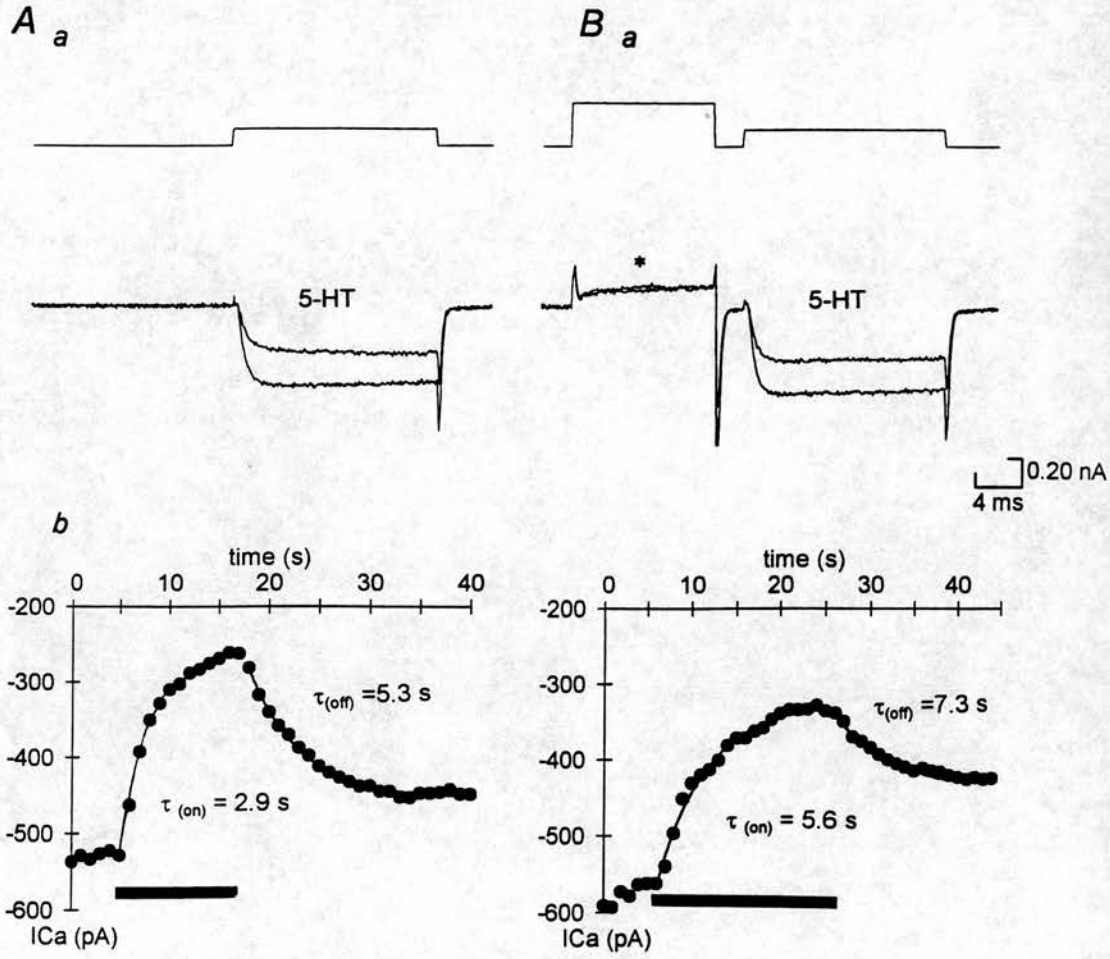
potentials (-2.5 ± 0.9 mV in control , vs. 5.4 ± 1.9 in 5-HT agonists, n=6, $p<0.05$) The inhibition also made the voltage-dependence of activation in the Boltzmann relation less steep than recordings in control (k= 7.2 ± 1.2 in control vs. k= 12.6 ± 1.4 in 5-HT agonists, n=6, $p<0.05$; Fig 4.4 C). In some neurons (eg. Fig 4.4 C), the maximum whole cell conductance was not changed by 5-HT or L-694,247, indicating that the inhibition was almost totally reversed at the larger depolarizing test potentials. In these same neurons, the change in the kinetics of HVA channel activation was also totally reversed by a depolarizing prepulse (Fig 4.4 C, n=4).

In other neurons where inhibition was produced by either 5-HT or the 5-HT_{1A} agonist, 8-OH-DPAT, the tail currents were reduced in a manner that did not change either the slope of voltage-dependence of activation of the Ca^{2+} conductance (Fig 4.5 C, k= 6.2± 1.7 in control, vs. k= 5.9± 2.3 for 8-OH-DPAT; n=5) or shift the voltage of half-activation (e.g. Fig 4.5C). Also unlike the voltage-dependent inhibition, the maximum conductance was reduced in 4 out 5 cells (e.g. Fig 4.5C). Prepulses did not reverse the reduction of tail currents, but in some neurons, the tail currents and maximum conductance were even further reduced (Fig 4.5 B & C). Nevertheless, the prepulse had no effect on the slope of voltage-dependence of activation (n=3). This suggests that in those neurons where suppression of HVA currents was not associated with ' kinetic-slowing', the reduction of HVA currents was not achieved by a change in the voltage-dependence of channel activation, but instead occurred through a less well described mechanism that involves reducing the maximum conductance of the whole-cell Ca^{2+} currents.

Since prepulse experiments suggested that two different biophysical mechanisms may contribute to the serotonergic inhibition of HVA currents, I examined whether the voltage-dependent and -independent mechanisms occurring in the same neurons could be distinguished on the basis of their rate of onset and recovery during wash. Tests were performed in neurons where the inhibition was

Figure 4.6 Rate of onset and recovery of 5-HT differs between voltage-dependent and -independent inhibition

A a) Calcium currents evoked at an interval of 1s with test pulse to + 20 mV to monitor the rate of onset of and recovery from inhibition of Ca^{2+} currents produced by 5-HT (10 μM). b) Onset and recovery time constant (τ_{on} , τ_{off}) were obtained by fitting single exponential equations to the relevant points. B) Calcium currents evoked by same protocol but with a prepulse to +120 mV. Recordings were made from the same neurone.


A a
B a
5-HT
5-HT
*
0.20 nA
4 ms
b
time (s)
0
10
20
30
40
-200
-300
-400
-500
-600
τ(off) =5.3 s
τ (on) = 2.9 s
ICa (pA)
time (s)
0
10
20
30
40
-200
-300
-400
-500
-600
τ(off) =7.3 s
τ (on) = 5.6 s
ICa (pA)

partially relieved by a prepulse. In presence of the prepulse, both the rates of onset and recovery of the inhibition of HVA currents by 5-HT were slower than without the prepulse (Fig 4.6 A, $\tau_{onset} = 5.2 \pm 0.3$ vs. $\tau_{onset} = 2.9 \pm 0.4$, $p<0.05$; $\tau_{recovery} = 5.5 \pm 1.5$ vs. $\tau_{recovery} = 3.5 \pm 0.9$, $p<0.1$, $n=4$). These differences in the time course of response suggest that two different signal transduction pathways may underlie the voltage-dependent and voltage-independent inhibition in the same neuron.

4.43 Activation of 5-HT_{1A} and 5-HT_{1D} receptors produced differential inhibition of HVA currents

In the primary sensory (Rohon-Beard) neurons of *Xenopus* larvae, I previously demonstrated the existence of 5-HT_{1A} and 5-HT_{1D} receptors, that mediated voltage-independent inhibition of HVA currents (Sun & Dale, 1997). In this earlier study, I demonstrated that 5-HT_{1A} and 5-HT_{1D} receptors in *Xenopus* could be selectively activated by 8-OH-DPAT and L-694,247, respectively, and selectively blocked by NAN-190 (or p-MPPI) and GR127935, respectively. Furthermore a receptor with high amino-acid similarity to the mammalian 5-HT_{1A} receptor has been cloned from *Xenopus* (Marracci , Cini & Nardi, 1997). Although 5-HT_{1D} receptors have not been cloned from *Xenopus,* the pharmacological properties of this receptor are very similar to those in mammals (Sun & Dale, 1997). In addition, the 5-HT_{1B} agonist, CGS-12066B had no effects on HVA currents (Sun & Dale, 1997). I therefore examined whether these same two receptors may be differentially involved in the inhibition of HVA currents in non-sensory neurons.

At a test potential of +10 mV, 62% (29/46) of the neurons tested with the selective 5-HT_{1D} agonist , L-694,247 (Beer, Stanton, Bevan, Heald, Reeve, Street, & Matassa, 1993), exhibited inhibition of Ca^{2+} currents that was

Figure 4.7 Activation of 5-HT_{1A} and 5-HT_{1D} receptors produced differential inhibition of HVA currents

A, Effects of selective 5-HT_{1A} and 5-HT_{1D} agonists on Ca^{2+} currents. a) Ca^{2+} currents elicited by steps to +10 mV from holding potential of -50 mV in control (not labeled), 5-HT (1 μM), 8-OH-DPAT (DPAT, 100 nM, 5-HT_{1A} agonist), and L-694,247 (100 nM, 5-HT_{1D} agonist). b) Time series measurements in the same cell (symbols correspond to measurements at times indicated in a). Inhibition by 5-HT and L-694,247 was accompanied by voltage-dependent slowing of activation (current inhibition at 5 ms is greater than at 70 ms). The inhibition by 8-OH-DPAT was not accompanied by kinetic-slowing (inhibition at 5 ms is same as 70 ms). c) Summary of the inhibition of HVA Ca^{2+} currents by 5-HT (1 μM), L-694,247 (1 μM) and 8-OH-DPAT (1 μM). Filled bars represent mean inhibition of Ca^{2+} currents by agonists, open bars represent percentage of cells where inhibition was accompanied by voltage-dependent slowing of kinetics (**: $p<0.01$ vs. DPAT examined by χ^2 test). B a) HVA Ca^{2+} currents elicited by steps to +20 mV from holding potential of - 50 mV, in control (not labeled) and 100 nM L-694,247. A prepulse to +120 mV (asterisk) partially relieved the inhibition by L-694,247. b) However, the suppression of HVA currents by 8-OH-DPAT, was not relieved by prepulse (asterisk). c) Summary showing the inhibition by L-694,247 was significantly ($p<0.05$, n=8) reduced by applying prepulse, whereas the inhibition by 8-OH -DPAT remain unchanged (n=9).

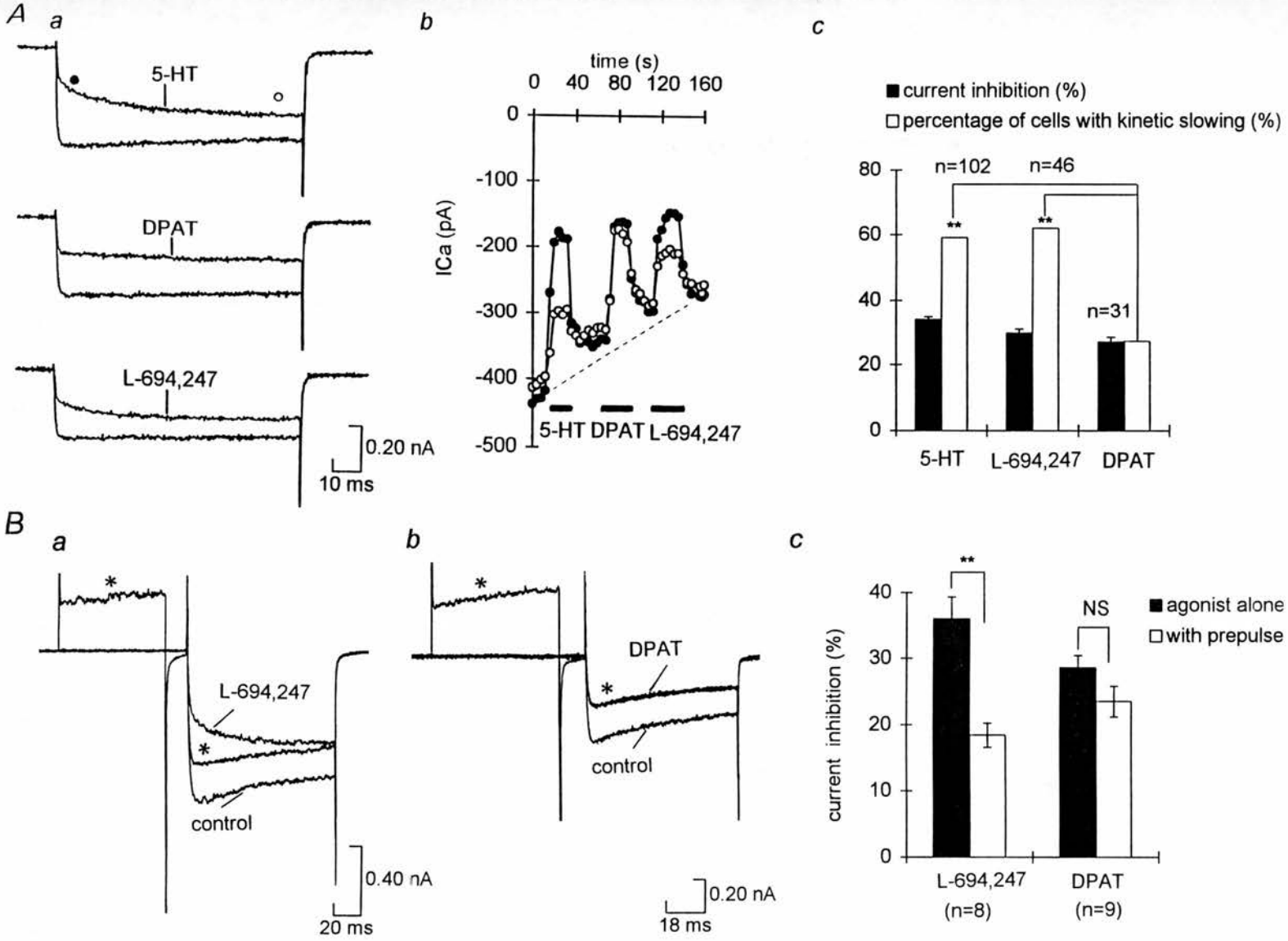
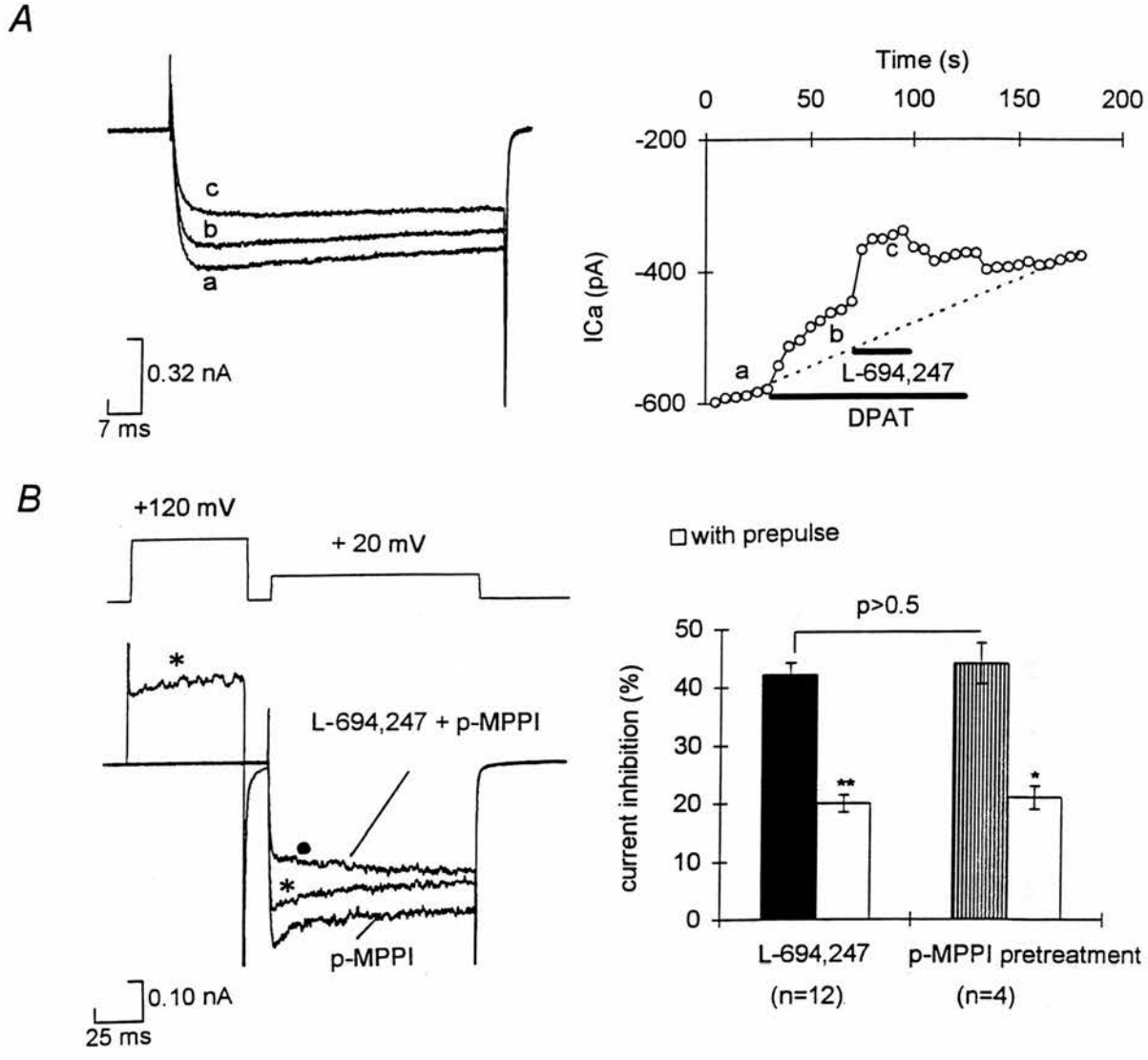

A
a
5-HT
DPAT
L-694,247
0.20 nA
10 ms
b
time (s)
0 40 80 120 160
0
-100
-200
-300
-400
-500
ICa (pA)
5-HT DPAT L-694,247
c
current inhibition (%)
percentage of cells with kinetic slowing (%)
n=102
n=46
n=31
**
**
80
60
40
20
0
5-HT
L-694,247
DPAT
B
a
*
L-694,247
*
control
0.40 nA
20 ms
b
*
DPAT
*
control
0.20 nA
18 ms
c
**
NS
agonist alone
with prepulse
current inhibition (%)
40
30
20
10
0
L-694,247
(n=8)
DPAT
(n=9)

Figure 4.8 L-694,247 and 8-OH-DPAT act through 5-HT_{1D} and 5-HT_{1A} receptors, respectively

A The effects of the two agonists were partially additive. Left) HVA currents elicited by steps of +10 mV from holding potential of - 50 mV in control, 1 μM 8-OH-DPAT, and 1 μM 8-OH-DPAT + 1 μM L-694,247. These example traces were (taken from the indicated time points) on graph at the left. B) Pretreatment of selective 5-HT_{1A} receptor antagonist p-MPPI did not alter the effects of L-694,247. Left) In presence of 1μM p-MPPI, 1μM L-694,247 still caused voltage-dependent inhibition (asterisk shows relief of inhibition by +120 mV prepulse). Right) Summary showing that the effects of L-694,247 in control (black bars; n=12) was similar to L-694,247 + p-MPPI (n=5, hatched bars). C) Selective 5-HT_{1A} antagonist, NAN-190, partially blocked the effects 5-HT (a); selective 5-HT_{1D} antagonist, GR127935, blocked the effects of L-694,247(b) and 5-HT(c) in different neurones.


A
c
b
a
0.32 nA
7 ms
Time (s)
0
50
100
150
200
-200
-400
-600
ICa (pA)
a
b
c
L-694,247
DPAT
B
+120 mV
+ 20 mV
*
L-694,247 + p-MPPI
p-MPPI
0.10 nA
25 ms
with prepulse
p>0.5
50
40
30
20
10
0
current inhibition (%)
**
*
L-694,247
(n=12)
p-MPPI pretreatment
(n=4)
C
a
time (s)
0
50
100
150
0
-40
-80
-120
NAN-190 (100 nM)
5-HT (1 μM)
b
0
125
250
-40
-90
-140
GR127935 (100 nM)
L-694,247 (100 nM)
c
0
100
200
-400
-600
-800
-1000
GR127935 (100 nM)
5-HT (1 μM)

associated with a slowing in activation kinetics (Fig 4.7A & Ba), In the remaining neurons, L-694,247 suppressed the HVA currents without ' kinetic slowing' . Furthermore, in neurons tested with a strong depolarizing prepulse, more than 50% of the total inhibition was relieved (Fig 4.7 Bb & c, $p<0.05$, n=8). This suggests that activation of 5-HT_{1D} receptors predominantly caused voltage-dependent inhibition of HVA currents. However there was also some voltage-independent inhibition.

However, when the selective 5-HT_{1A} agonist, 8-OH-DPAT was used , only 27% (9/31) neurons exhibited inhibition that was accompanied by ' kinetic-slowing' . This is a significantly lower proportion of neurons compared to both 5-HT and L-694,247 (Fig 4.7Ac, $p<0.01$). In neurons tested with conditioning prepulses (n=9), the inhibition by 8-OH-DPAT was not significantly relieved (Fig 4.7B b & c), suggesting that the inhibition of Ca^{2+} currents by 5-HT_{1A} receptors occurs predominantly through voltage-independent mechanisms.

To confirm that these two agonists were acting through different receptors, I tested whether the effects of L-694,247 and 8-OH-DPAT were additive. In 6 neurons, the actions of saturating doses of L-694,247 (1 μM) and 8-OH-DPAT(1 μM) were partially additive (Fig 4.8A). Furthermore, the effects of L-694,247 were blocked by GR127935 (Fig 4.8C b, n=4), a selective 5-HT_{1D} antagonist (Skingle, Skopes, Feniuk, Connor, Carter, & Clitherow, 1993; Sun & Dale, 1997), but were unaffected by simultaneous application of the 5-HT_{1A} antagonists, p-MPPI (n=5, Kung, Kung, Clarke, Mayani, Zhuang, 1994; Sun & Dale, 1997) and NAN-190 (n=3, Liau, Sleight, Pitha, Peroutka 1991; Sun & Dale, 1997). I also examined the effects of pretreatment with receptor antagonists. In neurons (n=4) pretreated with p-MPPI, L-694,247 still caused a very similar amount of inhibition to those that had not been pretreated (n=12). The amount of relief by the prepulse in presence of p-MPPI was also the same as L-694,247 alone (Fig 4.8 B). In conjunction with our previous studies (Sun &

Dale, 1997) our results suggest that 8-OH-DPAT and L-694,247 selectively activate the 5-HT_{1A} and 5-HT_{1D} receptors.

Since the effects of 5-HT were partially blocked by GR127935 (Fig 4.8 Ca, n=3), or NAN-190 (Fig 4.8 Cc, n=4), our results suggest that both 5-HT_{1A} and 5-HT_{1D} receptors are involved in the inhibition of HVA Ca^{2+} currents. Neither ketanserin nor clozapine had any effects on the actions of 5-HT (n=15), suggesting that 5-$HT_{2A/2C}$ receptors are not involved in the modulation of HVA currents.

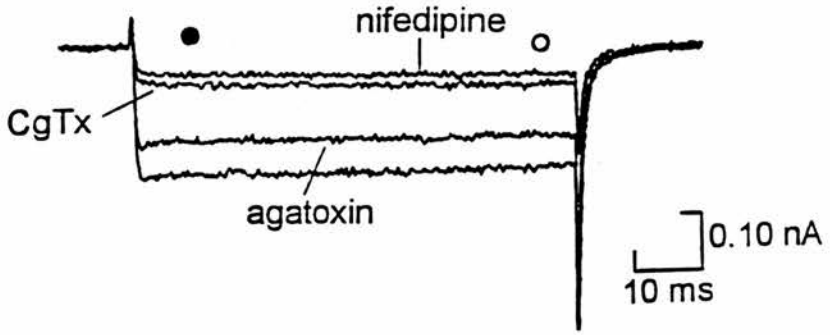
4.44 N- and P/Q- type Ca^{2+} channels are targeted by 5-HT

In *Xenopus* larvae spinal neurons, current carried by N-type channels, which can be blocked by ω-conotoxin-GVIA (ω-CgTx, 1 μM to 3μM), comprises 66.3±1.1% (n=21) of the total HVA Ca^{2+} current (Fig 4.9). ω-agatoxin-IVA sensitive currents (100 nM) comprise a further 22±0.9% (n=24) of the total (Fig 4.9). The Ca^{2+} channel blockers, ω-contoxin-MVIIC (1 μM) and ω-Agatoxin-TK (100 nM) blocked similar amounts of currents (23±2.9, n=3, and 25 ±2.5, n=5, respectively, not illustrated). The effects agatoxin were irreversible, saturated at doses less than 70 nM, and did not show obvious inactivation during both short (70 ms) and long (1 s) test pulses (not, illustrated), suggesting the agatoxin-sensitive currents maybe carried mainly by P-type channels (Randall & Tsien, 1995). Nifedipine at 10 μM, blocked less than 5% of the total Ca^{2+} currents (n=5, Fig 4.4), suggesting *Xenopus* spinal neurons possess only small number of L-type channels. There was also a very small amount of current that was not blocked by any of these blockers, but could be blocked by the non-selective Ca^{2+} channel blocker Cd^{2+} (100 μM, Fig 4.9).

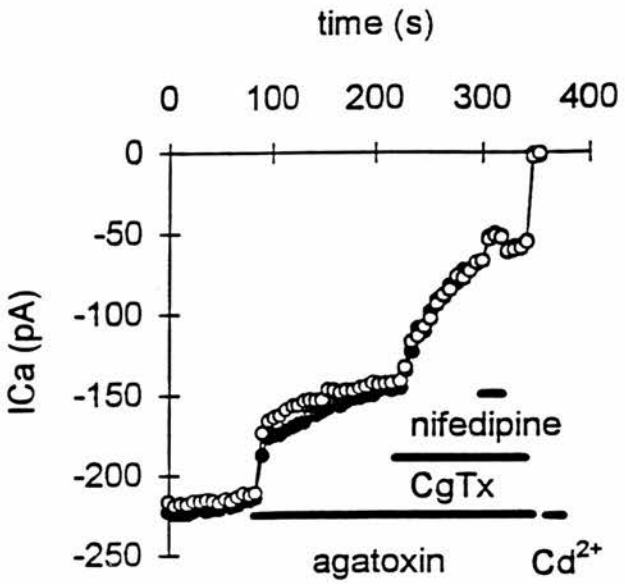
Figure 4. 9 Composition of the HVA currents in *Xenopus* spinal neurones

A a) HVA Ca^{2+} currents, elicited by +20 mV test pulse from holding potential of -50 mV, in control, agatoxin (200 nM), ω-CgTx (1 μM) and nifedipine (10 μM). A b) Time-course of block measured at the points indicated in (a). B) Summary showing agatoxin blocked 21.8±0.9% of total HVA currents (n=24, P/Q-type), ω-CgTx blocked 66.3±1.1% of total HVA currents (n=21, N-type), and nifedipine blocked 4.2±0.8% (n=5, L-type).

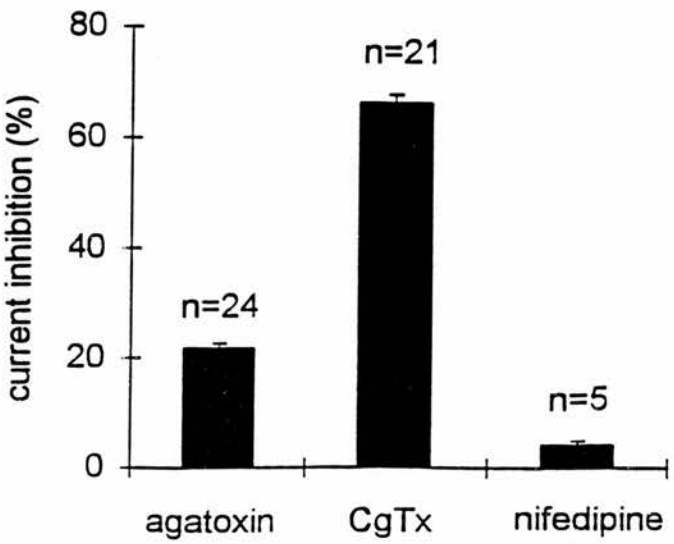
A a


nifedipine
CgTx
agatoxin
0.10 nA
10 ms

b


time (s)
0
100
200
300
400
0
-50
-100
-150
-200
-250
ICa (pA)
nifedipine
CgTx
agatoxin
Cd2+

B


80
60
40
20
0
current inhibition (%)
n=24
n=21
n=5
agatoxin
CgTx
nifedipine

To examine the identity of the Ca^{2+} channels inhibited by 5-HT receptors in *Xenopus* spinal neurons, I tested the effects of 5-HT alone and once again in the presence of ω-CgTx (1 μM) and (or) agatoxin (100 nM) to determine whether the block was additive or occludent. 5-HT(1 μM) inhibited 33.9±1.6% (n=102, P<0.01 vs. control) of the HVA currents when applied alone. In the presence of ω-CgTx (1 μM), 5-HT produced 9.1±0.8% further inhibition (n=16, P<0.01 vs. ω-CgTx, Fig 4.11 & 4.12). This partial occlusion (around 70%) suggests that both N-type and non-N type channels were inhibited by 5-HT.

When 5-HT was applied in the presence of agatoxin (100 nM), it produced a reduction of 23.9±4.7 % (n=25), which was significantly less than 5-HT applied alone (36.2±3.8%, p<0.01; Fig 4.10 & 4.12), suggesting that P/Q-type channels were also targeted by 5-HT .

In the presence of both ω-CgTx (1 μM) and agatoxin (100 nM), 5-HT produced virtually no further inhibition (2.1±1.1%, n=7, not significant, Fig 4.12), suggesting that 5-HT acts only on N-type and P/Q-type Ca^{2+} channels.

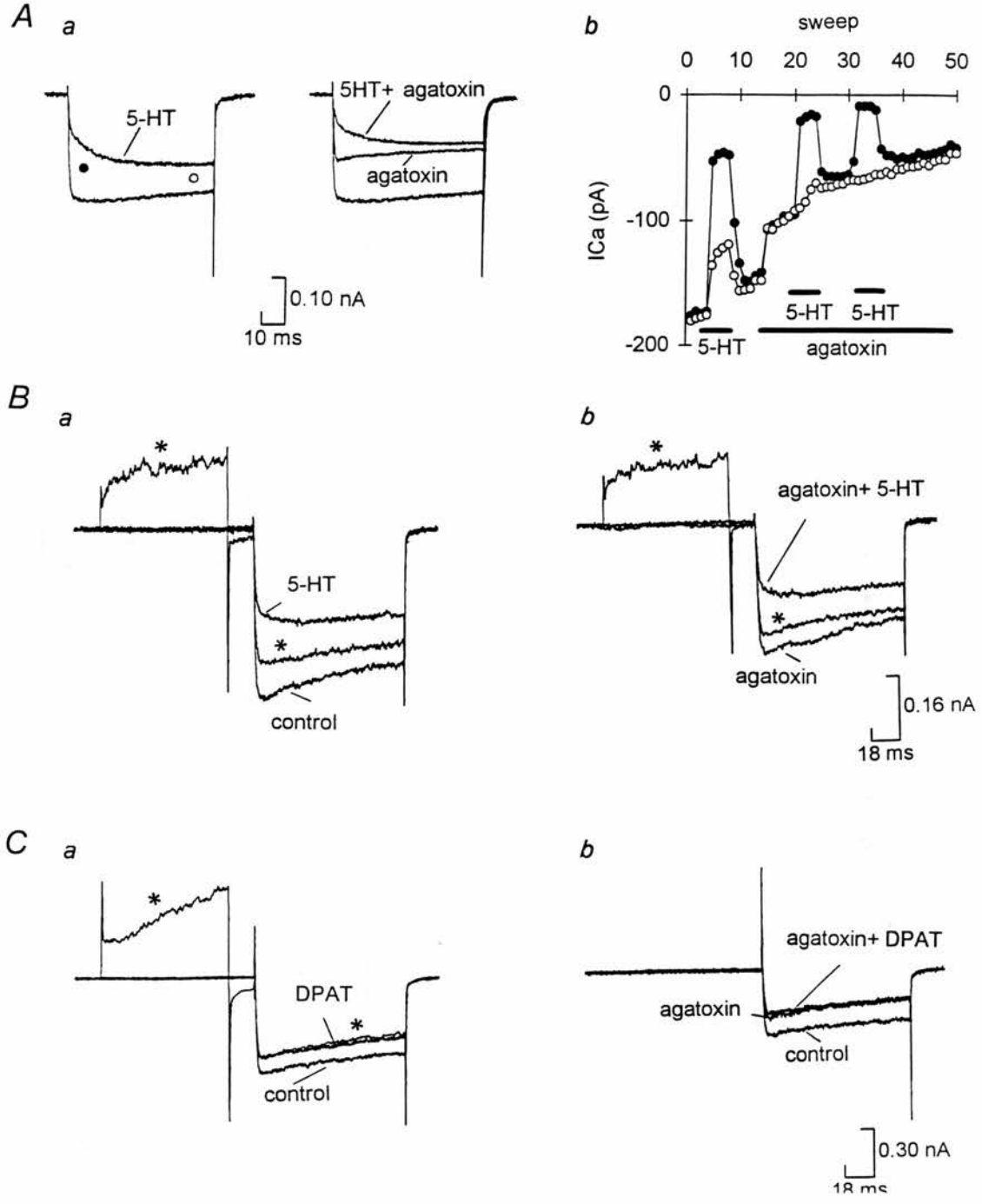
4.45 P/Q- channels were inhibited only by voltage-independent mechanisms

In our initial experiments, I found that the shape change usually associated with voltage-dependent suppression was not altered by agatoxin. Instead, agatoxin seemed only to occlude the 'steady-state' inhibition (Fig 4.10A, n=10).

I examined this further by testing whether the inhibition of P/Q type currents could be relieved by a prepulse. If P/Q-type currents were modulated in a

Figure 4.10 P/Q-type Ca^{2+} currents are reduced only through voltage-independent mechanisms

A) Agatoxin occluded the voltage-dependent block by 5-HT. Example traces of an experiment are shown in (a) and the time course measurements at 5 ms (filled circle) and 70 ms (open circle) are shown in (b). In agatoxin (100 nM), 5-HT caused a ' pure' voltage-dependent reduction which almost totally reversed at the end of the test pulse. B a) A prepulse to + 120 mV partially reversed the 5-HT inhibition in control (asterisk, prepulse). b) Agatoxin blocked around 20% of the total HVA current, and in the presence of agatoxin, the prepulse relieved almost same amount of currents (asterisk). However some voltage-independent inhibition remained. C a) Ca^{2+} currents elicited by a test pulse to + 10 mV from holding potential of -50 mV, the reduction of Ca^{2+} currents by 8-OH-DPAT is not changed by prepulse (asterisk), suggesting voltage-independent inhibition. b) In the presence of agatoxin (100 nM), 8-OH-DPAT (1 μM) did not produce further inhibition in the same cell.


A
a
5-HT
5HT+ agatoxin
agatoxin
0.10 nA
10 ms
b
sweep
0
10
20
30
40
50
0
-100
-200
ICa (pA)
5-HT
5-HT
5-HT
agatoxin
B
a
5-HT
control
b
agatoxin+ 5-HT
agatoxin
0.16 nA
18 ms
C
a
DPAT
control
b
agatoxin+ DPAT
agatoxin
control
0.30 nA

voltage-dependent manner, the amount of current inhibition that could be relieved by a prepulse should be reduced after treatment with agatoxin. Our results showed that the amount of current inhibition that could be relieved by a prepulse was unaffected by agatoxin (see Fig 4.10 & 12 for examples, Fig 4.13 A for summary). In contrast the amount of inhibition that was resistant to relief was significantly reduced by agatoxin (see Fig 4.10 & 4.12 for examples, Fig 4.13A for summary). Our results thus suggest that P/Q type currents were modulated only via voltage-independent mechanisms.

Since activation of 5-HT_{1A} receptor preferentially caused voltage-independent inhibition (Fig 4.7), I examined whether these receptors preferentially target P/Q type channels. In 8 neurons examined, the modulation of HVA currents by 8-OH-DPAT was not accompanied by shape changes and was not relieved by +120 mV prepulse, as observed above. The inhibition by 8-OH-DPAT of HVA Ca^{2+} currents (21.4± 3.8%) was mostly occluded by agatoxin (additional inhibition in the presence of agatoxin 7.9 ± 1.7%, $p<0.05$ vs. agatoxin alone; see Fig 4.10 C for example). This therefore suggests that P/Q -type currents were the main target of the voltage-independent suppression of HVA currents by 8-OH-DPAT. However, a small amount of the inhibition by 8-OH-DPAT was produced via non-P/Q channels, presumably N channels. Thus 5-HT_{1A} receptors not only preferentially cause voltage-independent inhibition, but do so by acting mainly on one subclass of Ca^{2+} channels.

4.46 N-type channels were reduced by both voltage-dependent and voltage-independent mechanisms

To examine the mechanisms of modulation of N-type channels, I studied the effects of ω-CgTx on the actions of 5-HT on the HVA currents . In those cases where 5-HT caused kinetic slowing, 1 μM ω- CgTx greatly reduced the amount

Figure 4.11 N-type Ca^{2+} currents are modulated through both voltage-dependent and -independent mechanisms

A a) Ca^{2+} currents in control and 5-HT (1 μM); right: Ca^{2+} currents in control, ω-CgTx (1 μM) and 5-HT (1 μM) in the presence of ω-CgTx; b) time series measurements in the same cell showing that 5-HT produced more inhibition at 5 ms (filled circle) of test pulse than 70 ms (open circle) when applied alone. On top of ω-CgTx (1 μM), 5-HT inhibited the much smaller Ca^{2+} currents by similar amounts at the 5 ms and 70 ms of the step. B a) A positive prepulse partially relieved the inhibition of the HVA currents by 5-HT. b) In the presence of ω-CgTx (1 μM), 5-HT further reduced a much smaller amount of HVA currents, but the effect of 5-HT was not relieved by positive prepulse. Therefore in this neurone, ω-CgTx totally occluded the voltage-dependent inhibition but only partially occluded the voltage-independent inhibition. C a) Ca^{2+} currents in control, L-694,247(1 μM), ω-CgTx (1 μM), L-694,247 + ω-CgTx. b) Time series measurements from the same neurones showing that L-694,247 reduced HVA currents by around 35% in control. However, in the presence of ω-CgTx (1 μM), there was virtually no further reduction.

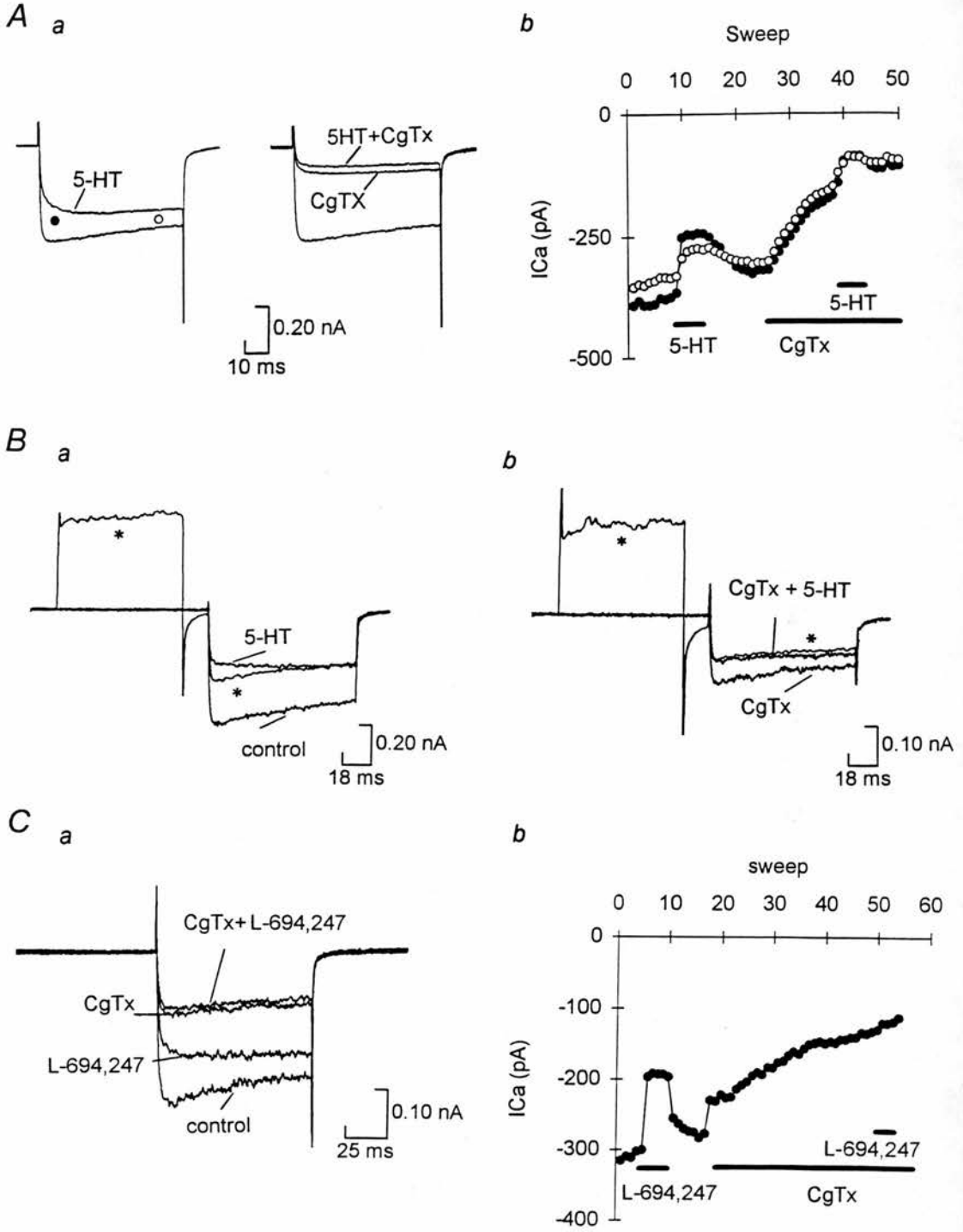

A
a
5-HT
5HT+CgTx
CgTX
0.20 nA
10 ms
b
Sweep
0
10
20
30
40
50
0
-250
-500
ICa (pA)
5-HT
5-HT
CgTx
B
a
5-HT
control
0.20 nA
18 ms
b
CgTx + 5-HT
CgTx
0.10 nA
18 ms
C
a
CgTx+ L-694,247
CgTx
L-694,247
control
0.10 nA
25 ms
b
sweep
0
10
20
30
40
50
60
0
-100
-200
-300
-400
ICa (pA)
L-694,247
L-694,247
CgTx

Figure 4.12 The inhibition of HVA currents by 5-HT was totally occluded by agatoxin and ω-CgTx applied together

A) HVA currents elicited in control (a), agatoxin(d) , and ω-CgTx (g). 5-HT caused different amounts of inhibition in presence of control (b), agatoxin (e), and agatoxin + ω-CgTx (h); B) Time series measurements from the same neurone. In control the inhibition was partially relieved by a prepulse to + 120 mV (arrow, open circles showing steady-state inhibition). After agatoxin was applied, the amount of voltage-dependent inhibition, relieved by prepulse (arrows) remained almost unchanged, but the voltage-independent inhibition (difference between open circles and solid circles at (f)) was reduced. In the presence of both toxins, 5-HT had very little effect on the currents

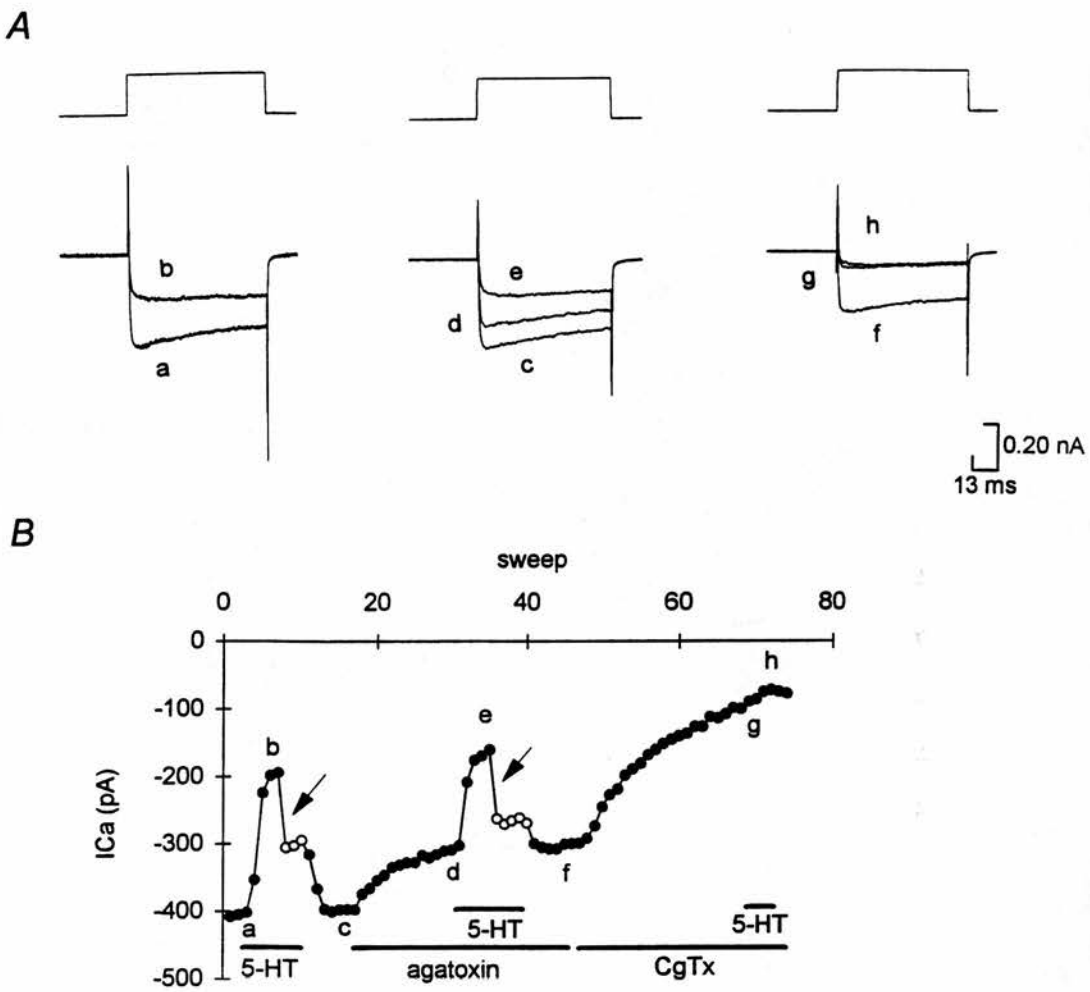
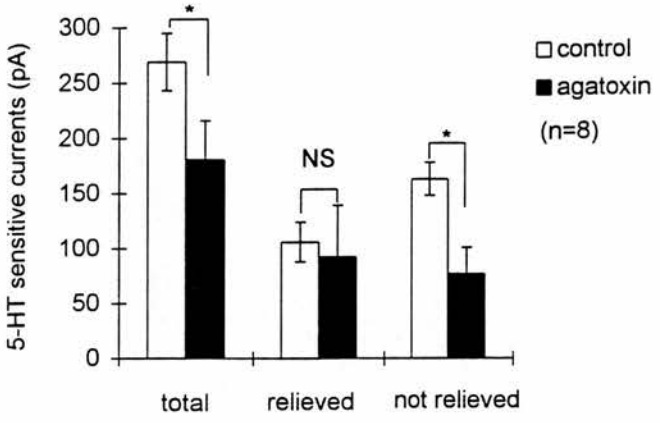

A
b
a
e
d
c
h
g
f
0.20 nA
13 ms
B
sweep
0
20
40
60
80
0
-100
-200
-300
-400
-500
ICa (pA)
a
b
c
d
e
f
g
h
5-HT
agatoxin
5-HT
CgTx
5-HT

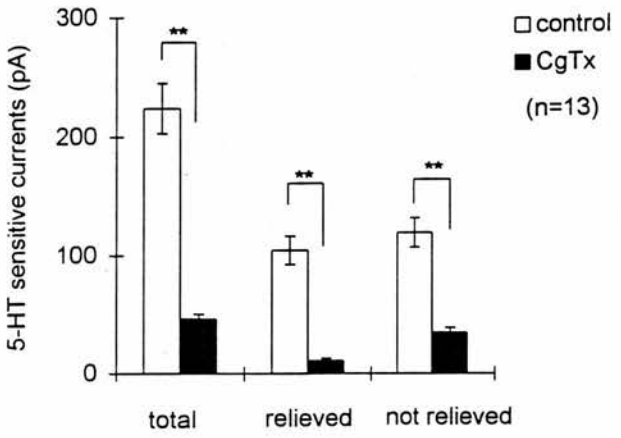
Figure 4.13 Summary of the effects of ω-CgTx and agatoxin on the inhibition of HVA currents by 5-HT

The 5-HT sensitive current was divided into two components: a 'voltage-dependent' component, which is relieved by a conditioning prepulse; and a 'voltage-independent' component, which was unaffected. A) Agatoxin reduced the 5-HT sensitive current, predominately through the reduction of the voltage-independent component ($p<0.05$). Its effect on the voltage-dependent component was not significant. B) In contrast, CgTx almost completely occluded the voltage-dependent component of the 5-HT sensitive currents ($p<0.01$) and had a large effect on the voltage-independent component ($p<0.01$).

A


5-HT sensitive currents (pA)
300
250
200
150
100
50
0
*
NS
*
control
agatoxin
(n=8)
total
relieved
not relieved

B


5-HT sensitive currents (pA)
300
200
100
0
**
**
**
control
CgTx
(n=13)
total
relieved
not relieved

of slowing by subsequent application of 5-HT (Fig 4.11 A, n=10), suggesting the modulation of N-type channels by 5-HT occurred through voltage-dependent mechanisms. To test this further, I utilized prepulses to examine the proportion of voltage-dependent and -independent inhibition more directly. In those neurons where modulation of HVA currents by 5-HT was associated with 'kinetic-slowing' and relieved partially (or totally) by a prepulse, ω-CgTx almost totally occluded the voltage-dependent block (see Fig 4.11C & 4.12 for examples, Fig 4.13 A for summary), ω-CgTx also occluded a large amount of the voltage-independent modulation (Fig 4.12 & 4.13 A). N- type currents were therefore modulated by a combination of voltage-dependent and -independent mechanisms.

Since activation of 5-HT_{1D} receptors preferentially caused voltage-dependent inhibition (Fig 4.7 & 4.8), I examined whether the effects of ω-CgTx could occlude actions of 5-HT_{1D} agonist, L-694,247. In 5/10 neurons examined, the modulation of HVA currents by L-694,247 was accompanied by 'kinetic-slowing' (Fig 4.11B), and was almost completely relieved by a prepulse to +120 mV. In these 5 neurons, where the inhibition occurred through a voltage-dependent mechanism, the inhibition of HVA Ca^{2+} currents by L-694,247 (30.4± 2.8%) was totally occluded by ω-CgTx (Fig 4.11C). However, in 5 other neurons, where L-694,247 produced both voltage-dependent and independent inhibition, ω-CgTx only partially blocked the effects of L-694,247 (around 70%, not illustrated). This suggests that although 5-HT_{1D} receptors preferentially modulate N- type channels, they may also act on non-N, presumably P/Q, channels.

4.5 Discussion

4.51 N- and P/Q Ca^{2+} channels were inhibited by 5-HT through different mechanisms

Currently, at least six different subclasses of Ca^{2+} channel can be discerned by voltage dependence, conductance, pharmacology and kinetics: low-voltage-activated Ca^{2+} channels (LVA, or T-type) and the high-voltage-activated channels (HVA) of the L-, N- , P-, Q-, and R-type. 5-HT has been reported to modulate some of these channels in CNS neurons, including the N- type channels (Bayliss et al, 1995; Del Mar, Cardenas, and Scroggs, 1994; Foehring, 1996; Koike et al, 1994; Penington, et al, 1991; Sun & Dale, 1997); P/Q channels e.g. in rat motor neurons (Bayliss et al, 1995), mammalian neocortical pyramidal neurons (Foehring, 1996) and sensory neurons (Sun & Dale, 1997). Our findings that N- and P/Q HVA channels are modulated by 5-HT are consistent with these reports. However, we found that the actions of 5-HT on P/Q-channels in *Xenopus* larval spinal neurons differed from its actions on N channels. Whereas the inhibition of P/Q channels was only through the voltage-independent pathway, the inhibition of N-type channels involved both voltage-dependent and independent mechanisms.

Perhaps the most common form of inhibition of N-type Ca2+ channels by activation of G-protein activated receptors is voltage-dependent (Hille, 1994; Dolphin, 1998). Nevertheless, N-type channels have also been reported to be reduced via both voltage-dependent and independent mechanisms (Beech, Bernheim & Hille, 1992; Diversé-Pierluissi and Dunlap, 1993; Luebke and Dunlap, 1994); or only through voltage-independent mechanisms (Sun & Dale, 1997). Thus our results of N- type currents being modulated through both voltage-dependent and -independent pathways is consistent with some of these previous reports.

P/Q type channels are identified pharmacologically as being blocked by agatoxin (Randall & Tsien, 1995), however it remains unclear which α subunit gene encodes these channels. In comparison to the N channels, there are fewer studies of modulation of P/Q channels by receptor activated G-proteins. In some case, they appear to be inhibited by mechanisms similar to the N-type channels (Mintz & Bean, 1993; Herlitze et al, 1996). A few reports suggest that P/Q type channels are inhibited to a lesser extent than N channels (Mintz & Bean, 1993; Bayliss et al., 1995; Bourinet, Soong, Stea, & Snutch, 1996; Currie & Fox, 1997). In adrenal chromaffin cells, the voltage-dependence of the inhibition of P/Q channels seems to differ from that for N-type channels (Currie & Fox, 1997), and in *Xenopus* sensory neurons, P/Q channels are modulated only via non-voltage-dependent ways (Sun & Dale, 1997). Nevertheless, P/Q-type channels have not been generally thought to be modulated via voltage-independent mechanisms. Therefore our findings of P/Q currents being inhibited through purely voltage-independent mechanism contrasts with most previous reports.

There is a general consensus that the voltage-dependent inhibition of N- and P/Q-type channels involves direct interaction between Ca^{2+} channels and G βγ subunits, (Ikeda, 1996; Waard, et al.,1997; Herlitze, et al., 1996). Since the inhibition of Ca^{2+} channels by direct application of G βγ is almost totally relieved by strong conditioning prepulses (Ikeda, 1996; Waard, et al., 1997; Herlitze, et al., 1996), it raises the possibility that voltage-independent modulation of Ca^{2+} channels may involve a different second messenger (c.f. Luebke & Dunlap, 1994). In some circumstances, the voltage-independent inhibition may be mediated via phosporylation of N-type channels by activation of protein-kinase C (Diversé-Pierluissi & Dunlap, 1993). However, PKC appears to inhibit Ca^{2+} currents only in sensory neurons (Rane, Walsh, McDonald, & Dunlap, 1989) whereas in sympathetic neurons Ca^{2+} channel activity is enhanced (Swartz 1993). Therefore the second messengers underlying the voltage-independent modulation of N- and P/Q channels remain uncertain.

Our results can be most easily understood if the voltage-independent inhibition of P/Q and N Ca^{2+} currents occurs via a novel modulatory pathway. Our evidence also suggests that two different 5-HT receptors were differentially associated with the voltage-dependent and voltage-independent inhibition, and that the rate of onset and recovery differ between these two forms of inhibition. These observations make the involvement of separate modulatory pathways more likely. However, it is remotely possible that both N and P/Q channels were modulated through the same direct interaction with G βγ subunits. If this were true, the agatoxin sensitive channels in *Xenopus* spinal neurons, must be a different subset of ' P/Q' channel types, possibly encoded by different α subunit genes, such that their interaction with G βγ subunits was without voltage-dependence.

4.52 Two receptors differentially coupled to different HVA channel types

In *Xenopus* spinal neurons, the inhibition of HVA currents by 5-HT was mediated by the 5-HT_{1A} and 5-HT_{1D} receptors. Our results suggested that these pharmacologically distinct receptors preferentially mediate different forms of inhibition. Whereas the 5-HT_{1A} receptor preferentially targeted the P/Q type channels via voltage-independent manner, the 5-HT_{1D} receptors mainly acted on the N-type channels via both voltage-dependent and -independent manner. However, this distinction was not absolute: not all of the current inhibition mediated by the 5-HT_{1A} receptor was voltage-independent and agatoxin-sensitive and likewise, not all of the inhibition mediated by the 5-HT_{1D} receptor was ω-conotoxin-sensitive.

Previously, HVA currents in CNS neurons have only been reported to be modulated through the 5-HT_{1A} receptor acting by a voltage-dependent

mechanism (Pennington, et al., 1991; Koike, et al., 1994; Bayliss, et al., 1995; Foehring, 1996; but Ciranna et al., 1996; Sun & Dale, 1997). The voltage-dependent inhibition of N- channels by the 5-HT_{1D} receptors has not been previously reported. However in an earlier study we found voltage-independent inhibition of N- and P/Q currents by 5-HT_{1D} receptors in the *Xenopus* sensory neurons (Sun & Dale, 1997). Our results suggest that, like the 5-HT_{1A} receptors, the 5-HT_{1D} receptor may also activate more than one signal transduction pathway to mediate the voltage-dependent and -independent inhibition. The preferential modulation of N and P/Q Ca^{2+} channels by different 5-HT receptor subtypes may be explained by segregation and colocalization of receptors and with their target Ca^{2+} channels.

Since 5-HT_{1A} and 5-HT_{1D} receptors target different Ca^{2+} channels, the varied forms of response caused by 5-HT might be explained by only one or a combination of both 5-HT receptor subtypes being expressed. Similarly, the limited reversal of voltage-dependent inhibition by 5-HT in some neurons may also be explained by total relief of only one component of inhibition. If this were true, endogenously released 5-HT could also produce selective effects in different types of neurons depending upon the postsynaptic receptors that it activated.

The diversity of Ca^{2+} channels types and their differential localization is of fundamental importance in control of Ca^{2+} signaling. In the synaptic terminals, N- and P/Q channels can play a critical role in triggering neurotransmitter release (Luebke et al, 1993; Turner, et al, 1993; Wheeler, et al, 1994). In the soma and dendrites Ca^{2+} channels influence the excitability of the membrane either directly or through subsequent activation of Ca^{2+} -sensitive conductances. In principle, differential modulation of HVA channels that depends on different subtypes of receptor, allows these functions to be separately regulated. Furthermore, the existence of two forms of modulation preferentially evoked by the 5-HT_{1A} and 5-HT_{1D} receptors has important implications for neural function. The 5-HT_{1A} receptor mediated modulation of P/Q channels is non-voltage-dependent so that

its magnitude will not vary with neuronal activity. In contrast, the 5-HT_{1D} receptor mediated inhibition of the N channels, which has voltage-dependence, could in principle be relieved during bursts of action potentials. In the context of a rhythmic motor pattern that involves neurons firing bursts of spikes, the transient relief of inhibition of the N type current could be of physiological significance.

4.53 Implications for serotonergic modulation of locomotion motor pattern

The embryonic *Xenopus laevis* (stage 37/38 , Nieuwkoop & Faber, 1956) is a model for studying the control of locomotion (Dale, 1995 a, b). Many types of neurotransmitters and neuromodulators, such as GABA (Wall & Dale, 1994), noradrenaline (McDearmid, Scrymgeour-Wedderburn & Sillar, 1997), ATP and adenosine (Dale & Gilday, 1996), and acetylcholine (Perrins & Roberts, 1994) modulate the locomotor pattern in *Xenopus*. Between the embryonic (37/38) and larval stages (40-42), the locomotor pattern recorded in the ventral roots changes from brief biphasic compound action potentials on each cycle to longer bursts of activity on each cycle. This change is mimicked by the application of 5-HT and its metabolic precursor, 5-hydroxytryptophan at certain stages in development (Sillar, Simmers & Wedderburn 1992). 5-HT mediates presynaptic inhibition of the mid-cycle glycinergic IPSPs during fictive swimming and also increases ventral root burst duration in larvae (McDearmid, et al., 1997). Although evidence strongly supports an involvement of the 5-HT_{1A} receptor (Wedderburn & Sillar, 1994), the possible roles of the 5-HT_{1D} receptor have not been reported. The majority of neurons we recorded are the glycinergic interneurons which are responsible for the mid-cycle glycinergic IPSPs. Therefore the inhibition of HVA channels in these neurons may well contribute to the reduction of mid-cycle IPSPs. Our results are the first to suggest that the 5-HT_{1D} receptors could, in addition to the 5-HT_{1A} receptors, be involved in modulation of

vertebrate locomotor activity. The two 5-HT receptors are differentially coupled to the two different Ca^{2+} channels via voltage-dependent and -independent mechanisms. This could be significant. For example, if inhibition of N channels by 5-HT was relieved during the burst of spikes in the locomotor cycle, both the amplitude of synaptic potentials and the neuronal excitability would increase throughout the burst. Since the $5\text{-}HT_{1D}$ receptor primarily acts through the N channels, the effects of activation of this receptor could vary with the phase of locomotor cycle. In contrast, the $5\text{-}HT_{1A}$ receptor acts through the P/Q channels, which are only inhibited via voltage-independent mechanisms. I would therefore predict that the activation of the $5\text{-}HT_{1A}$ receptor would cause tonic inhibition which could not vary throughout the locomotor cycle. Only further studies will elucidate the precise roles of voltage-dependent and -independent inhibition and the contrasting actions of the $5\text{-}HT_{1A}$ and $5\text{-}HT_{1D}$ receptors on locomotor activity in *Xenopus*.

Chapter 5 GENERAL DISCUSSION

In the introduction, I proposed to investigate the following 5 questions: 1) Which 5-HT receptor subtypes are possessed by spinal neurones of *Xenopus* larvae? 2) Which types of Ca^{2+} channels, and Ca^{2+} dependent potassium channels are expressed in the *Xenopus* spinal neurones? 3) How does 5-HT modulate the different subtypes of Ca^{2+} channels? 4) What are the possible biophysical and biochemical mechanisms underlying the serotonergic modulation of Ca^{2+} channels? 5) What are the possible functional consequences of the modulation of Ca^{2+} currents? I shall now summarize to what extent I have answered these questions and what needs to be solved in further studies.

I have shown unambiguously that spinal neurones of *Xenopus* larvae possess at least two functional 5-HT receptor subtypes, 5-HT_{1A} and 5-HT_{1D} receptors. I demonstrated that the spinal neurones of *Xenopus* larvae possess N, P/Q , T , and a small proportion of L and R type Ca^{2+} channels. In different neurones, notably sensory neurones and non-sensory neurones, although the same 5-HT receptor subtypes are expressed, these receptors are coupled to different sets of Ca^{2+} channels via distinct second messengers.

In primary sensory R-B neurones, 5-HT produced pure voltage-independent inhibition of N and P/Q type HVA currents and T-type currents. The modulation of N and P/Q currents was mediated via a pertussis toxin sensitive G-protein acting on a diffusible second messenger. However, the modulation of T-type currents was not mediated by G-proteins and may involve a receptor domain separate from that involved in activating G-proteins. The functional consequences of modulation of somatic T-type channels have also been explored.

In non-sensory spinal neurones, 5-HT_{1A} and 5-HT_{1D} receptors produced differential inhibition of N and P/Q currents but had no effects on other Ca^{2+} channels. Two forms of inhibition of the HVA Ca^{2+} currents were produced by 5-HT. One was voltage-dependent inhibition which was associated with both slowing of the activation kinetics and a shifting of the voltage-dependence of the HVA currents and was relieved by strong depolarizing prepulses. The second was

voltage-independent inhibition and was neither associated with slowing of the activation kinetics nor relieved by depolarizing prepulses. The 5-HT_{1A} receptors not only preferentially caused voltage-independent inhibition, but did so by acting mainly on the ω-agatoxin-IVA sensitive Ca^{2+} channels. In contrast, the 5-HT_{1D} receptors produced both voltage-dependent and -independent inhibition and were preferentially coupled to ω-conotoxin-GVIA sensitive channels.

In the following 6 sections, I shall compare the significance of my results with previous studies carried out on modulation of Ca^{2+} channels by neurotransmitters, especially by 5-HT. I shall also compare my results with previous studies in the *Xenopus* embryo and larvae on GABAergic and serotonergic modulation of ion channels, locomotion behaviours and sensory transmission, and discuss what are the functional consequences of the modulation of these Ca^{2+} currents in *Xenopus* larvae. Some suggestions for future work is also discussed in relevant sections.

In the appendix, I demonstrate that during development, spinal neurones of the larvae expresse at least three types of fast $IK_{(Ca)}$ (BK) channels in place of some of the voltage-gated potassium channels which are down-regulated. These changes of ion channel expression have endowed the larval neurones with properties more suitable for the more mature motor pattern. I shall discusse the possible link between Ca^{2+} influx via Ca^{2+} channels and BK, SK currents in *Xenopus* neurones; and how this could contribute to the modulation of locomotion by 5-HT in *Xenopus* larvae.

5.1 Ca^{2+} channel subtypes being modulated by neurotransmitters

Table 5.1 Summary of neurotransmitter inhibition of neuronal voltage-gated Ca^{2+} channels

Ca^{2+} channels	Voltage-dependence	Agonist	Second messengers	Preparations	References
N-type channels	1. V.D.	$GABA_B$ Noradrenaline	PTX-sensitive	DGR neurones	1
	2. V.D.	Noradrenaline	Membrane-delimited	DRG neurones	2
	3. V.D.+ V.I.	Muscarinic α-adrenergic	Unidentified diffusible second messenger	Rat sympathetic neurones	3
	4. V.D.	mGluR	G-proteins	CA3 Pyramidal neurones	4
	5. V.D.	LHRF	G-proteins	Bullfrog sympathetic ganglion neurones	5
	6. V.D.	Somatostatin	PTX-sensitive G-proteins	Rat sympathetic neurones	6
	7. V.D.+V.I.	NE	PTX-sensitive+ insensitive	same as above	6
	8. V.I.	Substance P	PTX-insensitive	same as above	6
	9. V.D	$GABA_B$	G(0)	DRG neurones	7
	10. V.D.	$GABA_B$	PTX-sensitive +insensitive	GH3 cell line	8
	11. V.D.	Orphanin (FQ)	PTX-sensitive	CA3 pyramidal neurones	9
P/Q-type Channels	1. V.D.	$GABA_B$	G-protein	Cerebellar Purkinje neurones	10
	2. V.D.	G βγ	G βγ	Sympathetic ganglion neurones	11
	3. V.D.	Enkephalin	PTX-sensitive	α1A channel expressed in *Xenopus* oocytes	12
	4. V.D.+V.I.	ATP, GTP-γ-S	Unidentified	Adrenal chromaffin cells	13
	5.V.D.	Muscarinic	PTX-sensitive	striatal neurones	14
L-type channels	1. V.I.	NE or NPY	PTX-sensitive	sympathetic neurones	15
	2. V.I.	Muscarinic	Diffusible and slow PTX-insensitive	Sympathetic neurones	16
	3. V.I.	Muscarinic	PTX-insensitive G-proteins	Striatal neurones	17
	4. V.I.	Muscarinic	PTX-sensitive+- insensitive G-proteins	Mouse pancreatic B-cells	18
	5. V.I.	Opioid-receptor	(-)	Bovine chromaffin cells	19
	6. Unidentified	Muscarinic mGluR	PTX-insensitive G-proteins	Various preparation	20, 21, 22
	7. V.I.D.	Neurotensin Substance P	(-)	Nucleus basalis neurones	23

Table 5.1 continued

	1. Unknown	Muscarinic	PTX-sensitive	Paratracheal ganglion cells	24
R-type	2. V.D	Muscarinic	PTX-sensitive	Intracardiac neurones	25
channels	3 V.D.	Somatostatin Carbachol	PTX-sensitive	α1E Channel expressed in GH3 cells	26
	4. Not modulated**	**	**	**	**
	1. Unknown	GABA	G-proteins	Rat DRG	27
T-type	2. Unknown	GTP-γ-S Baclofen	PTX-sensitive G-protein	neurones Rat DRG	28
channels	3. Unknown	Neurotensin Substance P	PTX-insensitive G-proteins	neurones	39
	4. Unknown	Nociceptin (orphanin FQ)	GTP-insensitive pathways	Nucleus basalis neurones Rat DRG neurones	30

NE: norepinephrine; NPY: neuropeptide Y; LHRH: luteinizing hormone-releasing hormone; mGluR: metabotropic glutamate; DRG: dorsal root ganglion neurones; V.D.: voltage-dependent inhibition; V.I.: voltage-independent inhibition; **: R- channels are generally thought not to be modulated by G-protein activation in several different preparations cf. Bourinet et al., 1996; Toth et al., 1993; Connor & Christie, 1998). A recent report demonstrated that different α1E channels (with different N-ternimus amino acids length), could be differentially modulated in a voltage-dependent way by G-protein activation (Page et al., 1998). References for the table, 1. Holz et al., 1986; 2. Forscher, et al., 1986; 3. Bernheim, et al., 1991; 4. Swartz & Bean , 1992; 5. Bolland & Bean, 1993; 6. Shapiro et al., 1994; 7.Menonjohansson et al., 1993; 8. Zong et al., 1995; 9. Knoflach et al., 1996; 10. Mintz & Bean, 1993; 11. Herlitze et al., 1996; 12. Bourinet et al., 1996; 13. Currie & Fox, 1997; 14. Howe & Sumeier, 1995; 15. Plummer et at, 1991; 16. Mathie et al., 1992; 17, Howe & Sumeier, 1995; 18. Gilon et al., 1997; 19. Albillos et al., 1996; 20 Sahara & Wesbrook, 1993; 21. Fisher & Johnston, 1990; 22. Sayer et al., 1992; 23. Mageta-Mitrovic et al., 1997; 24 Murai et al., 1998; 25 Jeong & Wurster, 1997; 26 Yassin et al., 1996; 27. Deisz & Lux, 1985; 28. Scott et al., 1990; 29 Mageta-Mitrovic et al., 1997; 30 Abdulla & Smith, 1997.

From Table 5.1, I can see that N and P/Q channels are most commonly subject to voltage-dependent inhibition. However, both classes of channel may occasionally be inhibited via a combination of both voltage-dependent and -independent pathways. By contrast, L-type Ca^{2+} channels seem to be modulated exclusively by voltage-independent mechanisms (except in very rare cases, cf. Ciranna et al., 1996). Studies on the modulation of R-type channels have given contradictory results so far. However this confusion may result from the lack of selective blockers for R channels and a rather small body of work that has been carried out on these channels. T-channels have not been extensively studied and information about the voltage-dependence of their modulation is largely lacking (Table 5.1).

The table also show that G-proteins that are sensitive to PTX are predominantly involved in the modulation of N and P/Q channels. Nevertheless PTX-insensitive G-proteins can also be involved (cf. Dolphin, 1998 for review). Similar to N and P/Q channels, the modulation of R channels is also mediated maibly by PTX-sensitive G-proteins. In contrast, the voltage-independent modulation of L-type channels seems to be mediated most commonly by PTX-insensitive G-proteins (but PTX-sensitive G-proteins can also be involved). T channels appear to be inhibited by both PTX-sensitive and -insensitive G-proteins.

5.2 Modulation of Ca^{2+} channels by 5-HT receptor subtypes

Table 5.2 Summary of serotonergic modulation of neuronal Ca^{2+} channel subtypes

Ca^{2+} channel type	Voltage-dependence	Receptor subtypes	Second messengers	Preparations	Reference
HVA	V.D.	(-)	(-)	Rat spinal neurones	Sah, 1990
N and non-N	V.D.	(-)	G-proteins	Rat dorsal raphe neurones.	Pennington et al., 1991
N	V.D.	5-HT_{1A}	G-protein	VMH* neurones	Koike et al., 1994
HVA	(-)	5-HT_{1A}	(-)	Rat DRG neurones	Del Mar et al., 1994
N , P/Q	V.D.	5-HT_{1A}	G-proteins	Rat hypoglossal motoneuronees	Bayliss et al., 1995
Q , L	V.I. (L) V.D (Q)	5-HT_{1A} 5-HT_{2C}	PTX-sensitive	Rat melanotrophs	Ciranna et al., 1996
N, P/Q	V.D. (50%) V.I. (50%)	5-HT_{1A}	G-proteins (membrane-delimited)	Neocortical pyramidal neurones	Foehring, 1996
N, P/Q	V.D.	5-HT_{1A}	PTX-sensitive	Rat raphe neurones	Bayliss et al., 1997
N, P/Q	**V.I.**	**5-HT_{1A} 5-HT_{1D}**	**PTX-sensitive diffusible**	***Xenopus* RB neurones**	**Chapter II & III**
N, P/Q	**V.D. (N) V.I. (N, P/Q)**	**5-HT_{1A} 5-HT_{1D}**	(-)	***Xenopus* non-sensory neurones**	**Chapter IV**
T (potentiation)	(-)	(-)	(-)	Spinal motor neurone	Berger &Takahashi, 1990
T (potcntiation)	(-)	(-)	(-)	CA1 interneurones	Fraser & MacVicar, 1991
T (inhibition)	**VI.**	**5-HT_{1A} 5-HT_{1D}**	**membrane-delimited non-G-protein**	***Xenopus* sensory neurones**	**Chapter II & III**

VMH*: ventromedial hypothalamic (VMH) neuronal; V.D.: voltage-dependent inhibition; V.I.: voltage-independent inhibition; DRG: dosal root ganglion neurones; my own results are printed in bold characters.

5.21 HVA channels

The predominant effect of 5-HT_{1A} receptors on HVA channels in mammalian preparations is voltage-dependent inhibition (Table 5.2). However, in *Xenopus* spinal neurones, 5-HT also produced voltage-independent inhibition on both N and P/Q channels. The voltage-independent inhibition can been see either alone or in combination with voltage-dependent inhibition, and is preferentially activated by the 5-HT_{1A} receptor subtype in non-sensory neurones. I have found that in sensory neurones, it is mediated by a PTX-sensitive G-protein and a diffusible second messenger, which differs from the membrane-delimited $G\beta\gamma$ mediated voltage-dependent inhibition. For voltage-dependent inhibition, the voltage activation of the whole cell Ca^{2+} currents is shifted and its dependence on voltage is less steep. This contrasts with voltage-independent inhibition where there is no kinetic slowing and no shift of voltage-activation. In addition, the whole-cell conductance seems to be reduced by the voltage-independent inhibition but not by the ' pure' voltage-dependent inhibition. These results agree with many previous results (cf. Luebke & Dunlap, 1994; Ciranna et al., 1996; Currie & Fox, 1997; Page et al., 1997), and strongly suggeste multiple mechanisms can be activated by the 5-HT_1 receptor to inhibit HVA channels.

As agreed by many researchers (cf. Dolphin, 1998 for review), the voltage-independent inhibition of HVA channels is an unique form of modulation that occurs either independently or in conjunction with the well-known voltage-dependent inhibition. In some reports, the voltage-independent inhibition produced by GTP-γ-S appears to target all of the HVA channels (α-1 A B & R channels) expressed in cultured neurones (Page, et al., 1997). However, several examples (cf. Luebke & Dunlap, 1994; Albillos et al., 1996; Ciranna et al., 1996; Currie & Fox, 1997; Gilon et al., 1997), and my results suggest that the voltage-independent mechanisms can target specific HVA channel subtypes selectively. The reasons why this could happen need to be explored further, preferably in a

reconstituted model system where various HVA channels and G-proteins are expressed (cf. Page et al., 1998).

5.22 T-type channels

Investigations on the modulation of T-type channels by 5-HT have yielded contradictory results. Whereas in some reports T channels are not modulated, (Bayliss et al., 1995; Table 5.2), in rat spinal motor neurones and hippocampal neurones, these currents were enhanced by 5-HT and other transmitters through an unknown mechanism (Berger & Takahashi, 1990; Fraser & MacVicar, 1991).

In *Xenopus* spinal CPG neurones (non-sensory), 5-HT had no effect on the T-type currents. However, in R-B neurones (sensory), 5-HT inhibited the T-type channels by some 30%. I have found that the inhibition of T-type channels is mediated by 5-HT_{1A} and 5-HT_{1D} receptors. These receptors act through a membrane-delimited pathway that does not involve G-proteins and is mediated by a functional domain of the receptor that is distinct from that which couples to G-proteins. Although apparently GTP-insensitive pathways may be involved in the Nociceptin (orphanin FQ)-mediated inhibition of T-type channels (Abdulla & Smith, 1997), the prevailing orthodoxy is that the superfamily of 7 transmembrane-segment receptors, that includes for example the muscarinic, adrenergic and serotonergic receptors, are coupled to G-proteins which mediates their major actions on ion channels or other proteins (but see Hall et al., 1998).

What might be the mechanism of the G-protein-independent inhibition of T channels? Two general altenatives are possible given what already know (that it is membrane delimited and non-voltage-dependent):

(1) The inhibition may be mediated by some other protein that interacts with both the receptor and the T channels. For example, the Na^+/H^+ exchanger is modulated by a regulatory factor, which is activated by β_2-adrenergic receptor (Hall et al., 1998). This regulatory protein binds to the β_2-adrenergic receptor and interacts specifically with the last few residues of the carboxyl-terminal

cytoplasmic domain of the receptor (Hall et al., 1998). The C-terminal sequence of the β_2 adrenergic receptor is very similar to the I4 peptide that I used in this study. That the I4 peptide was without effect on the T-type channels suggests that the modulation of these channels, although not involving G-proteins, may also be distinct from the mechanism that underlies modulation of the Na^+/H^+ exchanger.

(2) The receptor may interact directly with T channels. The lack of voltage-dependence of modulation suggests that if such a direct interaction were to occur, it would involve interaction between the intracellular domains of the receptor and the channels. Regrettably, I have not been able to identify the receptor domains involved. Future experiments using either more synthetic peptides from other regions of 5-HT_1 receptors or site directed mutagenesis approach to abolish the 5-HT_1 receptor mediated modulation of T-type channels would be more convincing.

Why might 5-HT_1 receptors modulate T-type channels in sensory neurones but not in non-sensory neurones? Three explanations are possible: (1) Since the modulation of T channels occured via a membrane-delimited pathway, the machinery (receptor, channel, intermediary proteins) involved in the modulation must be very closely colocalized. If such colocalization does not occur in the non-sensory neurons, the T channels may not be the target of modulation by 5-HT. (2) Even if receptor and channel were colocalized, if a further intermediary protein was involved, it may be lacking in the non-sensory neurones. (3) There may be two classes of T channels expressed in different types of neurones (cf. Huguenard, 1996). The motif that can be targeted by 5-HT receptors may be lacking in one class of T channels. In sensory neurones, both these postulated classes of T channels could be expressed. However in non-sensory neurones, only the non-modulatable class of T channels may be expressed. These possibilities could be examined by using single-cell RT-PCR techniques to compare the genes that encode for the T channels in these neurones.

In summary, the major modulatory effects of 5-HT on Ca^{2+} channels are inhibitory and only certain classes of Ca^{2+} channels are subject to modulation.

Nevertheless, in several regions of CNS and PNS, there are hints that 5-HT may have other effects too. For example, iontophoretic application of 5-HT facilitates Ca^{2+} -dependent spikes (Nedergaard et al., 1988). This excitatory effect may be mediated by $5\text{-}HT_{1A}$ receptors (Marzelec et al., 1988). Unfortunately, identity of the G-proteins and down-stream second messengers and Ca^{2+} channels involved in the facilitatory actions of 5-HT remains unknown.

5.3 Functions of different voltage-gated Ca^{2+} channels and consequences of their modulation

Voltage-gated Ca^{2+} channels are involved many aspects of physiological processes, including neurotransmitter and hormone secretion, neuronal excitability and firing pattern, gene expression, neurone development (Gruol, et al., 1992) and cell death (Eichler et al., 1994; Porter et al., 1997). They may also play roles in many pathological processes of the brain, such as Alzheimer's disease (Ikeda et al., 1992; Sen et al., 1993; Davidson et al., 1994; Sanderson, et al., 1997); Parkinson' s disease (Sen e tal, 1993); Huntingtons disease (Sen et al., 1993), epileptogenesis (Doyle et al., 1997; Hendriksen, 1997); neuropathic pain (McGuire et al., 1997). Nevertheless their most crucial function is in excitation-secretion coupling (Hille, 1992).

5.31 HVA channels

a) Triggering neurotransmitter release (terminal location)

Ca^{2+} influx via voltage-gated Ca^{2+} channels is a key step in excitation-secretion coupling in presynaptic terminals and in many neuroendocrine cells. Results gained particularly from pharmacological studies have made it clear that different Ca^{2+} channel subtypes contribute differentially to secretion of the various neurotransmitters and hormones in different tissue types. (Augustin et al., 1987; Sher, et al., 1991; Miller 1992; Olivera et al., 1994).

In the central nervous system, there is a general consensus that T-type or L-type Ca^{2+} channels are probably not involved in triggering transmitter release. As most studies have shown that a substantial fraction of release is blocked by ω-conotoxins or ω-agatoxins, the **N-** and **P/Q** type channels are the most likely candidates to mediate transmitter release. In most studies, using either brain slices or synaptosomes prepared from various brain regions, and employing either electrical stimulation or elevated K^+ to effect depolarization, ω-conotoxins-GVIA has been shown to inhibit potently but partially the release of glutamate (Leubeke et al., 1993; Turner et al., 1993), acetylcholine (Wessler et al., 1990), dopamine (Turner et al., 1993, Woodward et al., 1988), noradrenaline (Dooley et al., 1988), and glycine (Wall & Dale, 1994). Thus N-type Ca^{2+} channels mediate a substantial fraction, but not all, of transmitter release in the brain. P/Q type channels are also involved in this process. In some brain tissues, P/Q play a more important role than N-type channels (Turner et al., 1992). It appears that both N and P/Q channels were at least partially involved in the excitatory transmission in the hippocampus (Leubeke et al., 1993; Takahashi & Momeyama, 1993) as well as inhibitory transmission in the cerebellum and the spinal cord (Takahashi & Momeyama, 1993).

However, ω-conotoxin-GVIA and ω-agatoxin-IVA resistant Ca^{2+} channels are also involved in central synaptic transmission. In some tissue, these unknown channels may mediate around 17-41 % of the total (cf. Takahashi & Momeyama, 1993). Although the identity of these Ca^{2+} channels is unknown, available evidence in the hippocampus suggests that a similar proportion of N, Q, and **R-** type channels may be distributed around the transmitter release sites and these Ca^{2+} channel types trigger transmitter release with a similar efficacy (Wu & Saggau, 1995). Another study made from Calyx of Held synapses has shown that **R-** type channels contribute to release but with lower efficiency than other HVA channels (Wu et al., 1998).

In the peripheral nervous system, N-type channels are also the predominant channels which mediate neurotransmitter release. In both sympathetic neurones and parasympathetic systems, N-type channels were involved in triggering release of noradrenaline and acetylcholine, respectively (cf. Pruneau et al., 1990). At the mammalian neuromuscular junction, ω-conotoxin-GVIA had very little effects on evoked acetylcholine release (Wessler et al., 1990; Uchitel, et al., 1992) and further evidence suggests that P/Q-type channels appear to be responsible (Uchitel, et al., 1992).

L-type channels may involved in stimulating secretion in non-neuronal tissues. For example, in chromaffin cells DHP-sensitive channels are involved in stimulating the secretion of noradrenaline and adrenaline in response to a variety of secretagogues such as acetylcholine (Artalejo et al., 1992).

b) Voltage-dependent modulation of Ca^{2+} channels and presynaptic inhibition of transmitter release

Since HVA channels (particularly N and P/Q type) are widely reported to be involved in supporting synaptic transmission, their modulation by neurotransmitters could contribute to presynaptic inhibition of transmitter release. Although direct studies of presynaptic Ca^{2+} channels are almost impossible in most preparations (as the size of presynaptic terminals is usually inaccessible to patch clamp recording), there is still considerable indirect evidence in favor of the proposal that Ca^{2+} modulation at least partially contributes to the presynaptic inhibition (Toth et al., 1993; Stefani et al., 1994; Wu & Saggau, 1994; 1995; Gruner & Silva, 1994; Wall & Dale, 1994; Doze et al., 1995). In most of these studies, Ca^{2+} channel modulation has been studied with whole cell recordings from the cell bodies, and in some of the reports, the inhibition of evoked EPSPs by neurotransmitters was partially or totally occluded by ω-conotoxin-GVIA or ω-agatoxin-IVA (Toth et al., 1993; Wu & Saggau, 1995; Gruner & Silva, 1997).

In some preparations it is possible to record directly from the presynpatic terminals. Thus in the chick ciliary ganglion adenosine reduces both presynaptic Ca^{2+} current and transmitter release (Yawo & Chuma, 1993). By recording Ca^{2+} currents from a giant presynaptic terminal, the Calyx of Held, and EPSCs from its postsynaptic target in the medial nucleus of the trapezoid body of rat brainstem slices, baclofen has been found to suppressed both presynaptic Ca^{2+} currents and EPSCs via G-proteins (Takahashi et al., 1998).

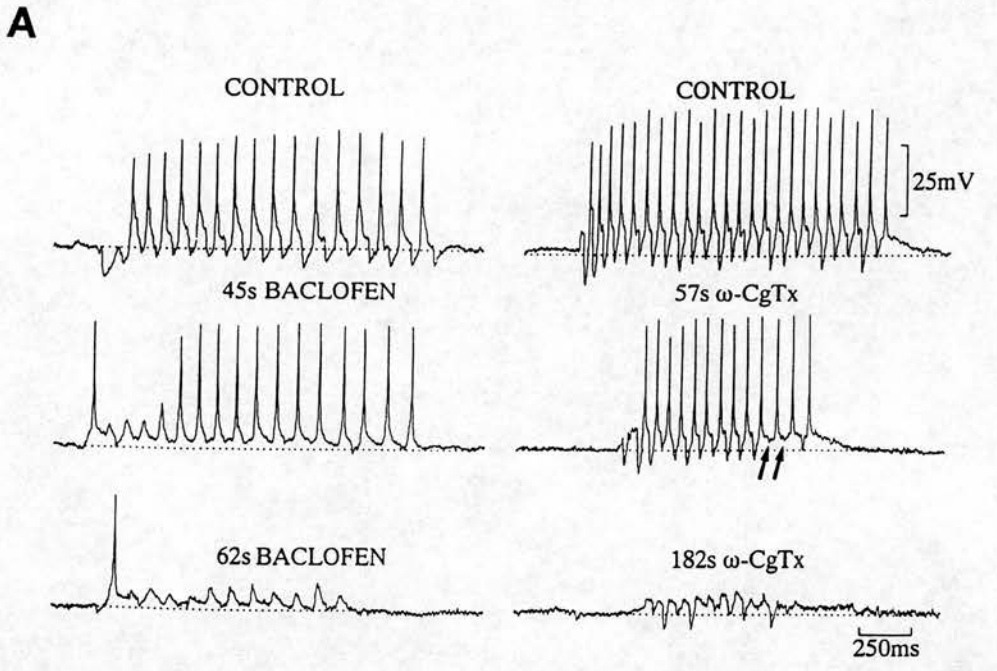
As the most common form for suppression of HVA channels is the voltage-dependent form which can be relieved by depolarizing prepulses, this inhibition could conceivably be reduced during a high frequency train of action potentials. Presynaptic inhibition could thus vary with patterns of presynaptic activity in physiologically significant ways. Studies using whole-cell recordings from frog sympathetic neurones have indeed provided evidence that this may occur in voltage ranges normally encountered during an action potential, and that a lessening of inhibition could be evoked during trains of action potentials (cf. Jones & Elmslie, 1996). This hypothesis was confirmed by experiments showing relief of inhibition of Ca^{2+} currents by action potential-like voltage-waveforms (Brody et al., 1997; Williams et al., 1997). However, these studies were carried out on isolated neurones. It remains to be demonstrated whether this also could occur at synaptic terminals and under physiological conditions.

c) Comparison with previous studies of neuromodulation in *Xenopus*

In the *Xenopus* embryo, the involvement of the ω-CgTX-sensitive Ca^{2+} current in supporting synaptic transmission in the intact spinal cord has been elucidated (Wall & Dale, 1994). Inhibitory interneurone axons were stimulated to evoke monosynaptic IPSPs in motoneurones, and recorded intracellularly. Since ω-CgTX blocked inhibitory transmission, an ω-CgTX-sensitive Ca^{2+} current plays

Fig 5.1 Effects of baclofen and ω-Conotoxin-GVIA on inhibitory IPSPs and motor pattern generation

A) Both baclofen and ω-Conotoxin-GVIA disrupt the swimming motor pattern. Fictive swimming was recorded in an intact embryo using a microelectrode and started by dimming the lights. During control fictive swimming episodes neurons fire an action potential once per cycle, recieve midcycle inhibition and are tonically depolarized. Application of 50 μM baclofen firstly caused a loss of mid cycle inhibition and then the dropout of action potentials. The application of 5 μM ω-conotoxin-GVIA initially caused dropout of the midcycle IPSPs and then a loss of action potential. B) Both ω-Conotoxin-GVIA and baclofen block inhibitory synaptic transmission in the intact spinal cord. Monosynaptic IPSPs were evoked by electrically stimulating the surface of the spinal cord and recorded in probable motorneurons with a intracellular microelectrodes. The amplitude of control IPSPs (trace 1) was greatly reduced in 50 μM baclofen, 5 μM ω-Conotoxin-GVIA, or 1 μM strychnine (trace 2). After wash the amplitude of the IPSPs recovered (trace 3). Records are an average of 10 consecutive sweeps (This figure is adapted from Wall & Dale, 1994).


A
CONTROL
CONTROL
25mV
45s BACLOFEN
57s ω-CgTx
62s BACLOFEN
182s ω-CgTx
250ms

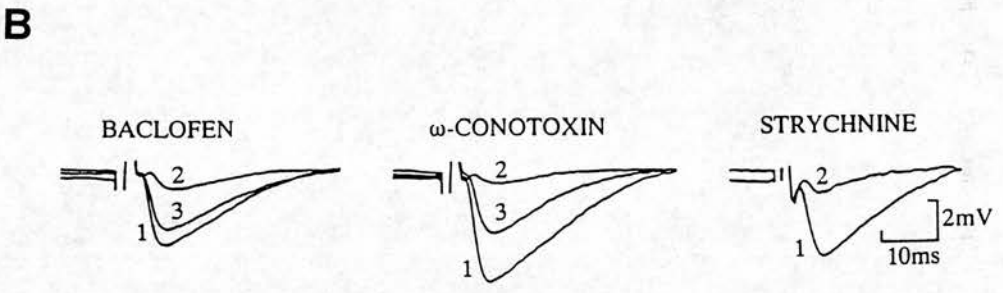
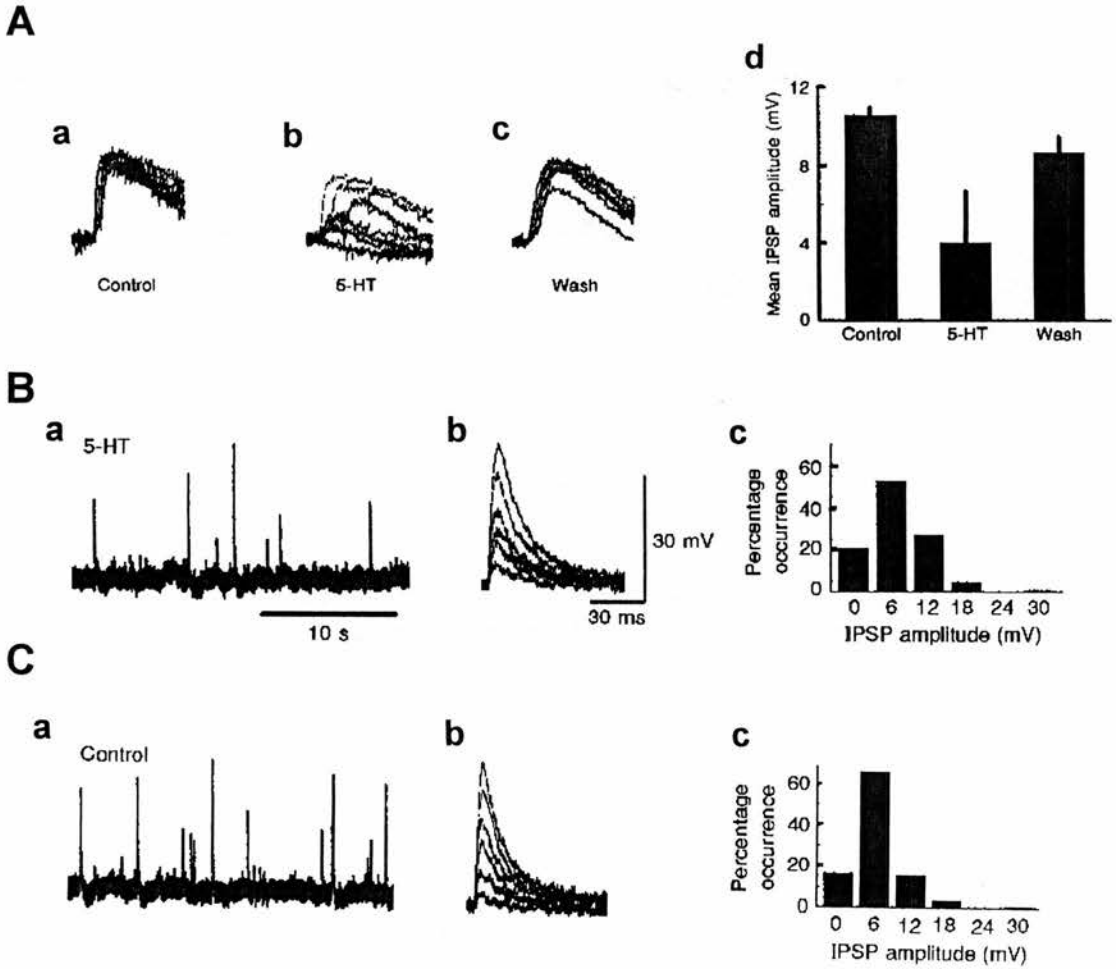

B
BACLOFEN
ω-CONOTOXIN
STRYCHNINE
2
3
1
2mV
10ms

Figure 5.2. Aminergic modulation of glycinergic mid-cycle IPSP amplitudes during fictive swimming occurs pre- not postsynaptically

A) 5-HT (1-10 μM) depresses mid-cycle IPSPs during fictive swimming. This panel (a-c) represents 10 superimposed consecutive cycles of swimming with an oscilloscope sweep triggered off mid-cycle IPSP. Note the IPSPs were depolarizing (KCl recording). In control saline, the mid-cycle IPSPs were relatively constant in amplitude (a). Under 5-HT, IPSPs were smaller, more variable in amplitude and could fail on some cycles (b). This effect was reversible after a 6 min wash (c). d) Summary of effects of 5-HT on mid-cycle IPSP amplitudes illustrated in a-c. Each bar represents the mean of 10 IPSPs plus standard deviation (S.D.). Effects of 5-HT was significantly different from controls (Student's t tests: $P < 0{\cdot}0001$ for 5-HT). B & C) In the presence of 500 nM TTX, spontaneous IPSPs (sIPSPs) varied in amplitude up to ca 30 mV and appeared to fall into distinct size categories (Ca-c). sIPSPs represent vesicular release of glycine from a commissural interneuron onto a motoneuron (Mn). 5-HT reduced the sIPSP rate (Ba) from $3{\cdot}84$ Hz to $1{\cdot}39$ Hz. The effect of 5-HT may be to directly decrease the probability of glycine release from commissural interneuron terminals. 5-HT had no effect on sIPSP amplitude distribution (Bb and c) suggesting a lack of effect of amines on postsynaptic glycine receptors (This figure is adapted from McDearmid et al., 1997).


A
a
Control
b
5-HT
c
Wash
d
Mean IPSP amplitude (mV)
12
8
4
0
Control
5-HT
Wash
B
a
5-HT
10 s
b
30 mV
30 ms
c
Percentage occurrence
60
40
20
0
0 6 12 18 24 30
IPSP amplitude (mV)
C
a
Control
b
c
Percentage occurrence
60
40
20
0
0 6 12 18 24 30
IPSP amplitude (mV)

an essential role in triggering transmitter release (Fig 5.1). Activation of $GABA_B$ receptors by baclofen in the *Xenopus* embryo causes a reversible reduction in the amplitude of Ca^{2+} currents via voltage-dependent and independent mechanisms. Baclofen also causes presynaptic inhibition of transmitter release from glycinergic spinal neurones and an increase in action potential threshold. Therefore baclofen reduces transmitter release by two coordinated mechanisms: one acting at the terminals to reduce vesicle fusion and other at the spike initiation zone to reduce the likelihood of firing an action potential. (Wall & Dale, 1994; Fig 5.1).

Like baclofen, 5-HT also produces inhibition of whole-cell N currents. Therefore it is highly probable that 5-HT could also, if the receptors are located near the terminals, produce presynaptic inhibition of transmitter release in *Xenopus* larvae spinal circuitry via mechanisms similar to that of baclofen. This speculation is supported by a recent finding, in which glycinergic inhibitory potentials occurring mid-cycle in motoneurones during swimming activity are reduced by 5-HT. The effects on inhibitory synaptic strength are mediated presynaptically where 5-HT decreases the probability of glycine release (McDearmid, et al., 1997, Fig 5.2). The presence of P/Q channels in *Xenopus* spinal cord has not been described before. The role that these channels appear to have in triggering transmitter release in CNS of other species makes it possible that they may play a similar role to the N channels. Note that 5-HT also produces voltage-independent inhibition of N and P/Q channels in *Xenopus*. The existence of two forms of modulation preferentially evoked by the $5\text{-}HT_{1A}$ and $5\text{-}HT_{1D}$ receptors has important implications for neural function. In principle, the modulation of specific HVA channels by separate subtypes of receptor allows these functions to be individually regulated. Furthermore, as the $5\text{-}HT_{1A}$ receptor- mediated modulation of P/Q channels is non-voltage-dependent, its magnitude will not vary with neuronal activity. In contrast, the $5\text{-}HT_{1D}$ receptor mediated inhibition of the N channels has voltage-dependence, so could in principal be relieved during bursts of action potentials (cf. Brody et al., 1997; Williams et al., 1997). In the context of a rhythmic motor pattern that involves neurones firing bursts of spikes, the transient relief of inhibition of the N- type current might be of

physiological significance. For example, if the suppression of mid-cycle IPSPs on the motor neurons by 5-HT were mediated by 5-HT_{1D} receptors , this could be relieved toward the end of an swimming episodes. This might explain why 5-HT has a much bigger effect on the motor bursts at the beginning of a swimming episode compared to the end (Q.Q. Sun & N. Dale, unpublished observations).

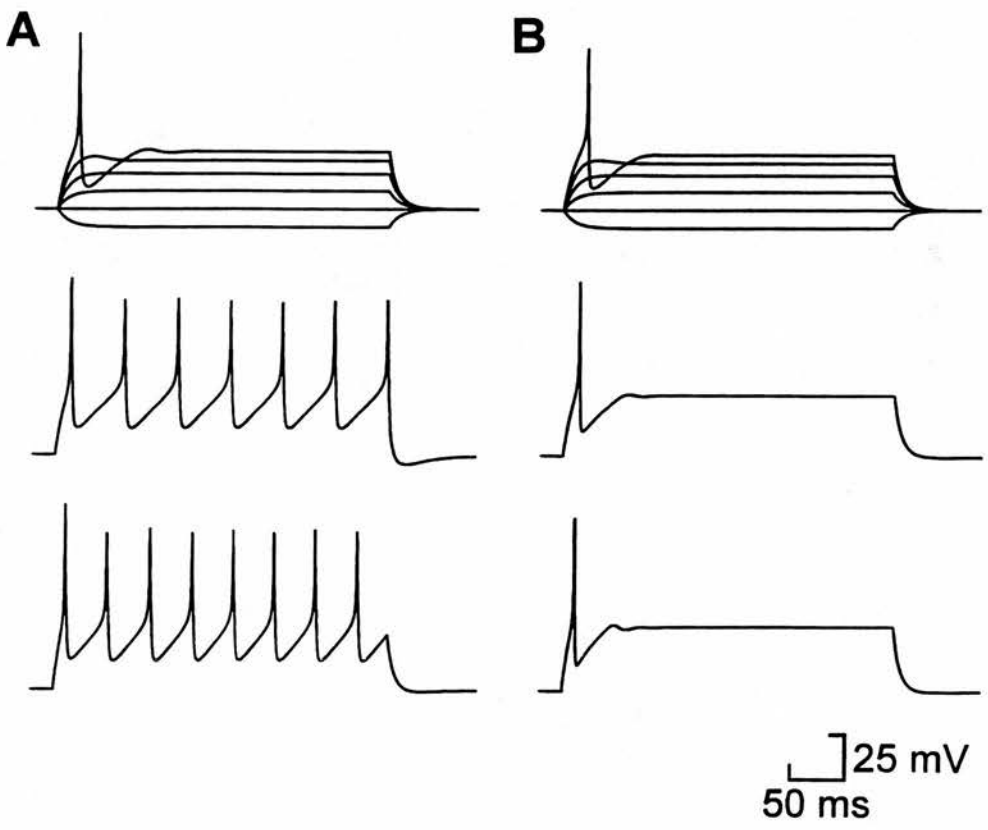
Whereas voltage-dependent inhibition of N and P/Q channels has been widely proposed as a key mechanism for presynaptic inhibition of neural transmitter release (cf. Dolphin et al., 1998), that voltage-independent inhibition of these channels could also act as a direct means to modify the exocytosis at synaptic terminals has not been generally appreciated. However, in a recent report, by recording directly from a brainstem giant synapse called Calyx of Held, the inactivation of P/Q channels has been found to contribute to synaptic depression (Forsythe et al., 1998). This suggests that the voltage-independent inhibition of presynaptic Ca^{2+} channels could be involved in tonic suppression of synaptic transmission. In an earlier report, Sillar & Simmers (1994) have found that 5-HT can also produce presynaptic inhibition of excitatory amino acid transmitter release from R-B neurones of *Xenopus* larvae (Fig 5.4). I have found that 5-HT reduced N and P/Q channels via a putative voltage- independent way in these neurones. Therefore this combined evidence suggests that the voltage-independent suppression of N and P/Q channels is likely to contribute to presynaptic inhibition. However, evidence to show whether the 5-HT mediated presynaptic inhibition can be occluded by N or P/Q channel blockers would be able to determine this more directly.

d) Setting action potential threshold (Somatic and dendritic location)

The threshold at which a neurone fires an action potential will depend on the relative contributions of the inward currents and outward currents. In the soma, HVA channels were also involved in setting action potential threshold. Dale (1995a & b) developed simulations of *Xenopus* embryo spinal neurones, which

Fig 5.3 Role of HVA Ca^{2+} currents in maintain repetitive firing of *Xenopus* model spinal neurons

The membrane properties of *Xenopus* model neurons were designed according to patch-clamp recordings of voltage-gated ionic currents. The model neurons have a rectifying membrane and can fire repetitively (A). The ability to fire repetitively is prevented if the HVA Ca^{2+} currents were removed (B). In A & B the bottom two sets of traces represent responses to successively increased current pulses to those shown in the overlapped traces. (This figure is adapted from Dale, 1995b).


A
B
25 mV
50 ms

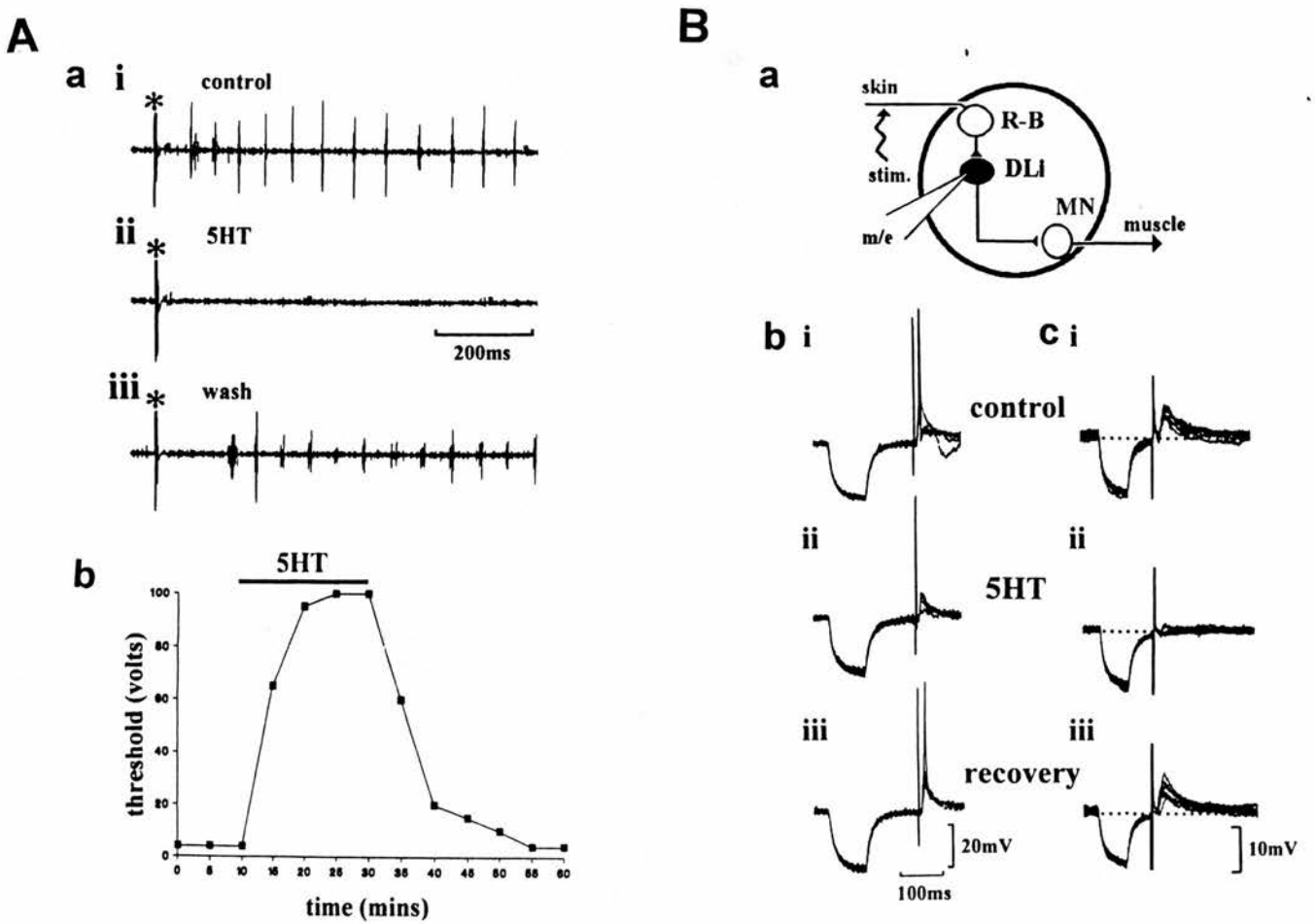
fire repetitively in response to current injection. The Ca^{2+} current seems essential for repetitive firing (Fig 5.3). The conclusions drawn from simulations have been confirmed by the observations that reduction of Ca^{2+} currents by the Ca^{2+} channel blocker ω-conotoxin-GVIA markedly reduced the number of spikes in response to current injection in isolated neurones (Wall & Dale, 1994; Fig 5.1). The $GABA_B$ agonist baclofen, which has been shown to inhibit N -type Ca^{2+} currents, can increase action potential threshold leading to possible loss of action potentials and de-recruitment of neurones during swimming (Fig 5.1). 5-HT, which has similar effects on the HVA currents as baclofen, was indeed found to reduce the number of spikes and raises the threshold of firing in response to current injection (Fig 5.5).

5.32 Function of T- type channels

The low-threshold Ca^{2+} current, or T current, has been demonstrated with voltage-clamp recordings in a large variety of central nervous system (CNS) neurones. It is especially prominent in the soma and dendrites of neurones with robust Ca^{2+} -dependent burst firing behaviours such as thalamic relay neurones and cerebellar Purkinje cells (cf. Hugunard, 1996). The kinetic properties of the T current allow it to play a role in spike initiation from negative resting potentials or subsequent to transient membrane hyperpolarization. Putative functional roles for T current include generation of low-threshold spikes that lead to burst firing, promotion of intrinsic oscillatory behaviour, boosting of Ca^{2+} entry, and synaptic potentiation (Huguenard, 1996 for review). The role of T-type channels in thalamic neurones has been most thoroughly studied. Llinas & Yarom (1981) found in thalamic projection cells, that T-type channels produced low-threshold Ca^{2+} potentials, which were important for generating bursts of action potentials and activated a conductance involved in maintaining oscillatory activity. This important role of T-type channels has been seen by other researchers (Suzuki and Rogawski, 1989; Huguenard' s group, cf. Huguenard 1996 for review). In other CNS neurones, T-type channels may also play similar roles, e.g. in mammalian dorsal root ganglion neurones T-type channels can generate an afterdepolarizing

Fig 5.4 Block of sensory pathways and presynaptic inhibition of primary afferent transmitter release by 5-HT in *Xenopus* spinal cord

A) Bath-applied 5-HT inhibits activation of fictive swimming by cutaneous stimulation in *Xenopus* laevis embryos. a) Swimming motor output (i) monitored from a single ventral root in response to a single brief electrical shock (3V*) to the caudal skin. In presence of 5-HT (2 μM, ii), the same stimulus voltage no longer activates swimming but is able to following washout in normal saline (iii). b) Time course of minimum skin stimulus voltage required to activate swimming before, during and after exposure to 2 μM 5-HT. B) 5-HT diminishes R-B mechanoreceptor-evoked EPSPs in dosal lateral interneurons (Dlis). a), Experimental schema involving intracellular recording from a Dli in the spinal cord while observing the effects of 5-HT on its mono-synaptic excitation from R-B cells in response to skin -stimulation. b & c) Single cutaneous stimuli are followed at constant latency by a compound EPSP that may trigger an action potential (bi). 5 min after exposure to 5-HT, the EPSP is markedly reduced (ii) to levels below threshold for DL impulse generation (e.g. Bii compare Bi) Control levels of Dli activation return upon wash in normal saline (iii). In both cases, the depression in sensory excitation by 5-HT is not associated with a change in membrane conductance, suggesting that 5-HT is acting presynaptically. (This figure is adapted from Sillar & Simmers, 1994)


A
a i
control
ii
5HT
200ms
iii
wash
b
5HT
threshold (volts)
time (mins)
B
a
skin
R-B
stim.
DLi
MN
m/e
muscle
b i
c i
control
ii
5HT
iii
recovery
20mV
100ms
10mV

potential that triggers burst firing (White et at., 1989). In rat hippocampal dentate granule neurones, activation of T-type currents contributes to a depolarizing afterpotential (Zhang et al., 1993). Although T-type channels are not generally involved in neurotransmitter release from the CNS, in chromaffin cells T-type channels may be responsible for the secretion of catecholamines (Morad, et al., 1989). T-type channels also contribute to the secretion of aldosterone by adrenal glomerulosa cells (see review by Ertel & Ertel, 1997). In both neurones and other tissues, T-type channels appear to be abundant in proliferating cells, which suggests that T-type channels may be important for cell growth and /or formation of new cell-to-cell interactions (Nargeot' s group cf. Ertel & Ertel 1997). T-type channels may also be important in cells such as endocrine cells that do not exhibit action potentials yet need to vary their intracellular Ca^{2+} for cellular functions. In such cells, small variations of the membrane potential would be sufficient to open T channels which pass enough Ca^{2+} influx to trigger further chemical responses (cf. Ertel & Ertel, 1997).

T-type channels have also been found in sensory neurones, including mammalian dorsal-root ganlion neurones (Deisz & Lux, 1985; Scott et al., 1990; Formenti et al., 1993; Christenson et al., 1993; Abdulla & Smith, 1997), *Xenopus* primary mechanosensory neurones (Chapter II & III) and lamprey touch-sensitive (T) primary mechanosensory neurones (Christenson et al., 1993). However, the role of T-type channels varies between the different preparations. In mammalian sensory neurones, removal of T-type channels by Ni^{2+} or nociceptin caused attenuation of the afterdepolarization and reduced bursts of spikes (Abdulla & Smith, 1997.) Lamprey touch-sensitive neurones have a significantly shorter time delay to the spike onset on a rebound of a hyperpolarizing current pulse than the pressure sensitive sensory neurones. In the short latency T-cells, blockade of T-type channels increased the latency under current clamp conditions, and inhibited the facilitatory effect on spike activation upon increased hyperpolarization. Therefore it is likely that the presence of low voltage-activated Ca^{2+} channels in T-cells are responsible for the differences observed between T- and P-cells (cf. Christenson et al., 1993). *Xenopus* R-B neurones have a very negative resting potential and do not fire repetitively in response to sustained current injection.

Therefore T-type channels are not involved in shaping the repetitive firing bursts. Instead, we have found that T-type channels are important in setting the firing threshold of sensory neurones, and selective block of these channels by Y^{3+} could reduce the probability of generating an action potential (Chapter II). This is a new locus of modulation of a sensory pathway which I shall discuss later (Fig 5.6 & 5.7).

5.4 Effect of 5-HT on potassium conductance and Ca^{2+} - dependent potassium conductances

In rat nucleus accumbens, 5-HT reduced the inward rectifier potassium conductance (North & Uchimara, 1989). This reduction produced a dose-dependent depolarization, and was mediated by 5-HT_2 receptors via activation of PKC (Uchimara & North, 1990). 5-HT has also been widely reported to inhibit the 'M' conductance in voltage clamped pyramidal neurones in human and guinea-pig cortex (McCormic & Williamson, 1989), rat hippocampus (Colino & Halliwell, 1987), and rat dorsal root ganglion neurones (Abramets et al., 1989). Although the receptors involved are not totally clear, the 5-$HT_{2A/2C}$ receptor may contribute to this response (Abramets et al., 1989). Reduction of potassium conductances such as these is likely to generate slow depolarizations and occur in many areas of the brain (see Anwyl 1990 for review).

In the neuronal soma, modulation of the Ca^{2+} currents may alter the activation of Ca^{2+}-dependent K^+ currents (Bayliss et al., 1997). These currents are powerful determinants of neuronal excitability and circuit function. Activation of Ca^{2+} dependent K^+ channels (K_{Ca} channels) depends on both membrane voltage and intracellular Ca^{2+} concentration. Some earlier reports suggest that K_{Ca} channels can be triggered by release of Ca^{2+} from intracellular stores (cf. Tautmann 1984). However a large body of work suggests that Ca^{2+} entry through voltage-gated Ca^{2+} channels, such as N-type HVA channels or P/Q-type channels is important in selectively activating K_{Ca} channels (Bayliss et al., 1995; Sah, 1995; Callister et al., 1997). Therefore Ca^{2+} influx following action potentials can produce a range

of fast and slow afterhyperpolarizations by activation of Ca^{2+}-dependent conductances. In mammalian motor neurones, the reduction of Ca^{2+} influx could secondarily produce a reduction of AHP (Bayliss et al., 1995 ; Bayliss et al., 1997), and in a variety of CNS tissues, 5-HT reduces both the Ca^{2+}-dependent K^+ conductance and the AHPs: e.g. in spinal cord neurones (Mtsushima & Grillner, 1992), rat caudal raphe neurones (Bayliss et al., 1997) and rat hypoglossal motorneurones (Bayliss et al., 1995). In CNS central pattern generator neurones, the reduction of AHP by 5-HT increases neuronal firing frequency (cf. Wikstrom, et al., 1995). 5-HT_{1A} receptors appear to be responsible for the modulation of Ca^{2+} -dependent potassium conductance in many cases (Van Dogen, et al., 1986; Wu et al., 1991; Bayliss et al., 1995 ; Bayliss et al., 1997; Wikstrom, et al., 1995). The roles of other 5-HT receptor subtypes have not been examined so far.

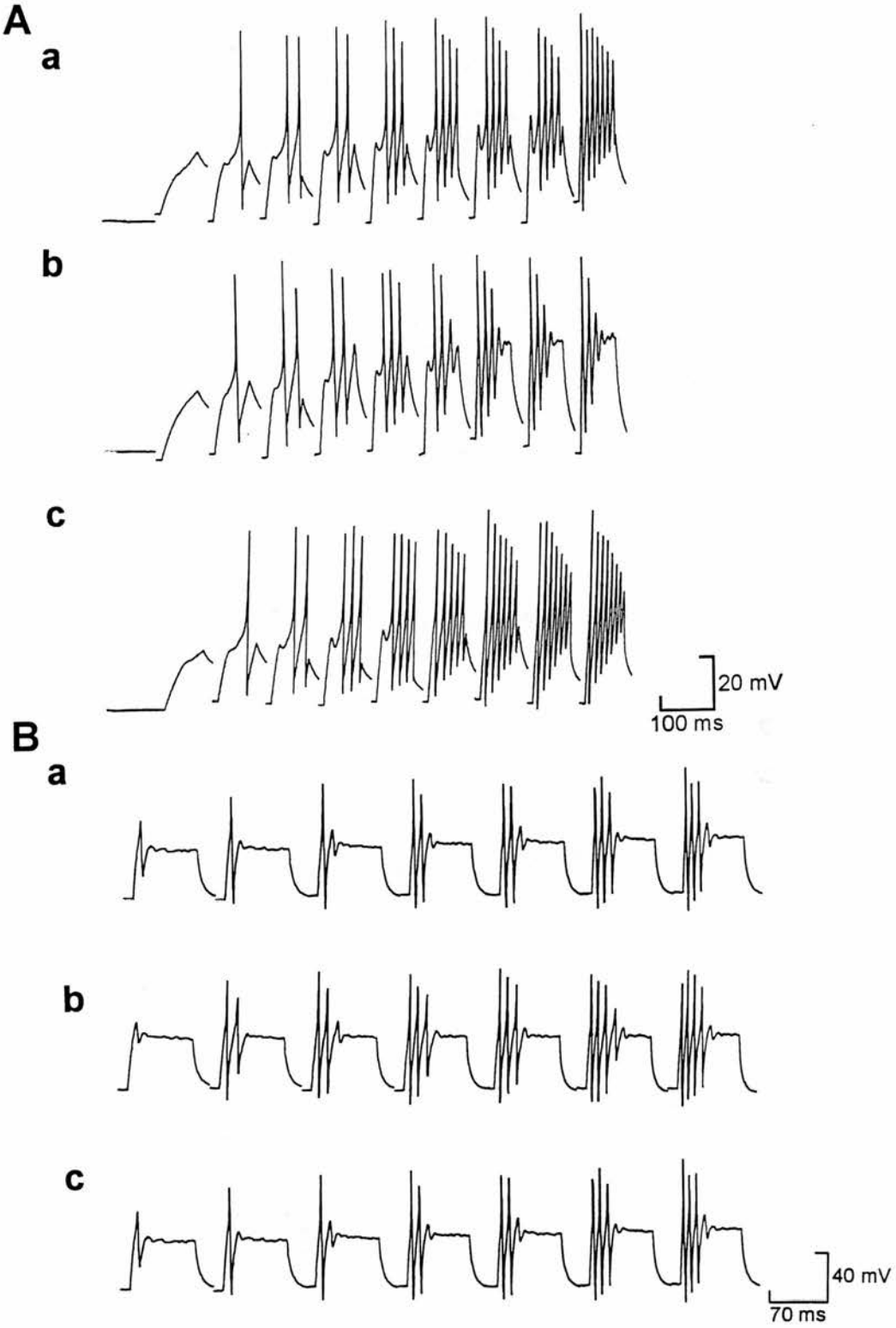
In the post-embryonic *Xenopus* spinal neurones, there are two different types of Ca^{2+} -activated potassium currents, a fast-activating, iberiotoxin-sensitive current (presumably carried by BK channels; appendix), and a slowly-activating apamin-sensitive potassium current (probably carried by SK channels, cf. Wall & Dale, 1995). The effects of Ca^{2+} channel blockers on the fast and slow- $IK_{(Ca)}$ were examined. N- and P/Q channels contribute to the slow-$IK_{(Ca)}$ in accordance with the proportion of Ca^{2+} currents carried via each channel (65% and 25%, respectively). However, the P/Q channel blocker ω-agatoxin-IVA blocked more than 50% of the fast-$IK_{(Ca)}$, and N channel blocker ω-contoxin-GVIA blocked less than 50%. This over-contribution of P/Q channels to the fast-$IK_{(Ca)}$ suggests that P/Q channels may be preferentially colocalized with the fast-$K_{(Ca)}$ channels (Q. Q. Sun & N. Dale, unpublished observations). In the acutely isolated spinal neurones, we found that N- and P/Q-type Ca^{2+} channels are differentially inhibited by 5-HT via voltage-dependent and voltage-independent mechanisms, respectively (Chapter IV). We have also found that 5-HT differentially reduced the two Ca^{2+}-dependent K^+ currents, probably via the reduction of Ca^{2+} influx via HVA Ca^{2+} channels. Whereas inhibition of the BK channels was voltage-independent, consistent with its activation by P/Q channels, the inhibition of SK

current was voltage-dependent and occurred via a voltage-dependent inhibition of the N type channels (Q. Q. Sun & N. Dale, unpublished observations).

As 5-HT modulates both inward and outward currents, its overall effect on excitability will depend on the relative balance of the two actions. If outward currents are reduced more than the inward then the net effect will be excitatory. If the reverse is true, then the net effect will be inhibitory. In acutely isolated stage 37/38 neurones, which only possessed the SK channels with very few BK channels (Wall & Dale, 1995), 5-HT always produced reversible inhibitory effects and was never found to have excitatory effects (n=10). These inhibitory effects presumably result from the inhibition of HVA Ca^{2+} currents, which have been found to play a crucial role in the excitation of *Xenopus* neurones (Dale, 1995 a b). In larval neurones the effect of 5-HT varies. In 4 out of 20 of these neurones (from stage 41 to 42), the effect of 5-HT was excitatory (Fig 5.5 B). In these neurones, 5-HT reversibly increased the number of spikes of firing in response to current injection (2.2 ±0.4 spikes in control vs. 3.5± 0.6, $p<0.05$, n=4; Fig 5.5 B), it also decreased the first interspike interval in these neurones (from 25± 5 ms to 18 ± 4 ms, n=4, $p<0.05$). These effects are very similar to the effects of the BK channel blocker iberiotoxin in *Xenopus* larval neurones (Appendix). However, 5-HT did not reduce the threshold of firing, as iberiotoxin did (Appendix). However, in the vast majority of neurones examined (16 out of 20), 5-HT decreased excitability by increasing the threshold for firing, reducing the number of spikes elicited by the same amount of current injection (2.9 ± 0.6 in control vs. 1.2 ± 0.4 in 5-HT; n=16; Fig 5.5 A), and decreasing the frequency of spiking (n=16, Fig 5.5 A). Therefore, although the predominant actions of 5-HT on soma firing in larval neurones are inhibitory (Fig 5.5 A), it did produce excitatory effects in a small proportion of neurones. Presumably this depended on balance between the reduction of inward currents (Ca^{2+} current) versus the outward currents (BK, Fig 5.5 B). This diversity of action on neuronal firing could play potentially important roles in modulating the motor activity, especially if the excitatory effects of 5-HT were present in only one subclass of neurones.

Fig 5.5 Dual effects of 5-HT on neural firing in non-sensory neurons of *Xenopus* larvae

The acutely isolated neurons fire repetitively in response to an asending series of current pulses. Whereas the main actions of 5-HT is inhibitory (A), in a small proportion of larval neurons, 5-HT also produced reversible excitatory effects on the neural firing (B). a) control, b) 5-HT 10 nM, c) wash.


A
a
b
c
20 mV
100 ms
B
a
b
c
40 mV
70 ms

5.5 Possible mechanisms underlying serotonergic modulation of fictive locomotion in *Xenopus*

Between the embryonic and larval stages (40-42), the locomotor pattern changes from a simple pattern involving brief biphasic ventral root impulses on each cycle of activity (ca. 7 ms in duration) to a more complex pattern involving bursts of discharge (ventral root discharge consists of bursts lasting around 20 ms per cycle, Sillar et al. 1992a, Fig 1.7). This change is mimicked by the application of 5-HT and its metabolic precursor, 5-hydroxytryptophan at certain stages in development (Sillar et al., 1992b; Fig 1.7). In addition, bath application 5-HT also reversibly shortens the length of swimming episodes. By studying the serotonergic modulation of the ionic currents present in *Xenopus* spinal neurones, I have provided new insight into the mechanisms by which this neuromodulator modifies locomotor activity.

5.51 Modulation of Ca^{2+} currents

In section 5.4, I summarized the consequences of modulation of calcium currents in *Xenopus* spinal neurons and showed that the major action of 5-HT is to diminish the excitability of neurons (Fig 5.5). Theoretically, this may have the following effects on the locomotion motor pattern: (1) a reduction in the number of spikes during motor bursts; (2) shortening of the swimming episodes. These speculations are supported by previous observations made from GABAergic modulation in *Xenopus* embryo, where baclofen reduces both N currents and swimming episodes (cf. Wall & Dale, 1993 & 1994). However, the major action of 5-HT on the motor pattern is excitatory- the duration of motor bursts become longer. Therefore a paradox arises: 5-HT reduces excitability of single neurons but nevertheless increases burst duration during swimming. This suggests that other mechanisms and greater complexity must be involved.

Glycinergic inhibitory potentials occurring mid-cycle in motoneurones during swimming activity are also found to be reduced by 5-HT. The effects on inhibitory synaptic strength are mediated presynaptically where 5-HT decreases the probability of glycine release from inhibitory terminals (McDearmid, et al., 1997). In my experiments, the majority (>80%) of neurones examined were probably the glycinergic commissural interneurones that generate this midcycle inhibition in the intact system. If 5-HT were to modulate N and P/Q channels in the terminals of these neurons, this could contribute to the presynaptic inhibition and a reduction of inhibitory synaptic strength in *Xenopus* (cf. Dolphin, 1998; Chapter IV). If 5-HT were to have less effect on the excitatory synaptic drive, the selective reduction of inhibitory synaptic drive alone may allow the neurones on the opposite sise to fire longer and hence contribute to the increase of burst duration by 5-HT. However, these ideas need to be tested experimentally.

5.52 Modulation of Ca^{2+} activated potassium currents and soma firing

One of the functional consequences of the voltage-dependent serotonergic inhibition of HVA channels is to decrease the Ca^{2+} -dependent AHP, through which the soma excitability is increased. Therefore although the effects of 5-HT on Ca^{2+} currents are inhibitory, the net effect on soma excitability could still be excitatory in some neurones. (cf. Bayliss et al., 1995 & 1997). In larval neurones, 5-HT reduced both HVA currents and the $K_{(Ca)}$ currents. The larval *Xenopus* spinal neurones expressed both BK and SK channels, which were both reduced by 5-HT. The BK channel activates very rapidly and is large enough to quickly recharge the membrane therefore it could contribute to both spike repolarization (e.g. Sah, 1995) and burst termination during locomotor activity (Appendix). In section 5.4 of this chapter, I have demonstrated that although the predominant actions of 5-HT on soma firing in larvae neurones are inhibitory (Fig 5.5 A), it also produced excitatory effects in a small proportion of neurones (20%). Presumably this depended on balance between the reduction of inward currents

(Ca^{2+} current) versus the outward currents (BK, Fig 5.5 B). This excitatory effect is only likely to contribute to the excitatory effects of 5-HT on the motor pattern if it occurs in only one subclass of neurones (such as excitatory interneurons). Such an effect could enhance the effect of the reduction of the midcycle inhibition. This effect could be enhanced if the net effect of 5-HT on the excitatory interneurons was to increase their excitability through reduction of the BK currents. This would only be known if *in situ* patch clamp recordings were performed, where electrophysiological data can be matched with neuroanatomical evidence.

The slow-$IK_{(Ca)}$ often produces a long-lasting Ca^{2+} dependent after-hyperpolarization (AHP) which plays a role in spike frequency adaptation in mammals (for review see Blatz and Magleby, 1987). In lamprey, the Ca^{2+} dependent AHP current may be required for the termination of activity during each cycle of swimming (Hill, et al., 1992). However, in embryonic *Xenopus*, the apamin sensitive slow-$IK_{(Ca)}$ plays a role in termination of swimming but has little effect on the swimming motor pattern (Wall and Dale, 1995). Thus the inhibition of slow-$IK_{(Ca)}$ by 5-HT may not contribute to the modulation of motor pattern by 5-HT.

5.53 Summary of possible mechanisms underlying modulation of *Xenopus* motor pattern by 5-HT

My experiments from acutely isolated spinal neurones suggests the effect of 5-HT on the motor pattern for swimming in *Xenopus* larvae may be produced by the following mechanisms:

(1) Inhibition of Ca^{2+} influx via HVA currents in the soma, which may reduce excitability and raise threshold for firing of spinal CPG neurones, and mainly contributes to the shortening of swimming episodes and indirectly to reduction of transmitter release (cf. Wall & Dale ,1995; Dale 1995 a b).

(2) Inhibition of Ca^{2+} influx via N and P/Q channels in the synaptic terminals could contribute to presynaptic inhibition of the mid-cycle IPSPs. This may contribute to the increase in burst duration during motor activity in spinal neurones.

(3) Inhibition of BK channels may increase the excitability of some spinal neurones, and thus partially contribute to the broadening of motor burst duration in certain classes of neurones (Appendix).

Other unidentified mechanisms may also exist. Therefore the actions of exogenously applied 5-HT on motor pattern is not produced by a simple component, but rather by a summation of multiple mechanisms involving both changes in neuronal membrane properties and circuitry properties through which neurones are connected.

5.6 Serotonergic modulation of sensory transmission in *Xenopus*

In paralyzed animals, exogenous 5-HT at 1-10 μM reversibly inhibits (within 1-2 min) the activation of fictive swimming in response to electrical stimulation of R-B free nerve endings in the skin. At threshold stimulus intensities for swimming under control conditions, intracellularly recorded EPSPs in contralateral motoneurones are completely abolished by 5-HT without any obvious change in neuronal conductance or membrane potential. However, increasing the stimulus voltage can activate swimming with enhanced motor burst discharge on each cycle (Sillar et al., 1992, Fig 5.4). This suggests that 5-HT inhibits the swim-initiating pathway in addition to the motor rhythm-generating circuitry itself (Sillar et al., 1994). The effects of 5-HT on the spontaneous release from R-B neurons was examined, by recording postsynaptically from dorsal lateral interneurones. Bath application of 5-HT dramatically reduces the rate of occurrence of these spontaneous EPSPs, consistent with a presynaptic locus for the inhibitory effects of 5-HT. This

suggests that one major site at which 5-HT inhibits R-B sensory pathway function is via a direct activation of 5-HT receptors located on the R-B afferent terminals, which leads to a reduction in the probability of transmitter release onto dorsal lateral interneurones (Sillar & Simmers, 1994; Fig 5.4).

However, the cellular actions and pharmacological profile of 5-HT is not clear. In acutely isolated R-B neurones, we found 5-HT produced voltage-independent inhibition of N and P/Q type HVA Ca^{2+} channels and T-type Ca^{2+} channels (Chapter II & III). N-, and P/Q- type Ca^{2+} channels are involved in supporting synaptic transmission in CNS and PNS (Leubke et al., 1993; Leubke and Dunlap, 1994; Wall and Dale, 1994; Wheeler et al., 1994; see Dunlap 1997, for review). Therefore, if both 5-HT receptors and N and P/Q Ca^{2+} channels were located at presynaptic terminals, the reduction of N- and P-type currents could contribute to the previously reported presynaptic inhibition of transmitter release from R-B neurones (Sillar & Simmers, 1994). Furthermore, since HVA channels in the soma also contribute to the excitability of the R-B neurone firing, their modulation also suggests an additional locus: an increase in the firing threshold.

However T-type channels are not generally thought to be involved in supporting synaptic transmission, instead they contribute to spike initiation. R-B neurones have a very negative resting potential (around -90 mV, Spitzer and Lamborghini, 1976), so the T-type Ca^{2+} current is unlikely to be in an inactivated state in these neurones at rest. Since the T-type currents are the only voltage-gated inward currents activated in these neurones between -60 and -30 mV, they may play an important role in triggering action potentials. Small reductions of the T-type Ca^{2+} current could therefore modulate the responsiveness of Rohon-Beard neurones to sensory stimuli. We found that both selective T-type channel blockers and low doses of 5-HT greatly reduced the probability of R-B neurone firing in response to threshold current injection at a holding membrane potential of -90 mV, but not at more positive membrane potentials of -50 mV, where the T-type channels were in inactivated. This strongly supports our hypothesis that T-type channels play a role in spike initiation in R-B neurones, and that the modulation of T-type

channels may raise the R-B firing threshold. We also found that the T-type currents were much more sensitive to serotonin agonists (IC_{50} = 0.1 nM for 5-HT) than HVA currents (IC_{50} = 40 nM for 5-HT). During 5-HT release, the T-type channels are thus likely to be modulated first when the concentrations of 5-HT are low. In our cell-attached recordings, I found that in patches located near the neuronal process, but not the nucleus, quasi-macroscopic T-type currents can be elicited. The most effective locus for the T-type channels to influence spike initiation would be in the peripheral neurites close to the site of mechanotransduction (Fig 5.6).

By considering the kinetic parameters of TTX-sensitive Na channels (cf. Dale, 1995a) and T channels (chapter II), I proposed that current through T channels located in peripheral neurites would be activated by stretch-activated non-selective cation channels. Their activation causes further depolarization of the membrane potential to levels which can activate Na channels and elicit action potentials (Fig 5.7). Itherefore propose that both the 5-HT receptors and T-type channels are in the peripheral neurites and that modulation of these channels alters the sensitivity of the sensory pathway (Fig 5.5 & 5.6).

In summary, our experiments from acutely isolated R-B neurones suggest the effects of 5-HT on sensory transmission of *Xenopus* larvae may be produced by the following mechanisms (Fig 5.6). (1) Reduction of N and P/Q channels by 5-HT in the soma could reduce the excitability and raise threshold for firing. (2) Reduction of N and P/Q channels by 5-HT in the synaptic terminals could contribute to the presynaptic inhibition of EAA release and therefore prevent the initiation of swimming. (3) The T-type channels are activated at much lower membrane potentials and the modulation of T-type channels by 5-HT via a novel signalling pathway in the soma raises the threshold for firing and reduces the probability of firing. If these channels are also located in the periphery, where the generator potential is produced, the inhibition could serve as the first step to prevent excitation of R-B neurones from occurring.

Fig 5.6 Schematic graph showing the possible mechanisms underlying the serotonergic modulation of sensory transmission in R-B neurons.

I propose 5-HT_{1A} and 5-HT_{1D} receptors located at three different levels: (1) at peripheral neurites, where the generator potential is evoked by mechano-sensory stimuli, the T channels may involved in filling the gap between generator potential and threshold for firing and its modulation by 5-HT could raise the threshold for firing; (2) at soma, the inhibition of HVA and T channels by 5-HT have also raised the threshold for firing and serves as a second mechanism; (3) at synaptic terminals, inhibition of Ca^{2+} influx, particularly via N and P/Q channels could inhibit the release of EAA.

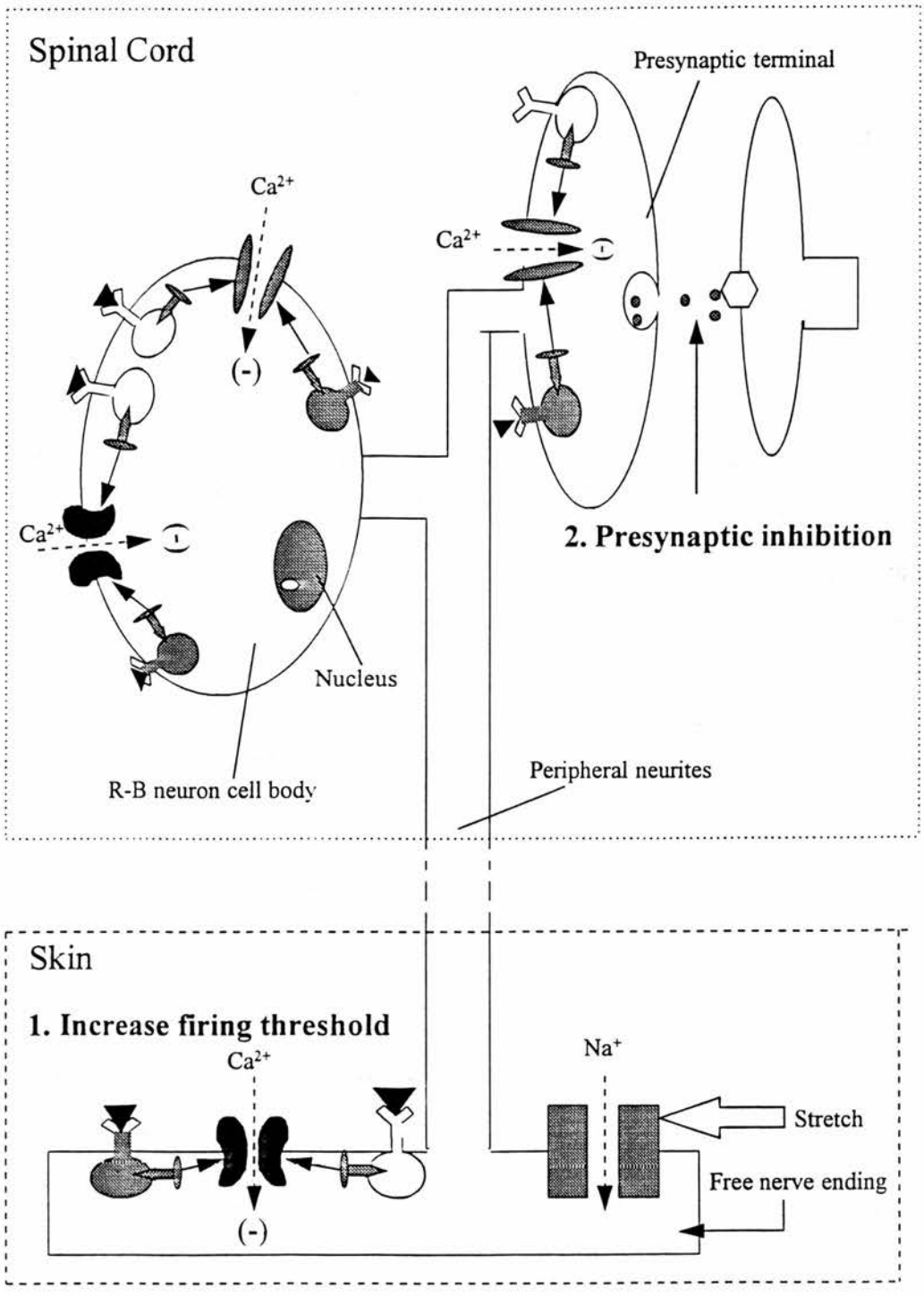
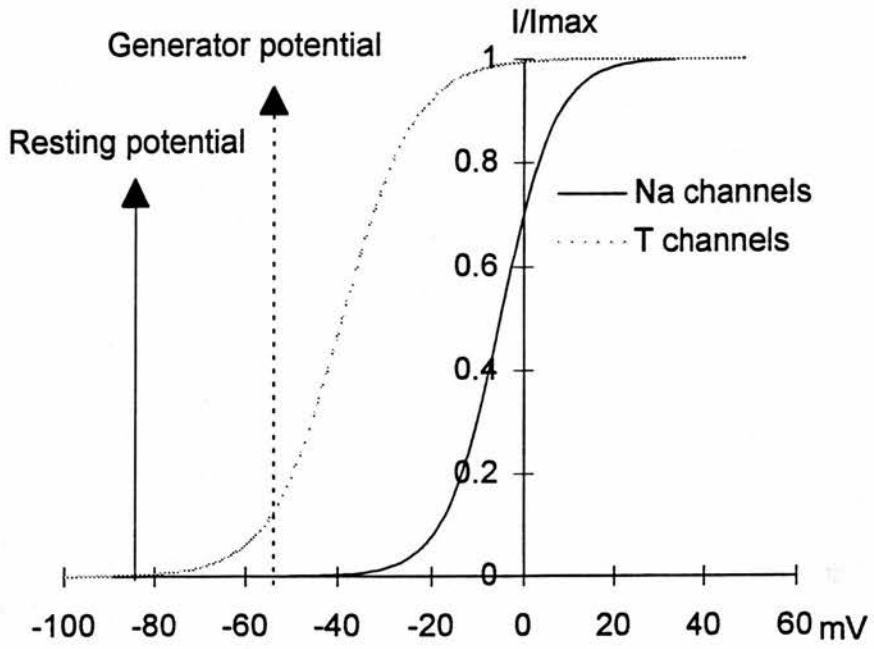

Spinal Cord
Presynaptic terminal
Ca^{2+}
Ca^{2+}
(-)
Ca^{2+}
2. Presynaptic inhibition
Nucleus
R-B neuron cell body
Peripheral neurites
Skin
1. Increase firing threshold
Ca^{2+}
Na^{+}
Stretch
Free nerve ending
(-)

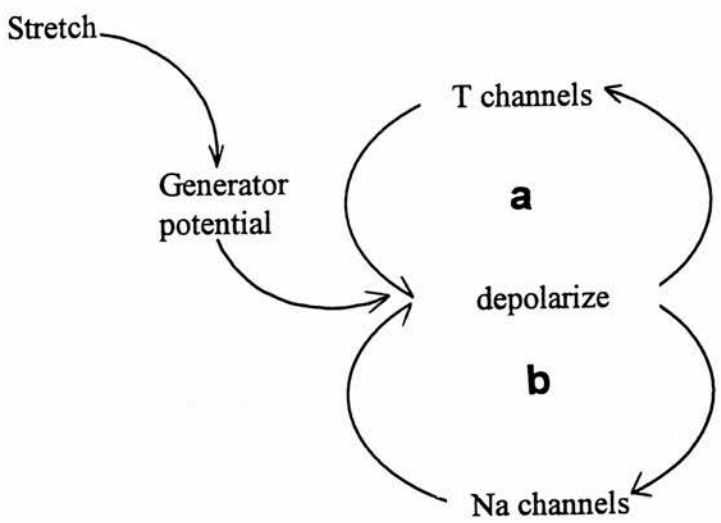
Fig 5.7 A role for T-type channels in generating action potentials of R-B neurons

A) Activation kinetics for T channels and Na channels were obtained from the best fit of the Boltzmann equation to experimental data for T channels and Na channels from Sun & Dale (1997) and Dale (1995a), respectively. B) Schematic graph showing how different ion channels, including the stretch activated cation channels, T channels and voltage-gated Na channels could be involved in the trigggering of action potentials in R-B neurons. During sensory stimuli, the T channels will normally be activated first and cause further regenerative depolarization of the the membrane loop (a), this in turn will activate Na channels and start regenerative loop (b), which will eventually led to generation of an action potential.

A


Generator potential
Resting potential
I/Imax
1
0.8
0.6
0.4
0.2
0
Na channels
T channels
-100
-80
-60
-40
-20
0
20
40
60 mV

B


Stretch
Generator potential
T channels
a
depolarize
b
Na channels

APPENDIX

DEVELOPMENTAL CHANGES IN EXPRESSION OF ION CURRENTS ACCOMPANY MATURATION OF LOCOMOTOR PATTERN IN FROG TADPOLES

Q-Q. Sun and N. Dale

***The appendix was writen in collaboration with Dr N. Dale. Q.-Q. Sun contributed to the whole-cell patch clamp work and Dr. N. Dale contributed to the experiments carried out in situ and the writing.**

SUMMARY

1. The K^+ currents of spinal neurons acutely dissociated from *Xenopus* larvae were studied and compared to those of neurons dissociated from *Xenopus* embryos.

2. The density of total outward current in the larval and embryonic neurons remained the same from stage 37/38 to stage 42.

3. Almost all neurons at stage 42 expressed a fast activating Ca^{2+}-dependent K^+ current (I_{KCa}) that was largely absent from embryonic neurons. Whereas I_{KCa} became larger and more prevalent during development, the delayed-rectifier K^+ currents were down-regulated.

4. About 53% of I_{KCa} was selectively blocked by iberiotoxin which had no effect on the delayed rectifier K^+ currents or the K^+ currents of embryonic neurons

5. The firing properties of neurons isolated from embryos were unchanged by iberiotoxin. However the toxin greatly increased the frequency of firing in larval neurons.

6. Iberiotoxin extended the duration of ventral root bursts during fictive swimming in larvae at stages 41 and 42 but had no effect at stage 40. The progressive expression of I_{KCa} thus contributed to burst termination.

7. Ihave found that changes in expression of outward current closely correlate with the maturation of the motor pattern during development. At a time when the motor pattern has a need for a burst-terminating mechanism, the larval neurons express a channel with properties appropriate for such a role.

INTRODUCTION

In a variety of animals the capacity to generate both locomotor and respiratory patterns arises early in embryonic development (Casasnovas & Meyrand, 1995; Ho & O'Donovan, 1993; Ho, 1997; Paton & Richter, 1995; Sillar, Wedderburn & Simmers, 1991; Sillar, 1994). As development proceeds, these motor patterns gradually mature. Although changes to network circuitry (Antal, Berki, Horvath & O'Donovan, 1994; Berki, O'Donovan & Antal, 1995; Paton, Ramirez & Richter, 1994) and the intrinsic properties of the neurons (Sillar, Simmers & Wedderburn, 1992) accompany this maturation, causal links between changes at cellular and network levels and maturation of motor patterns have been harder to establish.

In *Xenopus*, the embryonic motor pattern is characterized by neurons firing only a single spike per cycle. This changes to the larval pattern (stage 40 onwards) in which neurons fire bursts of spikes (Sillar, Wedderburn & Simmers, 1991). Although descending serotonergic reticulospinal fibres have been proposed as a trigger for this change (Sillar, Woolston & Wedderburn, 1995), the mechanisms underlying the change in the motor pattern remain unknown. Nevertheless the observation that neurons individually become more excitable (Sillar *et al*, 1992), suggests that changes in the expression of ion channels may be important. Previous studies have extensively characterized the ionic currents of embryonic spinal neurons at stage 37/38 (Dale, 1993; Dale, 1995a; Wall & Dale, 1995). Ihave extended this analysis to the K^+ currents of larval neurons to see whether new channels are expressed and whether embryonic channels may be down-regulated.

Ihave found that a process of ion current substitution accompanies the development of the larval motor pattern. While the total amount of depolarization-activated K^+ current remains the same in embryonic and larval neurons, the proportion that is Ca^{2+}-independent drops with development, and a

new fast-activating Ca^{2+}-dependent K^+ current, I_{KCa}, appears in the larval neurons. By using a specific blocker of I_{KCa}, iberiotoxin, Ihave shown that this current plays a role both in limiting the repetitive firing of the larval neurons and in the termination of bursts during the larval swimming pattern.

METHODS

All embryos and larvae were staged according to the external criteria of Nieuwkoop & Faber (1956). *Preparation of neurons* Acutely isolated neurons from the spinal cords of *Xenopus* embryos and larvae were obtained using methods previously described (Dale, 1991; Dale, 1995a). In brief, embryos were anaesthetized in MS222, and their spinal cords carefully dissected free. A combination of brief enzymatic treatment (30sec to 1 min in 8mg/ml pronase E, SIGMA) and mechanical dissociation reliably gave a good yield of healthy neurons. These were plated onto poly-D-lysine-coated dishes. Neurons derived from all developmental stages were left for about 1 hour before recording commenced.

Patch clamp recordings from isolated neurons Whole cell patch clamp recordings were made from neurons acutely isolated from the *Xenopus* embryo and larval spinal cord (Dale, 1991) that had multipolar and commissural neuron-like morphologies and would thus have constituted a mixture of motoneurons and excitatory and inhibitory premotor interneurons (Dale, 1991). All of these are involved in motor pattern generation. Electrodes were fabricated using a Sutter Instruments P97 puller from glass obtained from WPI (TW 150F). A List LM_PC amplifier together with a DT2831 interface (Data Translation) were used to record and digitize (at 40kHz) the voltage and current records. The recordings had access resistances ranging from 4 to 12MΩ. Between 70 and 85% of this access resistance was compensated for electronically.

The external medium contained 115mM NaCl, 2.4mM $NaHCO_3$, 3mM KCl, 10mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, pH 7.4 while the pipette solution contained 100mM $KMeSO_3$, 5mM KCl, 5mM $MgCl_2$, 20mM HEPES, 5mM ATP

and 2mM BAPTA, pH 7.4. To block the Ca^{2+}-dependent currents, the Ca^{2+} in the recording medium was substituted by an equimolar amount of Mg^{2+}. Drugs were applied via a multibarrelled microperfusion system. Iberiotoxin was obtained from RBI. Currents were measured from a holding potential of -50mV.

Extracellular Recordings *Xenopus* embryos and larvae were prepared for extracellular ventral root recordings in accordance with the UK Animals (Scientific Procedures) Act, 1986 using previously described methods (Sillar *et al*, 1991; Dale, 1995b). Drug access was facilitated by bilateral removal of the rostral myotomes and loosening the dorsal attachment of the remaining myotomes. The spinal cord was transected at the first or second post-otic myotome. The preparation was placed in a small bath (0.5ml volume) and continually superperfused with saline containing 115mM NaCl, 3mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 2.4mM $NaHCO_3$, 10mM HEPES, pH 7.4 at room temperature. Drugs were applied by superfusion of the bath. Electrical stimuli to the skin were used to trigger swimming with an interval of 3 minutes between the beginning and end of consecutive episodes.

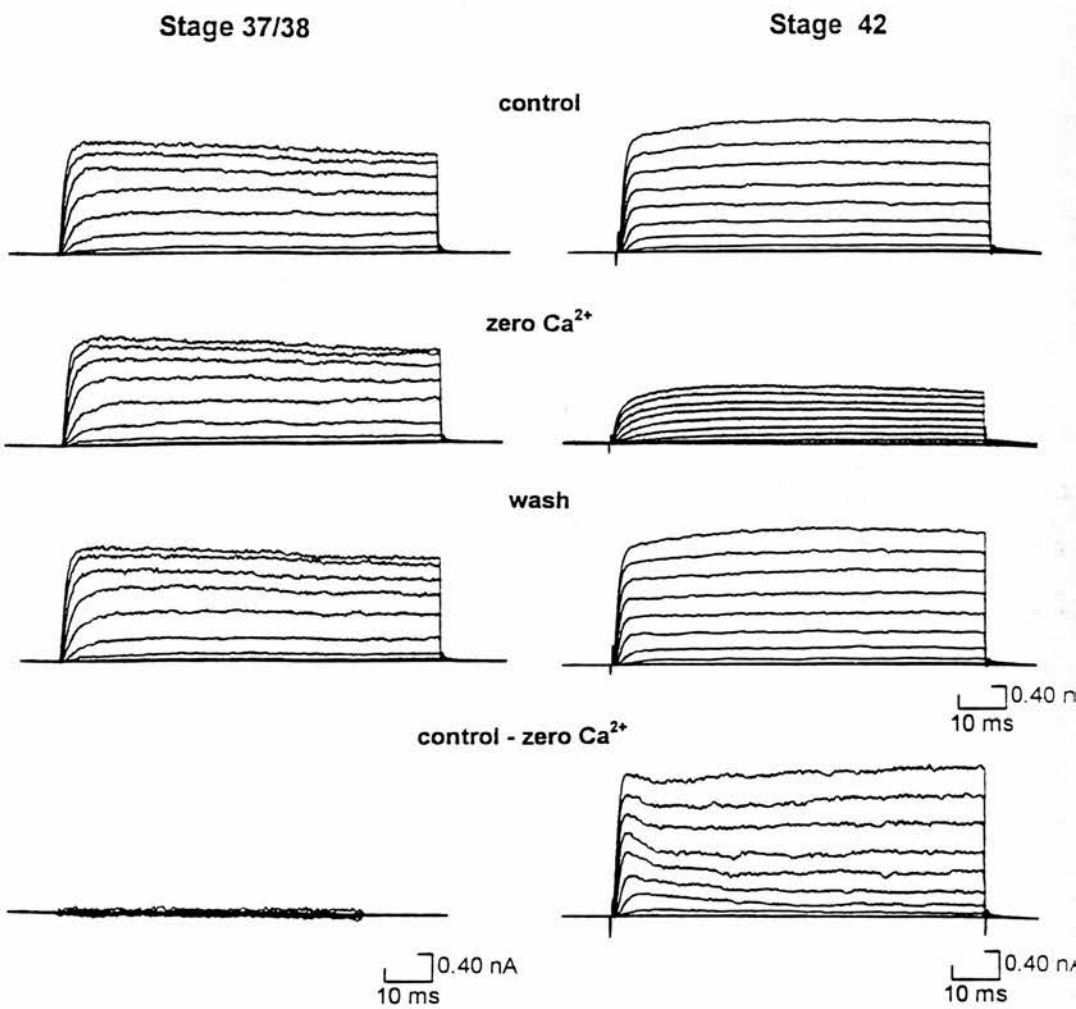
Analysis of burst duration By means of a Data Translation DT31EZ, swimming episodes were digitized and stored on a computer. The ventral root activity was then rectified and integrated with a 2ms period. Changes in burst duration were assessed in two ways. The first was a threshold crossing method in which a burst was defined as the time from the first crossing above threshold to the last crossing below threshold. The second method involved averaging the rectified and integrated ventral root bursts. Using the start of each burst as a reference point, the computer averaged a stretch of activity on either side of the burst. Both methods gave similar results, although the threshold crossing method tended to underestimate the duration of the bursts.

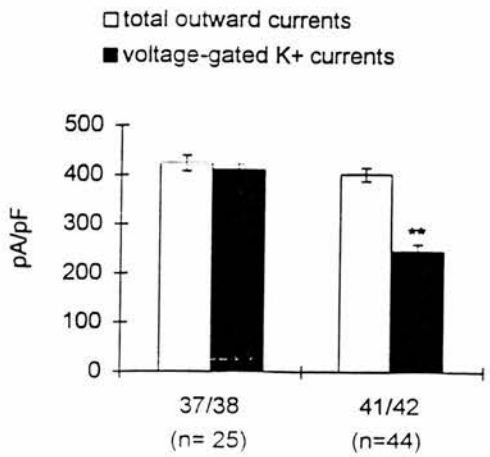
RESULTS

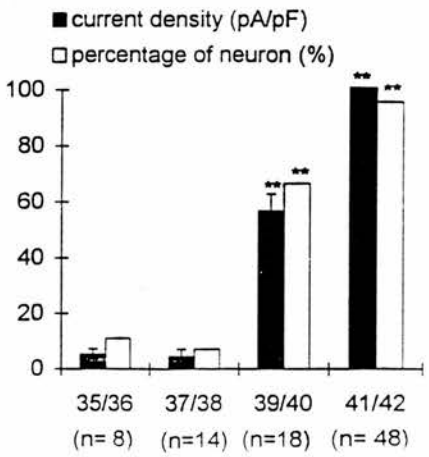
Larval neurons express a new I_{KCa}

Figure A.1 The delayed rectifier K^+ currents are down-regulated and I_{KCa} is newly expressed during development.

(a) The voltage-gated currents, elicited from a holding potential of -50mV by 10mV steps from -40 to +50mV, of a neuron acutely dissociated from a stage 37/38 embryo recorded in (from the top) control saline, zero Ca^{2+} saline, wash and the control-zero Ca^{2+} difference currents. There is no Ca^{2+}-sensitive current. (b) Voltage-gated currents (evoked by same voltage protocol as in a) for a neuron acutely dissociated from a stage 42 larva (traces equivalent to those in a). There is a large Ca^{2+}-sensitive current in this neuron which has both a transient and a sustained component. (c) The total and Ca^{2+}-independent outward current density (normalized to cell capacitance) shown for neurons from stages 37/38 and from stages 41 and 42 combined. Note that although the total current density remains the same the density of the Ca^{2+}-independent currents declines (** $P<0.01$ stages 37/38 vs. 41/42, t-test). (d) The development of I_{KCa}. Both the proportion of neurons that possessed the current and the density of the current increased with developmental stage. (** $P<0.01$ stages 39/40 vs. 37/38, and stages 41/42 vs. 39/40, t-test for current density and a χ^2 contingency test for percentage of neurons)


Stage 37/38
Stage 42
control
zero Ca2+
wash
0.40 nA
10 ms
control - zero Ca2+
0.40 nA
10 ms
0.40 nA
10 ms


total outward currents
voltage-gated K+ currents
pA/pF
500
400
300
200
100
0
37/38
(n= 25)
41/42
(n=44)


current density (pA/pF)
percentage of neuron (%)
100
80
60
40
20
0
35/36
(n= 8)
37/38
(n=14)
39/40
(n=18)
41/42
(n= 48)

The firing properties of *Xenopus* neurons change with development: the larval neurons are capable of more repetitive firing than their embryonic counterparts (Sillar *et al*, 1992). Itherefore examined the K^+ currents in acutely isolated spinal neurons of *Xenopus* embryos and larvae. To casual inspection, the currents in control appear very similar (Fig A.1a, b). To take into account the fact that larval neurons are smaller than their embryonic counterparts, Inormalized current density to cell capacitance. The density of the total outward currents was the same in embryonic and larval neurons (Fig A.1c, open bars).

However when the Ca^{2+}-dependence of outward currents was examined, a dramatic difference between larval and embryonic neurons was seen. Whereas very few embryonic neurons possessed I_{KCa}, virtually all neurons at stage 42 possessed this current (compare Figs A.1a and 1b). Thus at stage 42, the delayed-rectifier K^+ currents have been down-regulated (Fig A.1c, solid bars), and a new current, I_{KCa}, has been expressed which constitutes 29±2.5% (n=25) of the total outward current. By looking at a developmental sequence from stage 37/38 to stage 42, we found that both the density of I_{KCa} in individual neurons and the proportion of neurons possessing this current increased (Fig A.1d). The I_{KCa} is kinetically complex having both transient and sustained components and may be similar to the currents previously described in cultured *Xenopus* neurons (Blair & Dionne, 1985; O'Dowd, Ribera & Spitzer, 1988; Ribera & Spitzer, 1987).

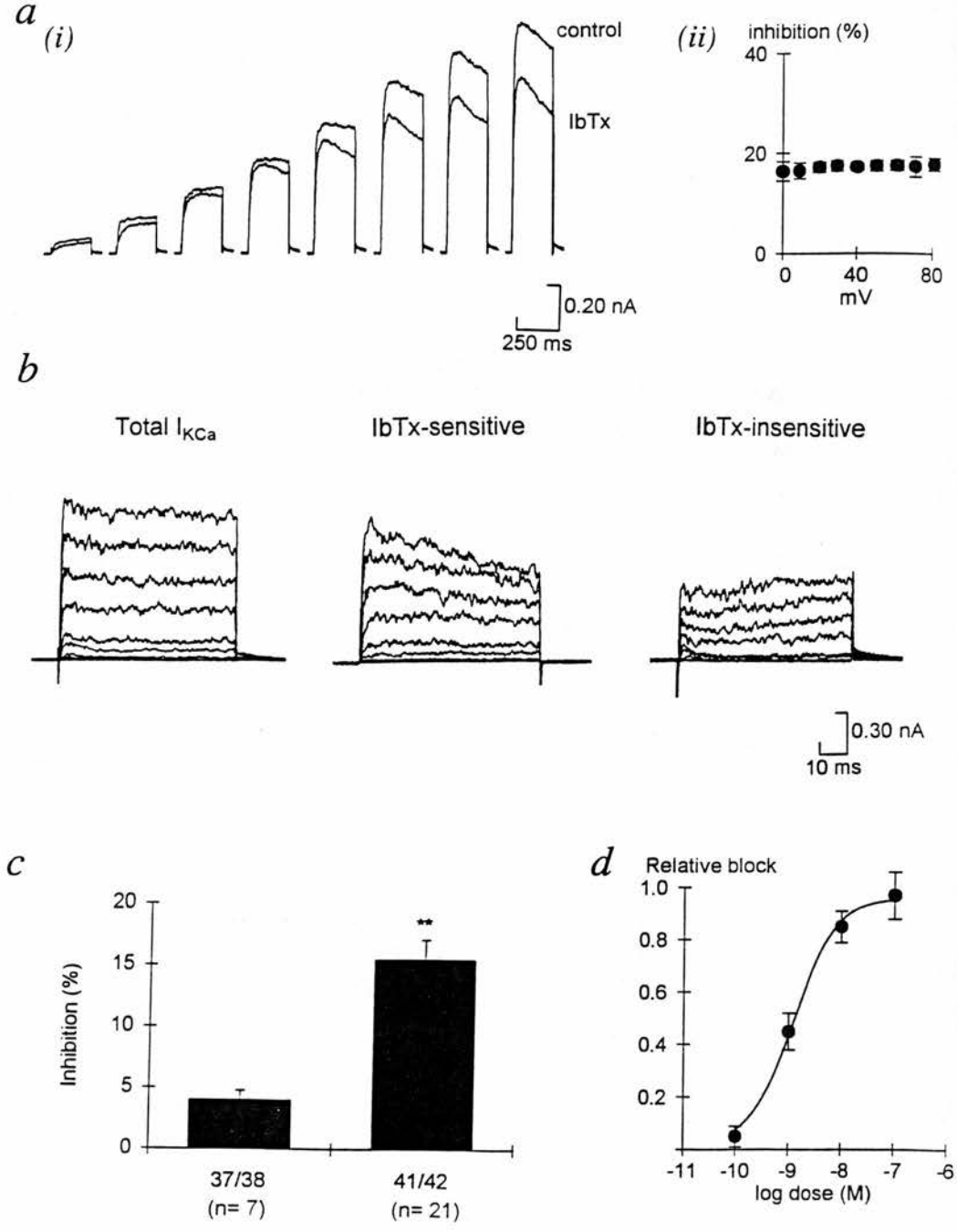
To check that the I_{KCa} was also present at more physiological levels of Ca^{2+}, I compared the Ca^{2+}-sensitivity of outward currents at 2 and 10mM external Ca^{2+}. The amount of I_{KCa} remaining at the lower level of 2mM was 64±6% (n=4) of that present at 10mM Ca^{2+}. This means that I_{KCa} is able to contribute to neural function under physiological conditions.

The new I_{KCa} is partially blocked by iberiotoxin

To test the functional roles of the newly-expressed I_{KCa}, we used iberiotoxin, a specific blocker of the B-K Ca^{2+}-dependent channels (Galvez, Gimenez-Gallego, Reuben, Katz, Kaczowrovski & Garcia, 1990). We found that iberiotoxin, at

Figure A.2 The new Ca^{2+}-dependent K^+ current is partially blocked by iberiotoxin.

(a) The effect of iberiotoxin (IbTx) on the outward currents evoked in a stage 42 neuron by 10mV steps from +10 to +80mV from a holding potential of -50mV. The total current is reduced at all potentials (i) and the percentage block (relative to the current in control) is constant at all potentials (ii) suggesting that the block is non-voltage-dependent (n=6). (b) A comparison of the total I_{KCa} with its IbTx-sensitive and -insensitive components (control minus zero-Ca^{2+}, control minus IbTx and IbTx minus zero-Ca^{2+} difference currents respectively). These were evoked in the same neuron, from a holding potential of -50mV, by 10mV steps from -40 to +40mV. The currents are characterized by large noise, suggesting that they are carried by relatively few high conductance channels. The slowly-activating component of the IbTx-insensitive current is almost certainly the S-K current that is present in these neurons. (c) The iberiotoxin-sensitive current shows the same development of expression as the I_{KCa}. Iberiotoxin had no significant effect on the embryonic stage 37/38 neurons, but significantly blocked neurons from stages 41 and 42 ($p<0.01$, t-test). (d) The dose response for iberiotoxin plotted together with the best-fitting Hill equation (line). The IC_{50} was 1.1±0.6 nM, n=4.


a
(i)
control
IbTx
0.20 nA
250 ms
(ii)
inhibition (%)
40
20
0
0
40
80
mV
b
Total I_{KCa}
IbTx-sensitive
IbTx-insensitive
0.30 nA
10 ms
c
Inhibition (%)
20
15
10
5
0
**
37/38
(n= 7)
41/42
(n= 21)
d
Relative block
1.0
0.8
0.6
0.4
0.2
-11
-10
-9
-8
-7
-6
log dose (M)

doses from 10-100nM, partially and selectively blocked I_{KCa} in larval neurons in a non-voltage-dependent manner (Fig A.2a,b,d). Consistent with their being generated by a large conductance channel, the iberiotoxin-difference currents were very noisy (Fig A.2b). We therefore suggest that the I_{KCa} is an example of a B-K current. By comparing the effects of iberiotoxin to those of zero Ca^{2+} in the same cell (Fig A. 2b), we found that the iberiotoxin-sensitive current comprised 53±10% (n=9) of the total Ca^{2+}-dependent K^+ current.

To check that iberiotoxin had no effect on the delayed rectifier and other non-Ca^{2+}-dependent K^+ currents, we blocked the Ca^{2+}-dependent currents by either removing external Ca^{2+} or applying Cd^{2+}. Under these conditions, iberiotoxin had no effect on the outward currents (mean change: 1.0±0.9%, n=6). As might be expected from the development of the I_{KCa}, we found that iberiotoxin had very little effect on the outward currents of the embryonic neurons (Fig A.2c).

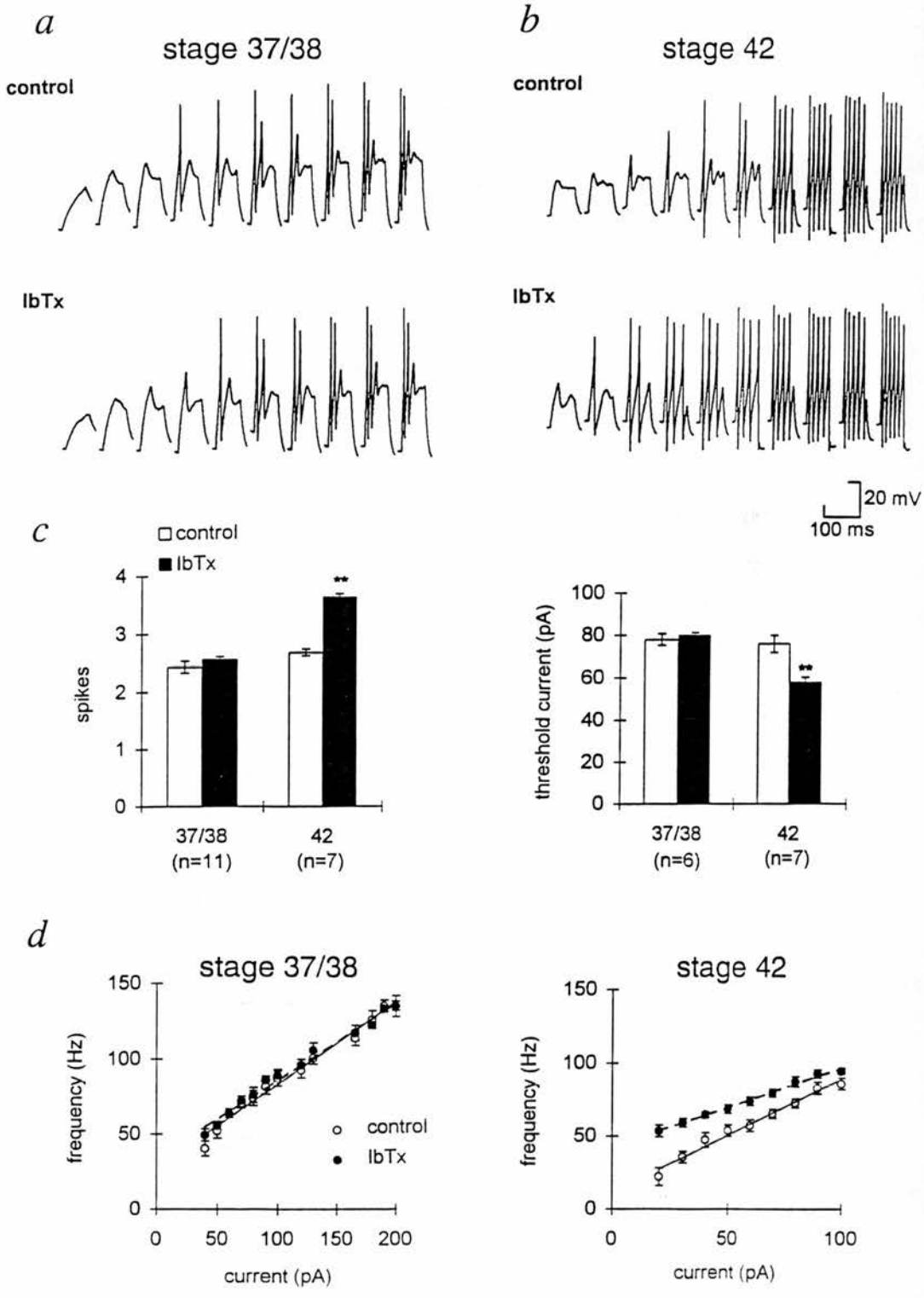
The role of I_{KCa} in control of repetitive firing and motor pattern generation

We next examined the effects of iberiotoxin on the repetitive firing characteristics of acutely isolated embryonic and larval neurons. In embryonic neurons, iberiotoxin had no effect on the number of spikes evoked by a series of increasing current pulses, the threshold current, or the frequency of firing (Fig A.3a,c,d). However in larval neurons, iberiotoxin increased the number of spikes evoked by current pulses, reduced the current threshold, and increased the frequency of firing (Fig A.3b,c,d). Clearly the newly expressed I_{KCa} plays an important role in controlling the excitability of the larval neurons.

We also examined whether iberiotoxin changed spike width. At stage 42 the time from the peak of the action potential to half repolarization for the first spike in a train was 1.2±0.2ms in control and 1.3±0.2ms in iberiotoxin (n=9, $p<0.01$). Although this small change is statistically significant it is probably of no functional importance. At stage 37/38 there was no significant change in spike

Figure A.3 I_{KCa} controls excitability of larval but not embryonic neurons.

(a) Current clamp recording from an acutely isolated embryonic neuron (stage 37/38) showing response to an ascending series of current pulses. Iberiotoxin (IbTx) had no effect on the firing in response to current injection. (b) Similar recordings from a neuron isolated from a stage 42 larva showed that blockade of the I_{KCa} by iberiotoxin both reduced the threshold and increased the number of spikes evoked by current injection. (c) Summary data for the effects of iberiotoxin on the mean number of spikes evoked during the sequence of current pulses shown in (a) and (b) (left) and the threshold current for firing (right). (d) Iberiotoxin had no effect on the frequency of firing in embryonic neurons (n=5), but enabled larval neurons to fire at a significantly higher rate (n=6). Frequency of firing was calculated as the inverse of the first interspike interval.


a
stage 37/38
control
IbTx
b
stage 42
control
IbTx
20 mV
100 ms
c
control
IbTx
spikes
0
1
2
3
4
**
37/38
(n=11)
42
(n=7)
threshold current (pA)
0
20
40
60
80
100
**
37/38
(n=6)
42
(n=7)
d
stage 37/38
frequency (Hz)
0
50
100
150
control
IbTx
0
50
100
150
200
current (pA)
stage 42
frequency (Hz)
0
50
100
150
0
50
100
current (pA)

width (means 1.3±0.2ms and 1.3±0.2ms for control and iberiotoxin respectively, n=6).

To test the role of the I_{KCa} in motor pattern generation, we applied iberiotoxin to the intact larva while monitoring ventral root activity. At stage 40, we found that iberiotoxin had no significant effect on burst duration or cycle period of the ventral root activity (Table A.1, Fig A.4a). However at both stage 41 and stage 42, despite blocking only around half of the total I_{KCa}, iberiotoxin significantly increased burst duration by 24-33% (Table A.1, Fig A.4b,c). As iberiotoxin had no significant effect on cycle period at stages 41 and 42, we conclude that the I_{KCa} contributes to burst termination during swimming and that block of this current not only increases absolute burst duration but also the proportion of the motor cycle that each burst lasts (Table 1).

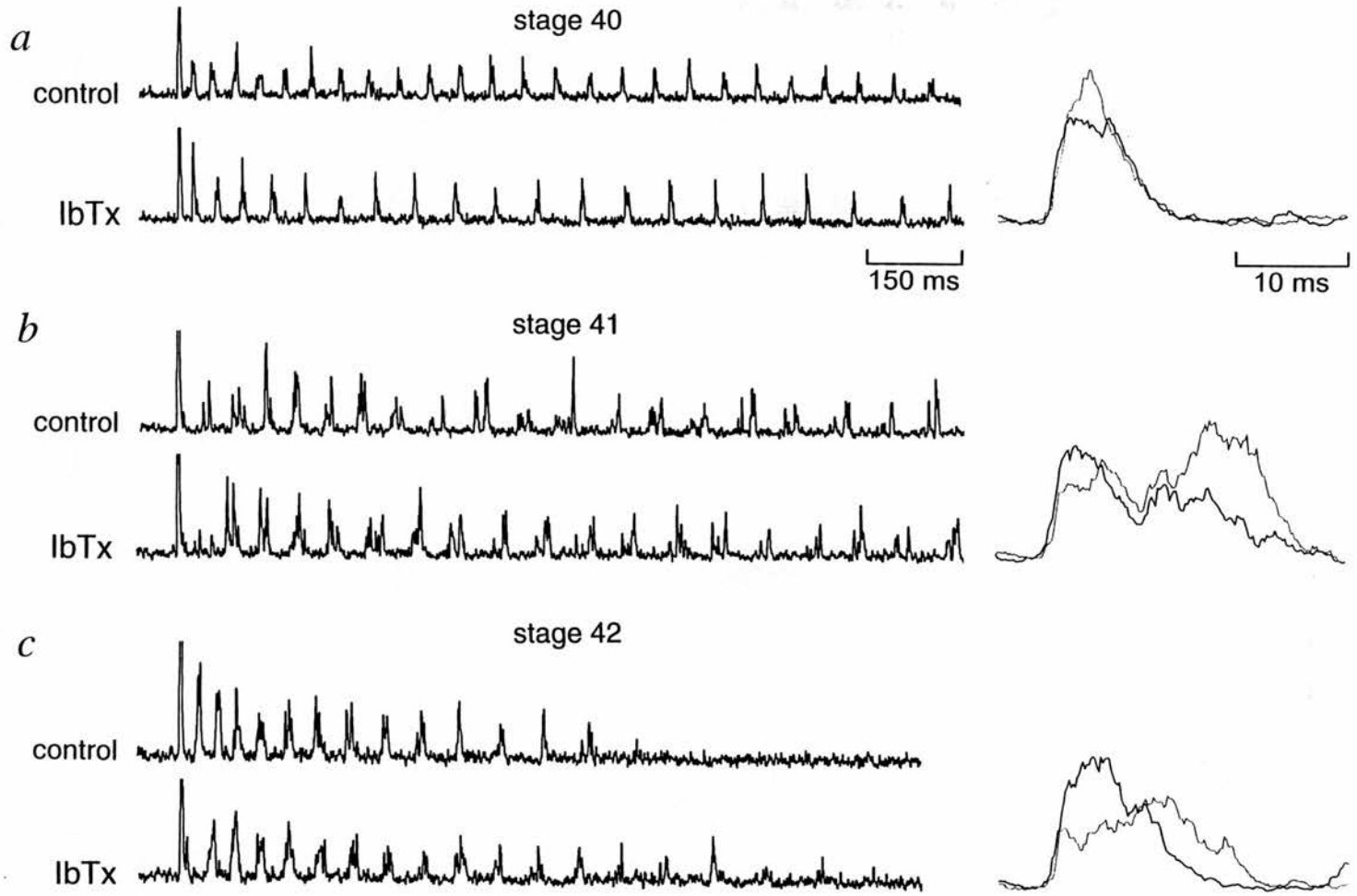
DISCUSSION

Our results show that as the motor pattern develops from the embryonic to the larval pattern, the expression of ion currents changes. The delayed rectifier currents are down-regulated and are partially replaced by the I_{KCa}. In earlier work, a similar transition in the Ca^{2+}-sensitivity of K^+ currents in cultured *Xenopus* neurons was interpreted as an alteration of channel structure to endow pre-existing K^+ channels with Ca^{2+}-sensitivity (Blair & Dionne, 1985). However, in the light of more recent knowledge about the genes that encode Ca^{2+}-dependent K^+ channels (Pallanck & Ganetzky, 1994), we interpret the acquisition of Ca^{2+}- and iberiotoxin-sensitivity as resulting from a switch in gene expression and synthesis of new B-K channel proteins. Given the massive conductance of B-K channels, the newly expressed channels may not be very numerous: for a whole cell conductance of 8nS only around 40-80 channels would be needed.

Since the fast and slow components of the delayed rectifier limit the rate of firing of the embryonic neurons and contribute to spike threshold (Dale, 1995a,b), down-regulation of these currents may increase the excitability of the larval

Figure A.4. 4 I_{KCa} contributes to termination of ventral root bursts during swimming in larvae.

(a) In a stage 40 larva, iberiotoxin (IbTx) had no effect on the ventral root activity (shown as rectified and integrated traces). The averaged ventral bursts in control and iberiotoxin (right) almost exactly overlapped (gray line shows averaged ventral root burst in iberiotoxin). In a stage 41 (b) and stage 42 (c) larva, iberiotoxin increases the burst duration during swimming which can be more easily seen in the averaged ventral root bursts (shown at the right). All averaged ventral root bursts were obtained from 2 swimming episodes in control and iberiotoxin.


a
stage 40
control
IbTx
150 ms
10 ms
b
stage 41
control
IbTx
c
stage 42
control
IbTx

neurons and contribute to the developmental increase in burst duration during swimming. Nevertheless other alterations to the network circuitry that also increase burst duration independently of ion channel expression are likely.

One effect of the switch in the expression of currents is to endow a significant proportion of the total outward current (around 30%) with a dependence on the levels of intracellular Ca^{2+}. This will accumulate in a cyclical fashion during rhythmic motor activity, owing to voltage-activation of Ca^{2+} channels and synaptic-activation of ligand-gated channels, most notably the NMDA receptor. Thus I_{KCa} is likely to exert a stronger repolarizing influence at the end of the motor cycle compared to the beginning (before significant accumulation of Ca^{2+}). When a motor pattern involves alternating burst activity between mutually antagonistic motor half-centres, mechanisms must be present to terminate the bursts in each half-centre so that alternation ensues rather than unceasing, high frequency firing in one dominant half-centre. Ca^{2+}-dependent K^+ currents are thought to contribute to such burst termination in the lamprey (El Manira, Tegnér & Grillner, 1994). Our results show very clearly that as the motor pattern matures in *Xenopus* and a need for burst terminating mechanisms arises (at stages 41 and 42), the delayed rectifier K^+ currents are partially substituted by Ca^{2+}-dependent K^+ currents that specifically meet this new requirement. The changes in ionic currents, and by implication, ion channel expression are thus closely matched to the requirements of the motor pattern.

Interestingly, *Xenopus* embryos express a Ca^{2+}-dependent K^+ current (probably an S-K channel; Wall & Dale, 1995) that activates some 3 orders of magnitude more slowly than I_{KCa} and is sensitive to apamin. This S-K current is present also in the larva and is therefore not subject to the same developmental regulation as I_{KCa} (Q-Q. Sun & N. Dale, unpublished observations). In the embryo the S-K current activates too slowly to play a role in the cycle-by-cycle regulation of the motor pattern. Instead it slowly accumulates over many cycles and contributes to the termination of swimming episodes (Wall & Dale, 1995). The role of this current in the larva has not been examined, however since its kinetics of activation remain slow compared to the duration of a single locomotor cycle, a similar role to that demonstrated in the embryo is plausible.

The identification of a current, whose expression is so closely matched to the maturation of the motor pattern, provides a new experimental approach to identifying the developmental cues that underlie this maturation. Any factors that change expression of this current in single neurons will be key candidates to underlie the triggering of a wider developmental programme that results in maturation of the motor pattern. Interestingly, cultured *Xenopus* neurons (isolated from the neural plate and allowed to develop *in vitro*), express Ca^{2+}-sensitive K^+ currents within a few hours of dissociation (Blair & Dionne, 1985; O'Dowd *et al*, 1988). This is probably 2-3 days in advance of when I_{KCa} is expressed *in vivo*. If the currents expressed in the cultured neurons are the same as those *in vivo*, this implies the existence of extrinsic mechanisms that delay expression of the new channels until an appropriate stage of larval development.

Table A. 1 The effect of iberiotoxin on locomotion mator pattern of *Xenopus* larvae

The effect of iberiotoxin on the mean duration of ventral root bursts during swimming at different stages of larval development expressed both as an absolute value and as a proportion of cycle period. Burst duration was measured by the threshold crossing method. Each value is shown with mean±sem, paired sample t-tests were used to assess changes in burst duration.

Stage	Burst duration (ms)				Burst duration (% cycle)			
	control	iberiotoxin	n	P	control	Iberiotoxin	n	P
40	8.8±1.2	8.5±1.3	5		15.5±2.1	14.1±2.4	5	
41	13.2±1.5	17.5±2.0	6	< 0.05	18.0±2.2	23.4±2.3	6	< 0.01
42	13.3±0.7	16.6±0.8	5	< 0.01	26.3±1.8	29.8±1.0	5	< 0.05

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