CHOLNERGIC EXCITATION OF DOPAMINE NEURONS IN THE VENTRAL TEGMENTAL AREA

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ABSTRACT

Ventral tegmental area dopaminergic neurons are critically involved in brain mechanisms of reward, motivation, and emotional arousal. The natural cholinergic agonist nicotine is highly addictive and believed to cause addiction through activating the central dopamine system. In this thesis, we used perforated patch-clamp recording to examine the effects of the cholinergic agonist carbachol on the excitability of dopamine neurons in brain slices. Results from these experiments revealed that bath application of carbachol (20 μM) for 1-2 min excited most cells in the VTA regardless of their membrane characteristics and neurochemical identities. Muscarinic and nicotinic receptors appeared to contribute equally to carbachol-induced excitation. A majority of cells putatively identified as dopaminergic based on the expression of hyperpolarization-activated current and dopamine-induced autoinhibition, responded to carbachol with depolarization and an increased rate of firing. Of these cells, 13% responded to the carbachol with a switching of firing pattern from tonic firing to bursting. Carbachol-induced excitation and bursting were all reversible and could be prevented by combined muscarinic and nicotinic antagonism.

The synaptic blocker cocktail containing 100 μM APV (NMDA receptor blocker), 10 μM CNQX (AMPA receptor blocker) and 100 μM picotoxin ($GABA_A$ receptor blocker) did not alter carbachol's effect on neuronal excitability and bursting. In addition, a small proportion of dopamine cells were spontaneously bursting in the slice. The characteristics of these bursts were very similar to those induced by carbachol. Both

spontaneous and carbachol-induced bursting could be blocked by the non-selective Ca^{2+}channel blocker cadmium and the L-type Ca^{2+}channel blocker nifedipine, while blocking the T-type Ca^{2+}channels did not have any effect on bursting. Spontaneous and induced bursting occurred on a slow, large amplitude membrane oscillation or hump potential which was dependent on Ca^{2+} entry through voltage-gated Ca^{2+} channels, and more specifically through the L-type Ca^{2+} channels.

In summary, the present investigation provides evidence that cholinergic excitation of dopamine cells is mainly postsynaptic. More importantly, cholinergic activation can serve as a trigger that switches the firing mode of dopamine cells and promotes burst firing. Burst firing induced as such is dependent on Ca^{2+} entry through the L-type Ca^{2+} channels, suggesting a new target for modulating the central dopamine system.

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TABLE OF CONTENTS

FIGURES

ABBREVIATIONS

ACh	acetylcholine
ASCF	artificial cerebrospinal fluid
AHP	afterhyperpolarizing potential
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
APV	D,L-2-amino-5-phosphonovaleric acid
ChAT	choline acetyltransferase
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DA	dopamine
DMSO	dimethyl sulfoxide
DOPAC	3,4-dihydroxyphenylacetic acid
EGTA	ethyleneglycolbis tetraacetic acid
GABA	gamma-aminobutyric acid
I_h	hyperpolarization-activated current
mAChR	muscarinic acetylcholine receptor
MLA	methyllycaconitine
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
NAcc	nucleus accumbens
TRP	transient receptor potential

RMP	resting membrane potential
TTX	tetrodotoxin
VTA	ventral tegmental area

Chapter 1 INTRODUCTION

The central dopamine (DA) system plays an important role in motor and affective expressions and autonomic regulation, functions that are carried out by DA released at the synaptic site (Girault and Greengard, 2004). Although DA can also be released from free nerve endings where no postsynaptic specializations exist, for clarity, we use the term synaptic release in this thesis to refer to DA release in axons rather than that occurs in dendrites. There are many mechanisms that regulate DA levels in its terminal fields and these factors often interact extensively to produce the final DA output. Increased DA output has been shown to alter motivation and affect, whereas decreased DA output has been implicated in motor disturbance. To maintain optimal levels of DA at the synapse, there has to be a balance between the release and reuptake machinery, with release being regulated at the terminal and somatic sites. This chapter will give an overview of the regulation of the midbrain DA system, with a special reference to cholinergic control of this system involving ionic mechanisms on the somata of DA neurons.

1.1. Enhanced DA Transmission and Disease

DA is one of the predominant catecholamine neurotransmitters in the brain. It is synthesized from the essential amino acid tyrosine in a biosynthetic pathway containing five enzymes in dopaminergic neurons. Those cells are distributed in specific areas in the brain and organized into four main dopminergic systems: the tuberoinfundibular, nigrostriatal, mesocortical and mesolimbic system. The tuberoinfundibular system is

involved in regulating hormone release from the pituitary; the nigrostriatal system controls voluntary movements through its dense projections to the dorsal striatum; the mesocortical and mesolimbic systems project to the nucleus accumbens and the prefrontal cortex (Fig. 1.1) which are involved in motivation, planning, attention, and social behaviour (Kiyatkin, 1995).

The midbrain DA systems play an important role in the regulation of emotion, reward mechanisms, and cognition through their projections from the ventral tegmental area (VTA) to the nucleus accumbens and the prefrontal cortex (Kiyatkin, 1995; Tzschentke, 2001). Specifically, enhanced DA output from the VTA has been implicated in schizophrenia and drug dependence (Koob, 2000; Knable and Weinberger, 1997). Schizophrenia is caused by multiple factors involving multiple transmitter systems, however, it is strongly believed that the central DA system plays a more significant role in this pathology. The clinical potency of an antipsychotic drug to suppress positive symptoms of schizophrenia and the ability of the drug to block dopamine D_2 receptors are strongly correlated (Seeman et al., 1976). Besides their proven effects on DA receptors at the synapse, antipsychotic drugs also have direct actions on the midbrain DA cells by modulating ion channels on the cell body. One of the channels that is affected is the T-type Ca^{2+} channel which possibly supports burst firing due to its slower kinetics than Na^{+} spikes. As discussed in later sections of this chapter, burst firing releases DA more effectively at the synapse. In line with this, the inhibitory actions of anti-psychotics on somatic T-type Ca^{2+} channels represent another potential modulatory force on this system (Santi et al., 2002).

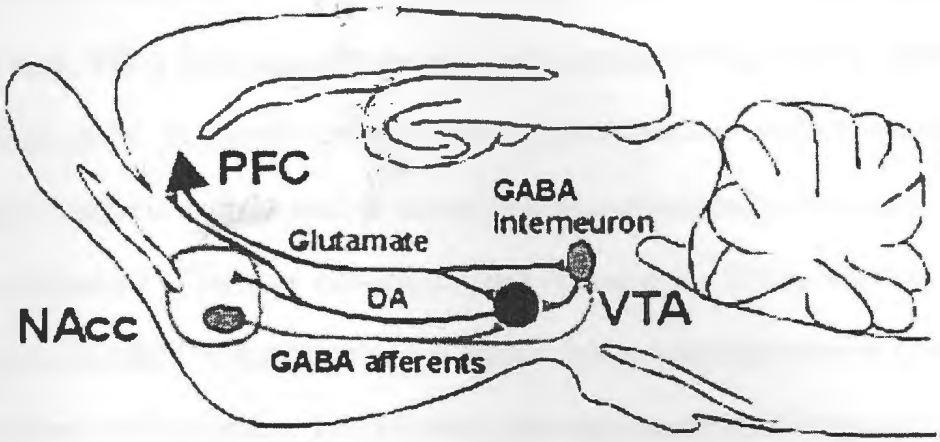


Fig. 1.1 The mesolimbic dopaminergic system. This simplified diagram shows reciprocal connections between the ventral tegmental area (VTA) and forebrain structures such as the nucleus accumbens and prefrontal cortex. NAcc, nucleus accumbens; PFC, prefrontal cortes; DA, dopamine; GABA, gamma-amino butyric acid.

Drugs of abuse also increase DA in VTA terminal sites such as the nucleus accumbens and the prefrontal cortex (Pan et al., 1996; Di Chiara and Imperato, 1988; Hurd et al., 1989) inducing euphoria and reinforcement of drug seeking behavior (Cami and Farre, 2003). In animals that are trained to self-administer cocaine, infusion of a DA receptor antagonist into the nucleus accumbens dose-dependently increases the frequency and total amount of the drug self-administered (Caine et al., 1995). Selective destruction of the mesolimbic DA system using the neural toxin 6-hydroxydopamine (Roberts et al., 1980; Dani and Heinemann, 1996) inhibits self-administration of drugs of abuse in rats. Although psychostimulants primarily enhance DA transmission through blocking DA reuptake or facilitating reverse transport in the terminal (Sulzer et al., 1995), they also involve the cell bodies by terminal D_2 autoreceptor inhibition and other compensatory changes associated with chronic use.

1.2. DA Release and Firing Pattern

DA release is a spike-dependent process as for other transmitters: higher frequency and longer duration of spiking increases DA release (Bean and Roth, 1991). The level of DA at the synapse depends not only on the rate at which it is released, but also on how efficient it is reuptaken back into the releasing terminal. Reduced reuptake, as in the case of psychostimulants, markedly boosts DA concentration in the synapse (Horn, 1990). Excitatory synapses on DA terminals also promote DA release (Sharma and Vijayaraghavan, 2003). Besides the actions at the terminal, the cell body of DA cells in

the VTA regulates DA output by changing firing rates and by responding to terminal as well as somatic autoreceptors (Bunney et al., 1973; Cubeddu and Hoffmann, 1982).

Firing pattern plays an important role in altering DA release. Dopaminergic neurons exhibit two common firing patterns: tonic spiking and bursting (Grace and Bunney, 1984). Tonic spiking refers to firing at regular frequency that is generally lower than that of burst firing. Burst firing refers to a cluster of spikes appearing on a hump potential which is a slow undulation of membrane potential. The frequency of this firing is irregular and a burst is usually followed by a pronounced post-burst hyperpolarization (Fig. 1.2). Based on the measurement of extracellular DA concentrations *in vivo* in VTA terminal sites, burst firing releases more DA from the terminal than the same number of evenly spaced spikes (Manley et al., 1992; Gonon and Buda, 1985). This is reported to be due to Ca^{2+} accumulation in the pre-synaptic terminal (Grace, 2000; Suaud-Chagny et al., 1992), or to saturated DA reuptake mechanism during burst firing (Chergui et al., 1994). The accumulation of DA in the synaptic cleft will have two significant effects: more pronounced postsynaptic effects at the synaptic site and stronger autoinhibition of DA cell bodies in the VTA by activation of the D_2 autoreceptors (Grace, 2000) (Fig. 1.3). Activation of release-modulating DA autoreceptors results in a feedback decrease in the release of DA (Wolf and Roth, 1987). These findings indicate that the activity of DA cell bodies can have far-reaching effects on DA synaptic transmission.

1.3. Firing Pattern Regulation

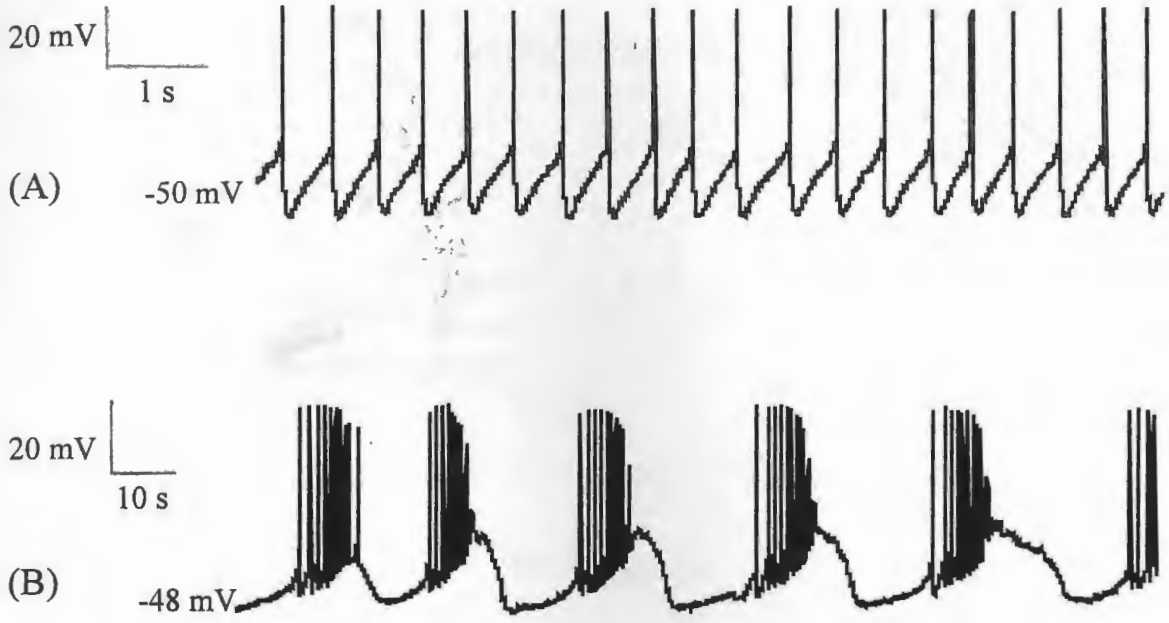


Fig. 1.2 DA cells fire in different modes in the VTA slice: Tonic and burst firing patterns. Traces are current clamp recordings from the VTA slice at room temperature. (A) The upper trace shows tonic firing, which occurs at a fixed frequency (2 Hz). (B) The lower trace shows a burst firing pattern. In a single cycle of bursting, cluster of spikes at higher frequencies appears on a depolarizing hump terminated by a pronounced post-burst hyperpolarization (the average frequency within the burst: 5.16 Hz).

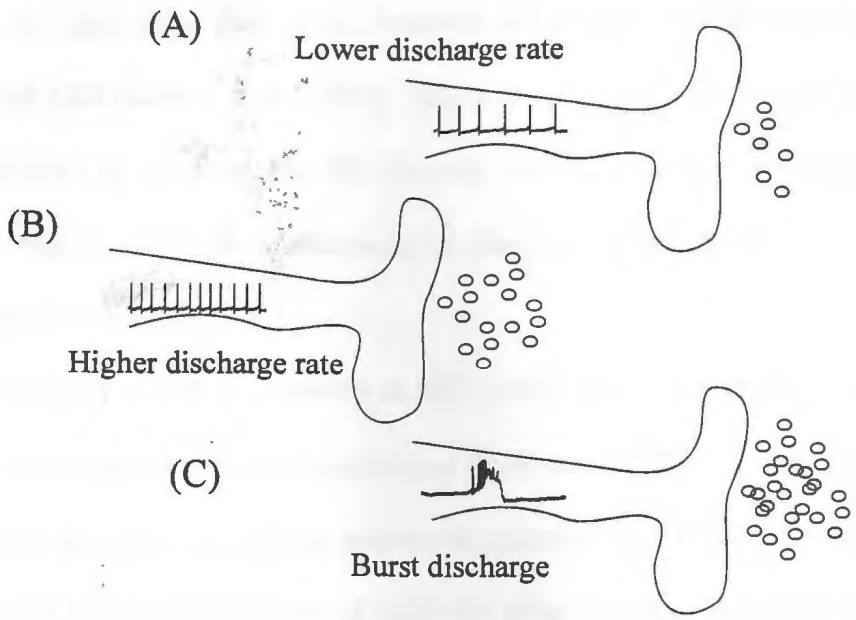


Fig. 1.3 Possible consequences of different firing patterns. (A) DA release is a spike-dependent process. Low frequency spiking induces a little DA release in the terminal. (B) During higher frequency spiking, more DA is released. (C) In the burst firing pattern, much more DA is released and causes stronger postsynaptic effects.

members of the small $K_{(Ca)}$ (SK) family channels that mediates sAHP were described as SK1, SK2 and SK3 (Kohler et al., 1996). SK2 and SK3 channels are very sensitive to apamin while SK1 is less sensitive. SK channels are linked to the Ca^{2+} binding protein, calmodulin, which leads to the conformational change in the channels and causes their opening (Schumacher et al., 2001).

SK2 expression is not as extensive as SK3 which is highly expressed in the VTA, while SK1 is not expressed at all (Stocker and Pedarzani, 2000). Functionally, the AHP has been found to affect the resting membrane potential, the firing frequency and the firing pattern of DA cells. Blockade of AHP has been shown to modulate spontaneous firing of DA neurons *in vitro* (Shepard and Bunney, 1991). In the substantia nigra, the SK channel blocker, apamin, changes the firing patterns of DA neurons from a regular pattern to bursting (Gu et al., 1992; Ping and Shepard, 1996). It is reported that in this nucleus, SK channels are activated by Ca^{2+} entry through the T-type Ca^{2+} channel and as such T-type Ca^{2+} channel blockade is sufficient to induce burst firing in DA neurons (Wolfart and Roeper, 2002). Regularly spiking DA cells in the VTA that display small AHPs respond to orexin A with bursting (Korotkova et al., 2003). All these reports indicate that AHP plays an important role in altering firing pattern in midbrain DA cells and may well be a major determinant for burst firing.

1.3.2. Extrinsic Factors

DA neurons exhibit a highly regular pacemaker-like firing pattern *in vitro*, while bursting of DA neurons is frequent *in vivo* (Grenhoff et al., 1988), suggesting that burst

receptors reduces ethanol intake of the alcohol-preferring rat (Nowak et al., 1998). Microinjection of the $GABA_B$ receptor agonist, baclofen, into the VTA reduces the rewarding effect of morphine (Tsuji et al., 1996). The cellular basis of these observed behaviors may be attributed to GABAergic influence on firing patterns of DA cells in the VTA. Selective $GABA_A$ antagonists, such as bicuculline, gabazine and picrotoxin, increase burst firing of DA neurons in chloral hydrate anesthetized rats (Grenhoff et al., 1988; Paladini and Tepper, 1999). Recordings in the rat midbrain slices have shown that baclofen can convert NMDA-induced burst firing to a single-spike firing pattern in DA neurons (Seutin et al., 1994). All these findings show that the GABAergic input from interneurons or fibers from other areas have effects on the firing pattern of DA neurons, which could have an impact on addictive and reinforcing behavior.

GABAergic effects on DA cells can be indirectly excitatory by disinhibiting local GABA interneurons because DA cells receive tonic inhibition from the interneurons. It has been shown experimentally that $GABA_A$-mediated synaptic responses in DA neurons exist spontaneously and can be prevented by tetrodotoxin (TTX) that blocks spontaneous action potential firing of GABA interneurons in the slice (Johnson and North, 1992a). Also, activation of $GABA_B$ receptors on presynaptic glutamatergic nerve terminals suppresses excitatory transmission onto GABA interneurons (Wu et al., 1999), relieving the tonic inhibition exerted by those neurons on DA cells.

for α_2–α_7 and β_2–β_4 subunits are expressed by both DA and GABA neurons in different quantities (Sgard et al., 1999; Klink et al., 2001).

Nicotinic receptors are well known to be important in the initial fast response of acetylcholine (ACh) and mediate addiction (Balfour et al., 2000). Acute systemic administration of nicotine increases intracranial self-stimulation rates (Druhan et al., 1989) or locomotor activity in rats (Picciotto, 1998) and infusion of a nicotinic antagonist in the VTA results in a significant reduction in nicotine self-administration (Corrigall et al., 1994) and in nicotine-induced locomotor activity (Louis and Clarke, 1998). Administration of nicotine either systemicly or locally within the VTA increases extracellular levels of DA in the nucleus accumbens (Pontieri et al., 1996; Nisell et al., 1994). Higher DA output following nicotinic activation may be due to increased firing rate or increased bursting of DA cells (Grenhoff et al., 1986).

All these experiments indicate that activation of nicotinic receptors in the VTA is closely related to the level of activity of DA cells and their terminal output. Furthermore, nicotinic receptor subtypes are differentially activated in this process. *In vivo*, nicotine stimulates DA release in the ventral striatum of wild-type mice but not in the β_2-mutant mice. *In vitro*, mesencephalic DA neurons from mice without the β_2-subunit no longer respond to nicotine. These results suggest that β_2-containing nicotinic ACh receptors (nAChRs) play an important role in nicotine-evoked DA release (Picciotto et al., 1998). Since $\alpha_4\beta_2$ is the most abundant nicotinic receptor on the soma of DA cells (Klink et al., 2001), these findings also support the importance of somatic activities of DA cells in the cholinergic modulation of the DA output. Although α_7 receptors are encountered in less

than half of DA and GABA neurons (Mansvelder and McGehee, 2000), its pre-synaptic effects on enhancing glutamate release are well documented in experiments which show that nicotine increases evoked and spontaneous excitatory synaptic currents in VTA DA neurons (Mansvelder and McGehee, 2000; Dani et al., 2001).

1.3.2.3.2. Muscarinic Receptors

Muscarinic receptors are G-protein coupled receptors and can be divided into five different subgroups, M_1–M_5. M_1, M_3 and M_5 muscarinic receptors are coupled to $G_{q/11}$ which activates phospholipase C leading to the hydrolysis of phosphoinositol; M_2 and M_4 muscarinic receptors are coupled to $G_{i/o}$, which inhibits adenylyl cyclase, and activates a K^+ channel (Hulme et al., 1990; Caulfield, 1993).

Muscarinic receptors are responsible for the prolonged DA cell responses to ACh (Lacey et al., 1990) and are related with hypothalamus-mediated reward such as eating and drinking. For example, infusion of atropine, a non-selective muscarinic antagonist, into the VTA is sufficient to stop self-stimulation and reduces food intake in male rats (Rada et al., 2000). Microinjection of atropine in the ventral tegmentum elevates frequency thresholds for lateral hypothalamic self-stimulation (Kofman et al., 1990). Electrical stimulation of the laterodorsal tegmental nucleus, a major source of cholinergic input to the VTA, results in a prolonged increase in DA release in the terminal region. In mutant mice with truncated M_5 receptors, the prolonged phase of DA release is absent which implies that M_5 muscarinic receptors on midbrain DA neurons mediate a prolonged facilitation of DA release in the nucleus accumbens (Forster et al., 2002). *In*

vitro, muscarine increases the rate of spontaneous firing and causes membrane depolarization in DA cells through action at the M_1-like receptors (Lacey et al., 1990). Besides direct excitatory postsynaptic effects through M_1 and M_5 subtypes, muscarinic agonists can potently increase the firing rate by reducing GABAergic transmission through presynaptic M_3 receptors (Grillner et al., 1999).

1.3.3. Calcium

Ca^{2+} plays an important role in regulating a great variety of neuronal processes such as regulating transmitter release, synaptic plasticity and gene transcription (Berridge, 1998). As neurons are the basic execution units which encode signals in the form of electrical activities, these changes in neuronal process are eventually reflected in how neurons fire action potentials. The involvement of Ca^{2+} in regulating firing patterns of DA cells has been well documented (Overton and Clark, 1997; Grillner and Mercuri, 2002).

Ca^{2+} influx affects neuronal excitability through different pathways. Firstly, Ca^{2+} influx causes direct membrane depolarization and excitation (Berridge, 1998). Secondly, Ca^{2+} influx activates Ca^{2+} dependent K^+ channels contributing to repolarization and hyperpolarizing afterpotentials (Bond et al., 1999). Thirdly, Ca^{2+} entry induces Ca^{2+} release from internal Ca^{2+} stores (Simpson et al., 1995; Dajas-Bailador et al., 2002). Finally, Ca^{2+} binding to calmodulin activates the calcium/calmodulin-dependent protein kinase phosphorylating other proteins including transmitter receptors and ion channels.

In the context of cholinergic modulation of DA cell excitability through Ca^{2+}, there are several ways that Ca^{2+} can enter the cell. Firstly, Ca^{2+} gets into the neurons through ligand-gated channels on cell membrane. For example, both α_7 nAChRs and NMDA receptors have high permeability to Ca^{2+}. As presynaptic nAChRs promote glutamate release (Schilstrom et al., 2000), Ca^{2+} entry through NMDA receptors could represent a considerable route of entry. In the meantime, ATP is often co-released, which activates P_{2x} purinergic receptors to allow Ca^{2+} entry into the cell. Secondly, Ca^{2+} enters the neurons through voltage-dependent Ca^{2+} channels to change the firing behaviour of DA neurons. Voltage-dependent Ca^{2+} channels can be divided into two categories: low voltage-dependent Ca^{2+} channels such as the T-type Ca^{2+} channel and high voltage-dependent Ca^{2+} channels, such as L, N, Q, and R type Ca^{2+} channels. Whereas T-type Ca^{2+} channels mediate burst firing in other brain regions, in some DA neurons in the substantia nigra, T-type Ca^{2+} channel inhibition is sufficient to induce intrinsic burst firing (Wolfart and Roeper, 2002). This is because Ca^{2+} entry through the T-type Ca^{2+} channels is coupled to the SK channels, its blockade therefore suppresses the expression of AHPs facilitating burst firing of these neurons. The L-type Ca^{2+} channels (Huguenard, 1996) thought to underlie burst activity are also found in DA cells in the VTA (Kang and Kitai, 1993a). Recently, it has been reported that subthalamic nucleus neurons switch from single-spike activity to burst firing through a mechanism that involves both the T-type Ca^{2+} channels and the L-type Ca^{2+} channels (Beurrier et al., 1999). This shows that both low and high voltage gated Ca^{2+} channels may play an important role in altering firing patterns. Recent evidence suggests a new family of channels, transient receptor

potential (TRP) channels, are implicated in receptor-operated and store-operated Ca^{2+} entry (Clapham, 2003; Zitt et al., 2002). Activation of muscarinic receptors activates TRP cationic channels and induces Ca^{2+} entry into the cells (Lee et al., 2003).

1.4. Proposed Work

A lot of work has been done on the responses of DA neurons to specific nicotinic and muscarinic agonists. In general, their actions on the postsynaptic cell are uniformly excitatory. Nicotine excites the DA cells and increases bursting (Grenhoff et al., 1986) through postsynaptic $\alpha_4\beta_2$ nAChRs (Picciotto et al., 1998). Activation of postsynaptic M_1-like receptors (Lacey et al., 1990) also increase the firing rate. However, their actions on the presynaptic site are more complicated. Nicotine receptors of the α_7 subtype enhance glutamate and GABA release (Mansvelder and McGehee, 2000), whereas presynaptic muscarinic M_3 receptor activation depresses both excitatory and inhibitory synaptic transmission to DA neurons (Grillner et al., 1999; Grillner et al., 2000). Because of the complexity of these cholinergic mechanisms, application of the native transmitter , ACh, on single midbrain DA neurons has produced variable results, with some reporting excitation and others no effect (Collingridge and Davies, 1981; Pinnock and Dray, 1982).We therefore used the non-selective cholinergic agonist, carbachol, to mimic ACh's actions at nicotinic and muscarinic receptors on both sides of the synapse to examine the contribution of ACh-induced excitation.

In addition, it has been shown that activation of ACh receptors induces Ca^{2+} influx directly through nAChRs and, indirectly, through voltage-dependent Ca^{2+} channels and

glutamate receptors of the NMDA type (Kulak et al., 2001; Grillner and Mercuri, 2002). The role of intracellular Ca^{2+} in burst firing of DA cells has not been understood. Burst firing can be elicited by intracellular calcium injection (Grace and Bunney, 1984). On the other hand, blocking Ca^{2+} influx through the T-type Ca^{2+} channels and blocking Ca^{2+}-dependent K^+ channels also enhance bursting (Wolfart & Roeper, 2002). It is, therefore, very important to examine how Ca^{2+} entry through various routes coupled to cholinergic pathways affects the overall excitability of DA cells. Specifically, experiments were conducted to test:

- Whether carbachol alters the rate and mode of firing;
- Whether presynaptic mechanisms contribute to carbachol's effects; and
- Whether Ca^{2+} acts as a major signalling molecule in carbachol's actions and how Ca^{2+} enters the cell to transduce carbachol's effects.

Chapter 2 MATERIALS AND METHODS

All experiments in this thesis were carried out on rat brain slices using the perforated patch clamp recording technique. Procedures involving animal handling and tissue harvesting were in accordance with guidelines set by the Institutional Animal Care Committee at the Memorial University of Newfoundland. Care was taken to use the minimum number of animals to achieve statistically significant experimental results.

2.1. Animals

Sprague-Dawley rat pups of either sex, aged 5-8 days upon arrival with their nursing mother, were obtained from the vivarium of Memorial University of Newfoundland. The pups were used in experiments between 8-20 postnatal days to examine whether burst firing was more frequent in specific developmental stages in pre-weaning rats as suggested by earlier reports (Mereu et al., 1997). Because of the practical difficulty of sexing them at certain ages, we decided to use both sexes in our experiments. All animals were kept in a controlled animal facility in the Health Science Center with trained staff providing animal care maintenance.

2.2. Chemical Materials

All chemicals used in this thesis study were commercially available. Commonly used chemicals, for example, components of extracellular and intracellular solutions used on a daily basis, were purchased from bulk distributors Fisher Scientific (Nepean, ON)

and VWR International (Missisauga, ON). All other chemicals were obtained from Sigma (St. Louis, MO) and Tocris (Ellisville, MO).

The composition of extracellular solution (which is often referred to as artificial cerebrospinal fluid, ACSF) was (in mM) 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose. In practice, a 10-fold stock solution was prepared and stored at room temperature, which was then diluted to working concentrations with sodium bicarbonate and glucose added before use. Working strength ACSF, when bubbled with carbogen that is a mixture of 95% O_2 and 5% CO_2, yielded a pH of 7.3-7.4.

Intracellular solution was made to the final concentrations as follows (in mM): 120 K-Acetate, 40 HEPES, 5 $MgCl_2$, and 10 EGTA, with pH adjusted to 7.35-7.4 using 0.1 N KOH. The solution was filtered and stored at 4°C until use. For perforated patch recording, the channel forming agent nystatin was added to the intracellular solution. Nystatin (4.5 mg) was first dissolved in 300 µl dimethyl sulfoxide (DMSO). Complete dissolving was often sped up with sonication. The resulting solution was then transferred to 10 ml of filtered intracellular solution to give a final nystatin concentration of 450 µg/ml and was stored in a dark container. While intracellular solution could be stored in a refrigerator for weeks to months, we only used the nystatin suspension for up to 5 days.

Other chemicals used were generally dissolved in deionized water, except for CNQX and nifedipine which were dissolved in DMSO as required. Stock solutions were aliquoted and kept at -30°C. Prior to application, an aliquot, or part of it, was diluted to working concentration and applied to the bath. Stock solutions of cadmium, cesium,

barium and nickel were kept at 4°C. Stock solutions of picotoxin were kept at room temperature. DA solution was made fresh daily with equimolar concentration of the antioxidant diodium metabisulfate. The drugs were applied to the slice through a gravity perfusion system.

2.3. Slice Preparation

Rats were deeply anesthetized with halothane and killed by crushing the chest. The skull was quickly opened to expose the brain, which was cooled *in situ* with ice-cold, carbogenated ACSF to reduce brain metabolism. The brain was then removed and placed in chilled and gassed ACSF. This step further reduced the metabolism; it also made the brain firmer and easier to slice. The whole process was completed within 1-2 minutes and, in the meantime, extreme care was taken not to stretch or press the brain tissue.

A block was then cut from the brain that contained the midbrain section sparing tissue at the rostral and caudal ends. The block of tissue was glued to a slicing stage with the base of the brain facing up. The stage was oriented in the slicing chamber, filled with cold and gassed ACSF, in such a way that cutting started from the caudal end of the block. In our experience, this allowed more accurate estimate of cutting distance before collecting VTA slices. 400 μm slices were cut on a LEICA vibratome (VT1000, Heidelberger, Germany) and usually two horizontal slices containing the VTA were collected from each brain. Collected slices were transferred to a beaker of carbogenated ACSF and allowed to recover at room temperature (22°C) for at least 1 hour prior to recording. Then, a slice was further trimmed to fit into a recording chamber of about 500

μl where it was submerged and continuously perfused with carbogenated ACSF (22°C) at a rate of 2-3 ml/min.

2.4. Nystatin-perforated Patch Recording

Recordings were obtained using the perforated-patch technique to avoid artifactual changes in the electrical properties of DA cells that can occur during conventional whole-cell recording. Nystatin was used as the pore-forming agent because this was found to produce reliable perforation and long-lasting, stable recordings (Levitan and Kramer, 1990). The recordings were made within the confines of the VTA under a dissecting microscope (LEICA MZ6, Heidelberger, Germany). In fresh slice, the VTA is a semi-transparent area between the two parts of substantia nigra, which are transparent and oval in shape. DA cells were more readily found in the caudal portion of the VTA, as has been shown by cytochemical visualization of DA cells in horizontal slice.

Patch electrodes were prepared from glass micropipettes (Garner Glass, Claremont, CA) (Glass type KG-33, O.D.1.5 mm, filament 0.10 mm) on a P-97 Brown-Flaming micropipette puller (Sutter Instruments, Novato, CA). The tips of the patch pipettes were filled with intracellular solution and then back-filled with the same solution containing 450 μg/ml nystatin and Pluronic F127 (dissolved in DMSO) yielding electrode resistances of 4-6 MΩ.

Minimal positive pressure was applied to the pipette before it was advanced to the bath. The pipette offset was corrected to zero. Then the pipette was positioned in the VTA by a 3-axis coarse manipulator; it was advanced into the slice using an inchworm

motor drive (Soma Scientific Instrument, Irvine, CA). The recording pipette was finally positioned close to a cell and a slight negative pressure was applied to form a seal. High resistance seals (1GΩ) were made using a Warner PC-505B amplifier (Warner Instruments Inc, Hamden, CT). The signals were amplified and fed to a DigiData interface 1320A (Axon Instruments, Foster City, CA) driven by pClamp software (Axon Instruments, Foster City, CA).

It usually took 10-20 minutes for complete partitions of nystatin into the membrane. In current clamp mode, access was reflected by the size of the action potentials, as many VTA cells were spontaneously active. After adequate access was attained, action potentials would overshoot 0 mV and the resting membrane potentials, which were obtained from pClamp software in current clamp mode, were around -50 mV. Most recordings were records of membrane voltages acquired in the current clamp mode to study firing patterns of VTA cells and their modulation by cholinergic agents. Episodic protocols were also used in both voltage and current clamp mode to induce I_h and derive other passive characteristics of the cell such as the current-voltage relationship and input resistance. Current and voltage pulses for I_h induction were of 1 s duration and the intervals between pulses were 8 s to allow complete recovery of I_h channels. In voltage clamp mode, the cell was held at -45 mV for maximum I_h induction with an initial level of -110 mV in 8 incremental steps of 10 mV. In current clamp mode, current was adjusted to hyperpolarize the cell to around -110 mV and the rest of the steps were increments of 20 pA.

Cells were identified based on their electrophysiologic properties. In general, DA cells have lower basal firing rates (1-3 Hz) (Johnson and North, 1992), wider action potentials (duration of action potential >2 ms in intracellular recordings) (Grace and Onn, 1989), stronger firing adaptation and a more prominent I_h expression than GABA interneurons. DA cells also respond to DA with a characteristic hyperpolarization (Richards et al., 1997). In practice, we adapted the expression of I_h and DA autoinhibition as major criteria for identifying DA cells.

2.5. Data Analysis

Data were analyzed offline with Mini Analysis (Synaptosoft Inc, Decatur, GA) and pClamp software (Axon Instruments, Inc). Basic properties used to identify recorded cells such as action potential width and basal firing frequency were averaged values of at least 1 min stable baseline recordings. Action potential half width referred to the time difference between the rising and falling phases of an action potential at its half amplitude. I_h was measured as the difference in voltage (or current in voltage clamp) between instantaneous and steady state readings. Analysis of firing behaviour was based on the interval between individual action potentials measured in the Mini Analysis program. Averaged, as well as instantaneous, firing frequencies were derived from those intervals. They were also used to express the regularity of action potentials and to detect burst firing, which was defined as more than two spikes in each burst and separated by an inter-burst hyperpolarization. Bursts were quantified in two aspects: intra-burst frequency

and inter-burst frequency. Intra-burst frequency is the averaged spike frequency within an individual burst, and inter-burst frequency is the frequency of the burst.

Data were expressed as mean ± standard error of the mean as absolute values. Statistical comparisons were performed using paired or unpaired two tailed Student's t tests as appropriate and they were considered significant when $p<0.05$.

Chapter 3 RESULTS

Nystatin-perforated patch recordings were made from 156 cells in the VTA. All cells were firstly obtained in the voltage clamp mode. The partitioning of nystatin into the membrane was monitored by current injection or the size of action potentials in current clamp mode, since many cells in the VTA were spontaneously active. Identification of cells was based on I_h expression and responsiveness to DA. Most data were gap-free current clamp recordings to examine the firing behaviour of the cells.

3.1. Electrophysiological Identification of VTA Cells

The VTA is a heterogeneous area that consists of DA cells and GABA cells, the latter group includes both projection neurons and interneurons. It has been shown in slice preparations that DA and GABA cells in the VTA have distinct electrophysiological properties that are well correlated with immunocytochemical identification (Richards et al., 1997). Of particular importance are two characteristics displayed by DA cells: autoinhibition by DA and expression of a prominent I_h. DA neurons are hyperpolarized in response to dopamine by activation of D_2 receptors (Fig. 3.1). GABAergic interneurons and projection neurons do not respond electrophysiologically to DA (Johnson and North, 1992b).

Of 86 cells on which DA was applied at 50 μM or 100 μM, 59 cells showed significant membrane hyperpolarization (-7.22±0.48 mV, n=59) and were identified as DA cells, whereas 27 cells showed no change in membrane potential and were therefore

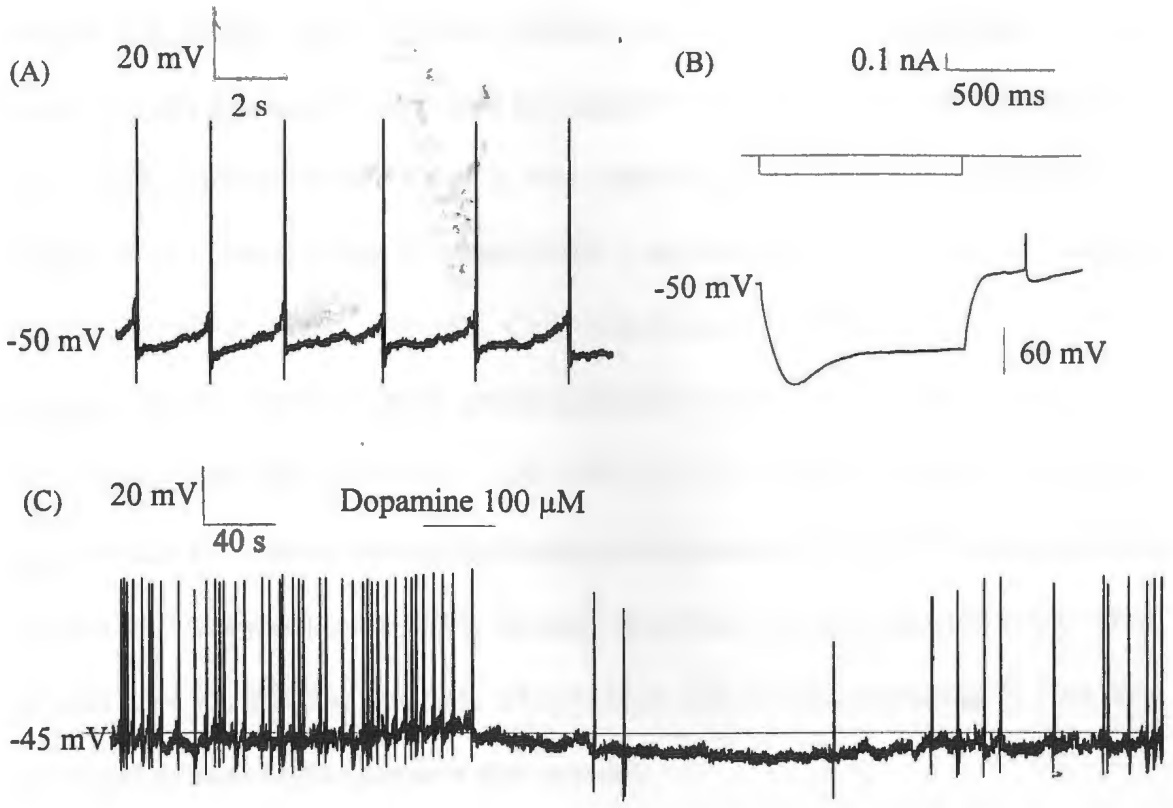


Fig. 3.1 Identification of DA cells. The data presented were obtained from a representative cell presumed to be dopaminergic. (A) Cell fires spontaneously in a pacemaker-like mode at low frequency (0.5 Hz) . (B) It also displays a pronounced time and voltage-dependent I_h during 1 s hyperpolarizing current pulses of -0.1 nA. (C) In addition, the application of DA (100 μM) causes a hyperpolarizing response.

identified as GABA cells. To further validate the use of DA autoinhibition as a main marker for DA neurons, we correlated DA-induced hyperpolarization with the magnitude of I_h. In the current clamp mode, I_h was induced by a series of 1 s pulses and the magnitude of voltage change associated with I_h was measured when the membrane was hyperpolarized to around -110 mV. Cells that responded to bath applied DA with a hyperpolarization expressed much greater I_h (22.6±2.66 mV, n = 5) than that displayed by cells that did not respond to DA (1.78±1.04 mV, n=4, $p<0.01$; unpaired t test). This indicates that DA-induced hyperpolarization and expression of a prominent I_h are parallel features of DA neurons in the VTA. As such, out of the 156 cells reported in this series, 98 cells were identified as DA cells, 33 as GABA cells and the remaining 25 cells were undecided because neither protocol was recorded.

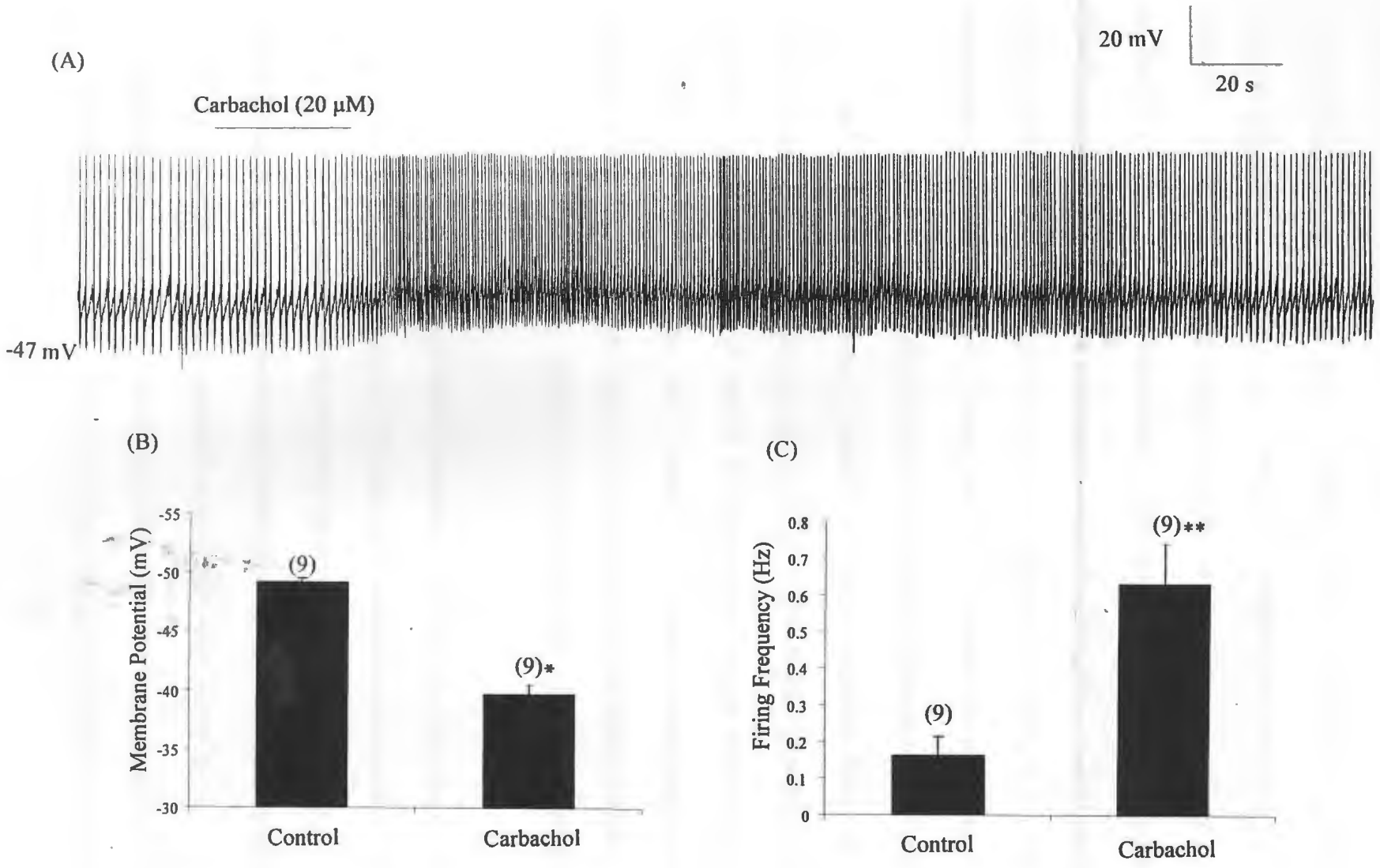
DA cells identified as such displayed different firing activities in current clamp mode (n=93, 5 remaining DA cells were all voltage clamp recording). 26% of the cells (n=24) were quiescent; 73% (n=67) were regularly or irregularly spiking and 2 cells (2%) showed spontaneous bursting. Cells in the spontaneous bursting DA group had similar RMP (-51.5 ± 0.86 mV, n=2) to the quiescent group (-51.1±1.18 mV, n=24), whereas tonically spiking cells had more depolarized RMPs (-47.5±0.55 mV, n=67, $p<0.05$, two-tailed unpaired *t* test). At room temperature, spiking DA cells had a low basal firing frequency of 0.42±0.04 Hz (n=67). GABA cells only had more negative RMPs than tonically spiking DA cells (-52.23±3.47 mV, $p<0.05$, two-tailed unpaired *t* test).

3.2. Carbachol Excites Most Cells in the VTA

Cholinoceptors have been found to modulate excitatory, as well as inhibitory, synaptic transmission in the VTA (Grillner and Mercuri, 2002). They also have direct effects on neuronal excitability in some regions of the brain (Raggenbass and Bertrand, 2002). In order to examine if cholinergic activation plays a role in controlling the firing pattern of DA cells in the area, we used carbachol (20 μM), a non-selective cholinergic agonist, as a test compound to unravel its ultimate effects on the firing behavior of DA cells in an *in vitro* setup. This was to mimic the actions of the native transmitter ACh, which acts on all types of cholinoceptors.

We tested carbachol's effects on firing behavior in a total of 146 cells in the VTA. Bath application of carbachol at 20 μM for 60 s to 120 s excited most of the cells tested (n=116) regardless of their neurochemical identity. In a group of cells, which were tested with both muscarinic and nicotinic receptor blockers, carbachol (20 μM) caused significant depolarization (-39.7±0.81 mV compared to -49.2±0.41 mV, $p<0.05$, n=9) and increased frequency of firing (0.634±0.11 Hz compared to 0.164±0.05 Hz, $p<0.05$, n=9) (Fig. 3.2).The increased firing rate was often accompanied by an apparent depolarization, and in some cases, it was enough to induce depolarization block. Both effects were reversible upon washout, though the time it took for recovery varied among cells, ranging from 15 to 30 min. When carbachol-induced effects fully recovered, re-application at the same dose induced responses of similar magnitude with no apparent desensitization (carbachol-induced depolarization by first application: 6.47±0.8 mV; by second

Fig. 3.2 Carbachol excites DA cells. (A)This is a current clamp-recording showing a cell spiking with a resting membrane potential of -47 mV. Bath application of carbachol (20 μM) for 60 s significantly depolarized the membrane and increased the firing frequency. After 10 min wash, the cell was fully recovered. (B) Histogram showing a significant depolarization induced by carbachol. Numbers in brackets are number of cells in the group. *$p<0.05$ vs. control. (C) Histogram showing that carbachol increases the firing frequency significantly in non-bursting DA cells. Numbers in brackets are number of cells in the group. **$p<0.05$ vs. control.


(A)
20 mV
20 s
Carbachol (20 μM)
-47 mV
(B)
Membrane Potential (mV)
-55
-50
-45
-40
-35
-30
(9)
(9)*
Control
Carbachol
(C)
Firing Frequency (Hz)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
(9)
(9)**
Control
Carbachol

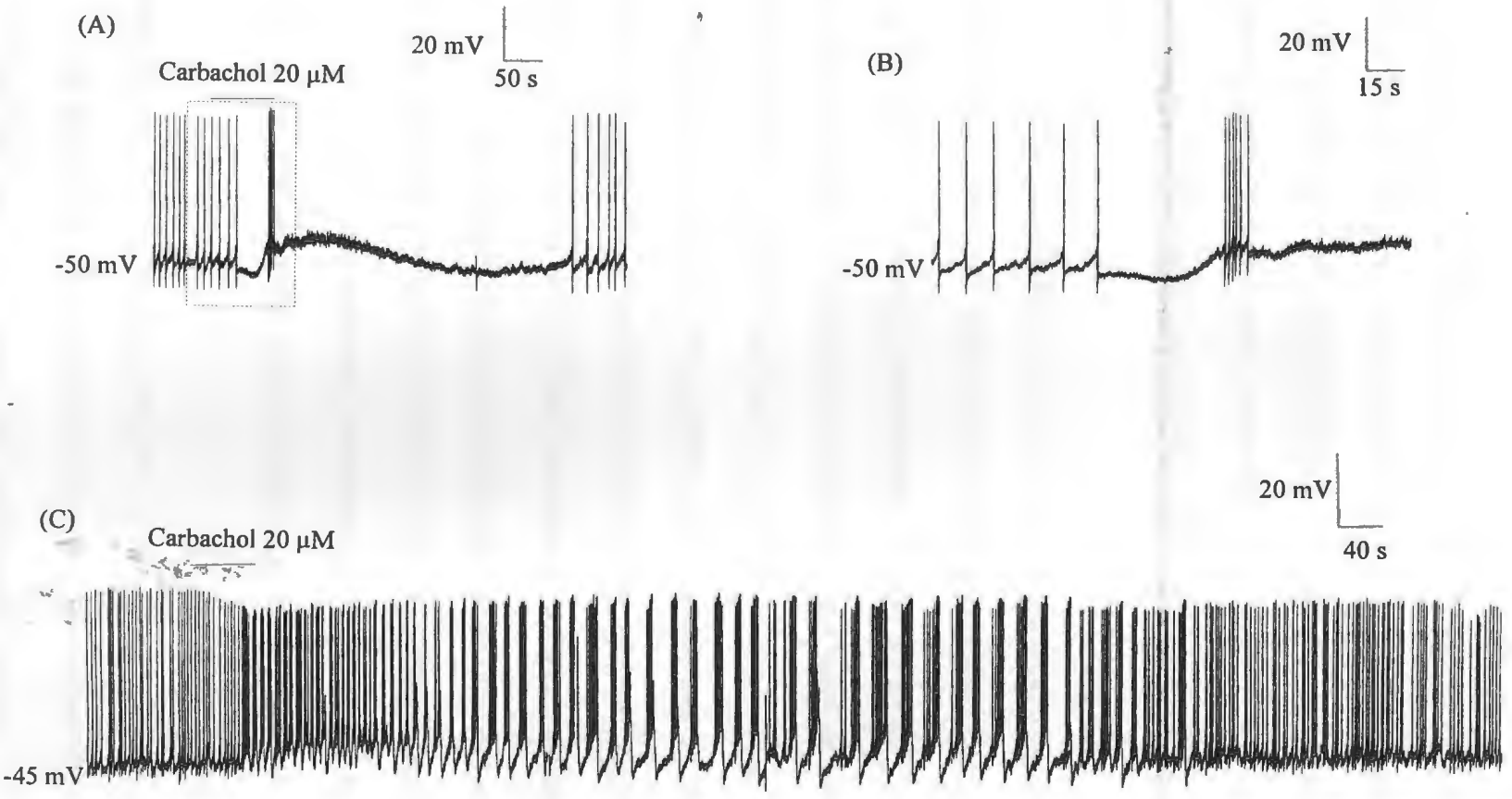
application at the same dose: 8.33±140 mV, n=4, p>0.1). Nineteen of the cells responded to 20 μM carbachol with bursting. A minority of cells on which carbachol was applied (11 out of 146 cells) responded with a significant hyperpolarization (-11±1.5 mV). Although not tested thoroughly, it appeared that carbachol-induced hyperpolarization in those cells was mediated by muscarinic receptors because muscarine (30 μM) induced similar hyperpolarization in two of the cells and 10 μM atropine, a muscarinic receptor blocker, completely blocked carbachol-induced hyperpolarization.

3.3. Features of Burst Firing Induced by Carbachol

Besides increasing the firing rate of VTA cells, carbachol may induce a different mode of firing to boost terminal DA release. One mode of firing, bursting, has been shown to be very efficient in releasing DA at axon terminals, we therefore examined whether carbachol could switch tonically firing VTA cells to bursting mode. Nineteen cells that were tonically spiking or quiescent showed a burst firing pattern after application of carbachol, representing 13% of cells tested.

Bursting usually started on the rising phase of carbachol-induced depolarization, followed by a period of depolarization block. Some cells resumed regular spiking afterward (Fig. 3.3) and some remained bursting for another 15-20 min before finally recovered to pretest firing. The average membrane depolarization induced by carbachol was 11.21±0.66 mV (n=19). The intra-burst firing frequency induced by carbachol (1.67±0.26 Hz) was much higher than the basal tonic frequency (0.15±0.04 Hz) and inter-burst intervals varied from 3 to 34 s. After 10-30 min washout, the inter-burst intervals

Fig. 3.3 Carbachol triggers DA cell bursting. (A) This is a current clamp-recording showing a cell with a resting membrane potential of -50 mV and in a slow pacemaking firing mode, which is a very common firing pattern in slice. Carbachol (20 μM) briefly hyperpolarized the cell before significantly depolarizing it by about 10 mV, and switched the firing pattern from regular spiking to bursting. Carbachol at this concentration depolarized the cells beyond the Na^+ deactivation potential and caused depolarization block. Induced bursting recovered to slow pacemaking activity after washout. (B) Extended time-scale showing the switching of firing mode from the same cell in A. (C) Tonically firing cell responded to carbachol with bursting which persisted for 20 min before recovery.


(A)
20 mV
50 s
Carbachol 20 μM
-50 mV
(B)
20 mV
15 s
-50 mV
(C)
20 mV
40 s
Carbachol 20 μM
-45 mV

prolonged and cells became quiescent or spiked tonically at the same frequencies as before carbachol application (0.17±0.05 Hz, compared to 0.15±0.04 Hz, $p>0.1$, n=19), accompanied by a complete recovery of membrane potential (-48.47±0.8 mV compared to -47.6±1 mV, n=19).

Carbachol-induced bursting cells appeared to be dopaminergic because all 5 cells tested with DA in this group showed significant hyperpolarization (-7.8±0.98 mV, n=5) or they displayed prominent I_h when the membrane was hyperpolarized to around -110 mV (19±3 mV, n=2). Their RMPs were similar to typical DA cells (-47.6±1 mV, n=19) in our experiments and displayed typical tonic spiking at low frequencies (0.15±0.04 Hz, n=19). However, this averaged basal firing frequency was lower than the average firing rate of a group of 21 non-bursting cells recorded from slices taken from littermates on adjacent days (0.37±0.06 Hz, n= 21, $p<0.05$, two-tailed unpaired *t* test). There was no difference in action potential half width between cells that responded to carbachol with bursting (6.07±0.27 ms, n=19) and non-bursting cells (5.59±0.44 ms, n=21, $p>0.1$, two-tailed unpaired *t* test). Carbachol-induced bursting cells were scattered throughout the recording period of 8-20 postnatal days. No apparent clustering was noted.

3.4. Carbachol's Effects are Mediated by Muscarinic and Nicotinic Receptors

To explore the types of receptors mediating carbachol's actions, we applied atropine (10 μM) and mecamylamine (10 μM) to block muscarinic and nicotinic receptors, respectively, to examine how they changed carbachol-induced firing. Carbachol (20 μM) applied for 60 to 120 s caused significant depolarization (9.5±1.13 mV, n=9). Nicotinic

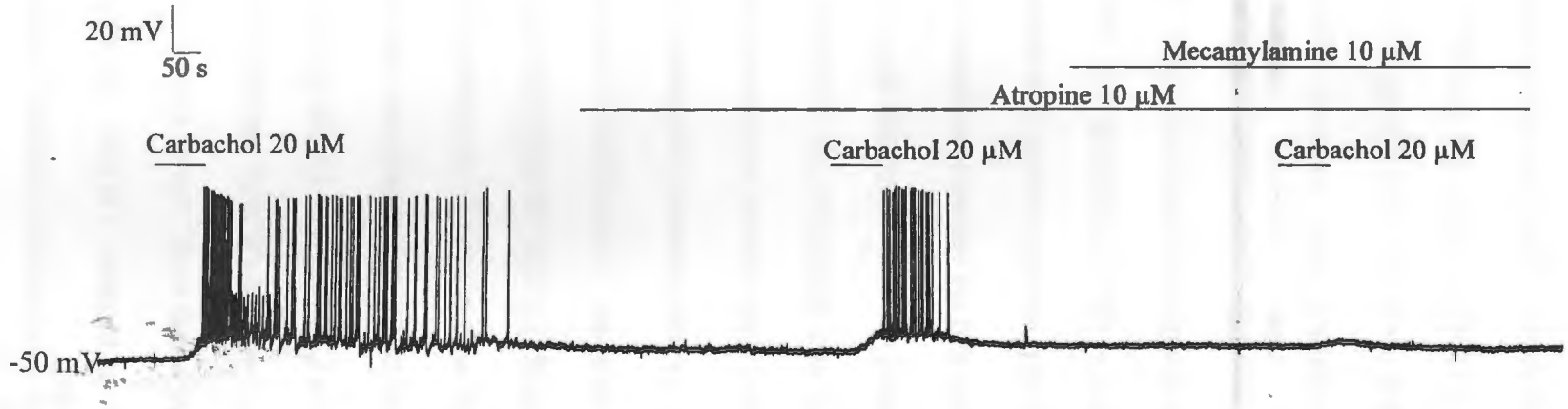
blockade appeared to be more effective in suppressing the initial depolarization induced by carbachol, while muscarinic blockade affected the slower phase of the response so that it recovered earlier. On average, atropine blocked 45.7±10% (4.17±0.91 mV) of the carbachol-induced depolarization, which was similar to the portion blocked by mecamylamine (41.1±7%, 3.73±0.88 mV) in the same group of cells (n=9).

In two tonically spiking cells (basal firing frequency: 0.15±0.11 Hz), 20 μM carbachol caused significant depolarization (9.0±2.1 mV) and at the same time induced burst firing. After 15-20 min washout, the cells recovered to pretest basal firing. When 10 μM atropine was applied for 4-5 min, carbachol's effects in the presence of atropine were reduced (depolarization: 7.3±1.9 mV, compared to 9.0±2.1 mV by carbachol alone). The response time was significantly reduced by atropine (350±35 s, compared to 1000±141 s by carbachol alone). However, the characteristics of bursting such as intra- and inter-burst frequencies remained unaffected by atropine. When 10 μM mecamylamine was applied with atropine, they almost blocked all the carbachol-induced depolarization and bursting (n=2) (Fig. 3.4). These experiments suggest that carbachol induced burst is mediated by both muscarinic and nicotinic receptors.

3.5. Carbachol Does Not Activate Presynaptic Mechanisms to Excite VTA Cells

Cholinoceptors of the muscarinic and nicotinic types have been found on both pre- and postsynaptic elements in the VTA (Weiner et al., 1990; Wooltorton et al., 2003) and their actions on both sides of the synapse lead to excitation. Specific to the presynaptic mechanism, muscarinic receptors are found to depress GABAergic transmission in the

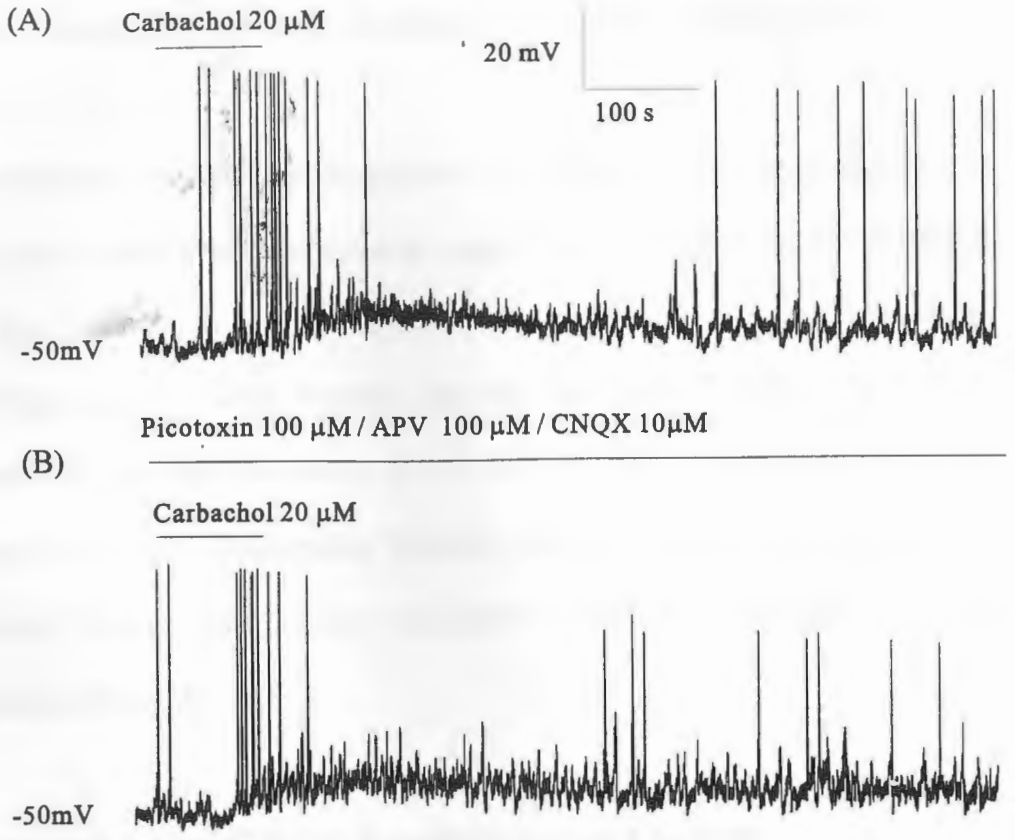
Fig. 3.4 Both muscarinic and nicotinic cholinoceptors are involved in carbachol-induced bursting. This is a continuous current clamp recording from a representative cell showing that carbachol (20 μM) induced depolarization and bursting. The response was qualitatively similar in the presence of the non-selective muscarinic blocker atropine (10 μM) except that the duration of the carbachol-induced response was much shorter. Combined application of the muscarinic and nicotinic receptor blockers almost prevented all of the carbachol-induced excitation.

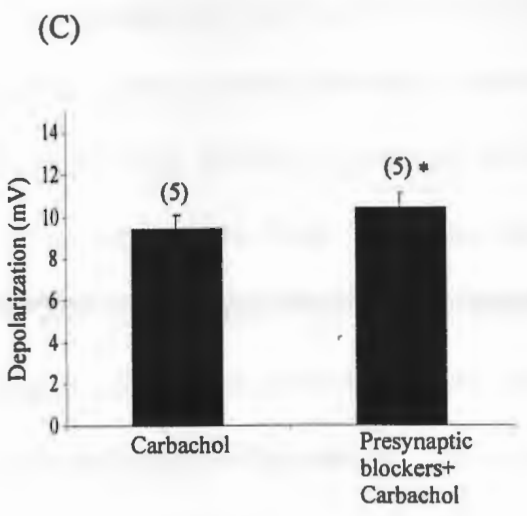

20 mV
50 s
Mecamylamine 10 µM
Atropine 10 µM
Carbachol 20 µM
Carbachol 20 µM
Carbachol 20 µM
-50 mV

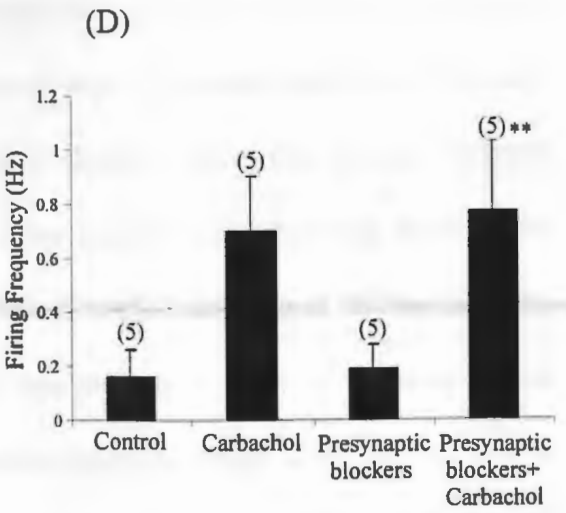
VTA (Grillner et al., 2000), and nicotinic receptors promote release of a variety of neurotransmitters including glutamate, GABA, noradrenaline, and acetylcholine (Wonnacott et al., 1990; Wonnacott et al., 1989; Vizi and Lendvai, 1999). In order to examine whether carbachol excites VTA cells through a presynaptic machinery, we applied the synaptic blockers APV (100 μM), CNQX (10 μM) and picrotoxin (100 μM) to see whether they could change carbachol's response. All synaptic blockers were shown to be effective in blocking responses evoked by their respective agonists.

In a group of 5 non-bursting DA cells identified by electrophysiological criteria, we tested whether carbachol activated a presynaptic mechanism that contributed to carbachol-induced excitation. Because repeated application of carbachol after complete recovery from the most recent dose caused comparable responses without apparent desensitization, carbachol was first applied and its responses were allowed to run their complete course. Then a cocktail containing 100 μM APV, 10 μM CNQX and 100 μM picrotoxin was applied for 5 min and the carbachol response was recorded in the presence of the cocktail. Carbachol (20 μM) caused a similar depolarization in the presence of the cocktail as compared to carbachol alone (10.4±0.68 mV compared to 9.4±0.67 mV by carbachol alone; n=5, p>0.1, two-tailed unpaired *t* test) (Fig. 3.5). The basal firing rate of the cells in this group was 0.16±0.1 Hz (n=5). After application of 20 μM carbachol, the average frequency increased 4 times (0.70±0.2 Hz, n=5). Application of the cocktail itself didn't change the basal firing frequency (0.19±0.09 Hz, n=5), nor did it change carbachol's effect (0.77±0.26 Hz compared to 0.7±0.20 Hz, n=5, p>0.1, two-tailed paired *t* test). These experiments suggest that carbachol's presynaptic effect is limited and does

Fig. 3.5 Presynaptic blockers do not block carbachol-induced excitation. (A) A representative cell responded to carbachol (20 μM) with membrane depolarization and increased firing, both of which recovered in 10 min. (B) The same DA cell was pre-treated with a presynaptic blocker cocktail containing picrotoxin (100 μM), APV (100 μM) and CNQX (10 μM) for at least 5 min, and carbachol-induced excitation was not altered in the presence of these synaptic blockers. (C) Histogram showing there was no significant difference between depolarization induced by carbachol and carbachol with presynaptic blockers. Numbers in brackets are number of cells in the group. *p>0.1 vs. carbachol group. (D) Histogram showing the same group of cells and the basal firing frequency and increased rates of firing after carbachol application alone or in the presence of synaptic blockers. Application of the cocktail itself didn't change the basal firing frequency, nor did it change carbachol's effect. Numbers in brackets are number of cells in the group. **p>0.1 vs. carbachol group.


(A)
Carbachol 20 μM
20 mV
100 s
-50mV
Picotoxin 100 μM / APV 100 μM / CNQX 10μM
(B)
Carbachol 20 μM
-50mV


(C)
Depolarization (mV)
14
12
10
8
6
4
2
0
(5)
(5) *
Carbachol
Presynaptic blockers+ Carbachol


(D)
Firing Frequency (Hz)
1.2
1
0.8
0.6
0.4
0.2
0
(5)
(5)
(5)
(5) **
Control
Carbachol
Presynaptic blockers
Presynaptic blockers+ Carbachol

not contribute significantly to carbachol-induced increase in firing rates in slice preparations.

The contribution of presynaptic mechanisms to bursting, spontaneous or carbachol-induced, was also tested. Preliminary results suggest that carbachol did not employ a presynaptic site of action to induce bursting of the VTA cells. For example, in the presence of the synaptic blocker cocktail, one cell was induced to burst by 20 μM carbachol with an intra-burst frequency of 1.72 Hz and fully recovered after 10 min washout (Fig. 3.6). Three spontaneously bursting cells in VTA whose identities were undecided, kept bursting with similar intra-burst frequencies (1.50±0.25 Hz, n=3) following synaptic blockade.

3.6. Bursting Depends on Ca^{2+} Entry through the L- type Channels

3.6.1. Ca^{2+}-dependency of Bursting

Spontaneous and carbachol-induced bursting cells reported here shared a common feature: a cluster of action potentials occurring on top of a hump potential to form one cycle of bursting. The hump potentials had much slower kinetics that allowed multiple action potentials to be fired. When the cells were in continuous bursting mode, these hump potentials merged to give rise to membrane potential oscillations. When the hump potentials occurred at lower frequencies they appeared as a series of potential waves intermingled with resting periods. The oscillation duration ranged from 7.81±1.16 s to 21.3±3.42 s, and peak-to-trough amplititude ranged from 9.22±1.07 mV to 21.05±1.8 mV (n=12). Slow kinetics and the self-supporting nature of the oscillations suggest the

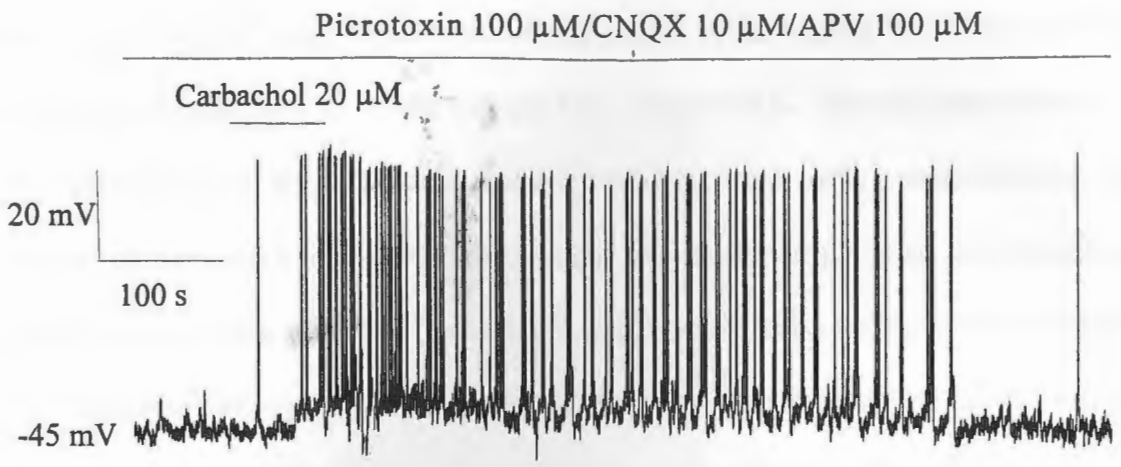


Fig. 3.6 Presynaptic blockers do not block carbachol induced bursting. After pretreatment with the presynaptic blockers picrotoxin (100 μM), APV (100 μM) and CNQX (10 μM) for 8 min, carbachol (20 μM) still depolarized the cell by nearly 10 mV and induced bursting in the presence of the blockers.

involvement of Ca^{2+} entry in the depolarizing phrase of the oscillation. In the meantime, increased intracellular Ca^{2+} could activate Ca^{2+}-dependent K^+ channels precipitating the repolarizing leg of the oscillation. Indeed, cadmium (400 μM), a non-selective Ca^{2+} channel blocker, applied for 3-5 min blocked all membrane potential oscillations and spontaneous bursting in 5 cells (including 2 putative DA and 3 undecided cells) without any change of resting membrane potential. In spontaneously bursting cells, cadmium blockade was reversible (Fig.3.7). In 2 cells on which carbachol (20 μM) was applied to induce bursting, following full recovery, reapplication of carbachol in the presence of 400 μM cadmium only caused 10 mV depolarizations, but no oscillations or bursting were induced (n=2). These experiments suggest that those oscillations are related to bursting and are Ca^{2+}-dependent.

Ca^{2+} entry through voltage-gated channels could be secondary to Na^+ spikes. In order to show that Ca^{2+} entry initiated membrane oscillation and, hence, burst firing, we blocked Na^+ spiking with 1 μM TTX and examined the effects of carbachol on the underlying oscillations. In 5 bursting cells with membrane potential oscillations, 1 μM TTX prevented firing, but membrane oscillation was still present in 3 cells. Oscillation disappeared in the remaining 2 cells after application of TTX and appeared again after application of 20 μM carbachol for 1-2 min in the presence of TTX. This suggests membrane oscillation does not depend on Na^+ spikes.

In 3 cells, when membrane potential waves were revealed by blocking action potentials with 1 μM TTX, application of carbachol (20 μM) significantly depolarized the cells from -47.66±0.88 mV to -41.67±1.67mV and changed the kinetics of the

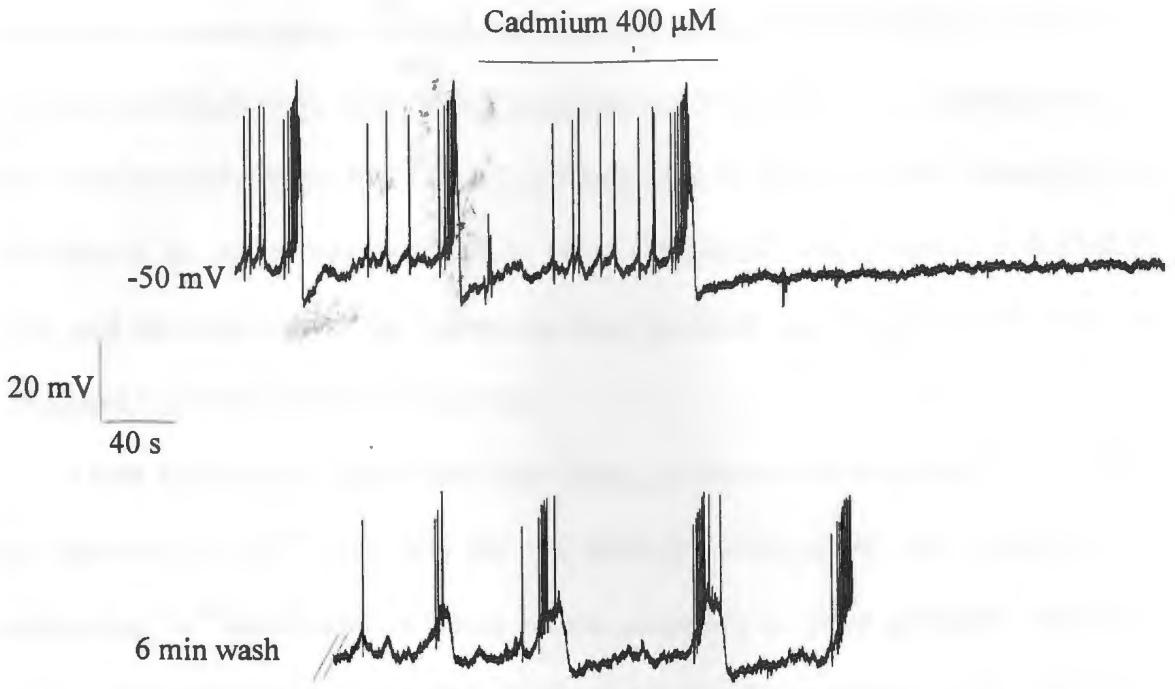


Fig. 3.7 Bursting depends on Ca^{2+} entry. A putative DA cell was spontaneously bursting with membrane potential oscillations. The Ca^{2+} channel blocker, cadmium (400 µM), applied for 100 s, completely eliminated burst firing and membrane potential oscillation. The cell gradually resumed bursting and oscillation upon washout for 6 min.

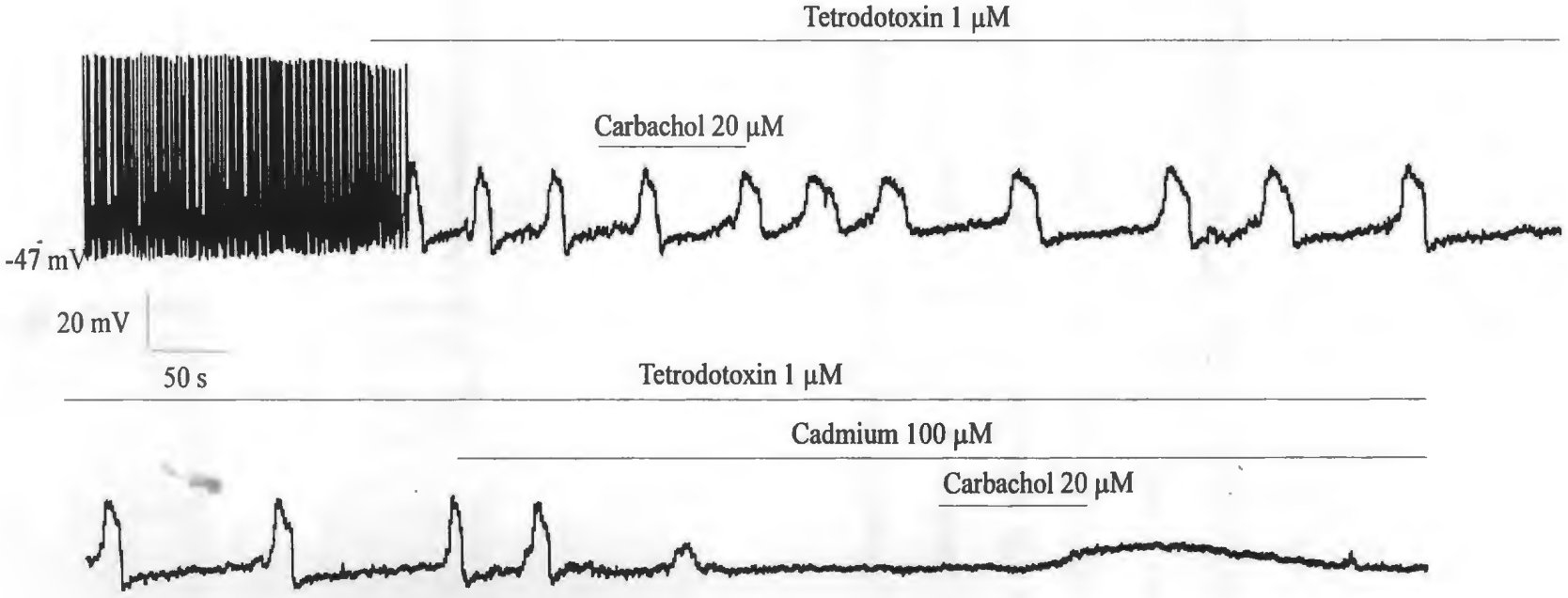
membrane potential waves. Afterhyperpolarization immediately following the waves was reduced by 68.8±4.52 % (-3±0.58 mV, compared to -9.67±1.45 mV). The amplititude of the wave was reduced by 30% (14±3.2 mV compared to 20.03±0.54 mV). However, the duration of the waves was increased by 60 % (10.33±2.85 ms compared to 6.67±2.73 ms), and the frequency of the waves was also increased significantly (0.16± 0.06 Hz, compared to 0.024± 0.001 Hz) (Fig. 3.8).

These experiments suggest that burst firing, spontaneously or induced by carbachol is dependent on Ca^{2+} entry into the cell through voltage-gated Ca^{2+} channels. The underlying Ca^{2+} oscillations or waves are not secondary to action potentials, but rather they initiate burst firing. Carbachol increases bursting by speeding up the underlying waves and is therefore also Ca^{2+}-dependent.

3.6.2. T–type Ca^{2+} Channels Do Not Mediate Bursting in DA Cells

Because membrane potential oscillation and bursting occurred at around -40 mV, at which T-type Ca^{2+} channels operate, we applied nickel (100 μM) to spontaneously bursting DA cells to see whether Ca^{2+} enters the cell through the T-type Ca^{2+} channel to support spontaneous or carbachol-induced bursting. In 2 spontaneously bursting cells, nickel applied at 100 μM for 3-4 min did not change the burst firing pattern, nor did it change the duration, amplititude and frequency of individual bursts. We applied 100 μM nickel to cells on which carbachol induced bursting. Comparing carbachol-induced bursting before and after nickel application, it was apparent that nickel did not block

Fig. 3.8 Carbachol speeds up membrane potential oscillations. In current clamp mode, a regular spiking dopamine cell is shown. After the Na^+ channel blockade with TTX (1.0 μM), the Na^+-dependent spiking disappeared revealing a subthreshold potential oscillation. Carbachol (20 μM) not only depolarized the membrane potential, but also increased the frequency of the oscillation. Cadmium (100 μM), a broad spectrum Ca^{2+} channel blocker, prevented the oscillation. Reapplication of carbachol (20 μM) only depolarized the membrane and no oscillation was induced.


Tetrodotoxin 1 μM
Carbachol 20 μM
-47 mV
20 mV
50 s
Tetrodotoxin 1 μM
Cadmium 100 μM
Carbachol 20 μM

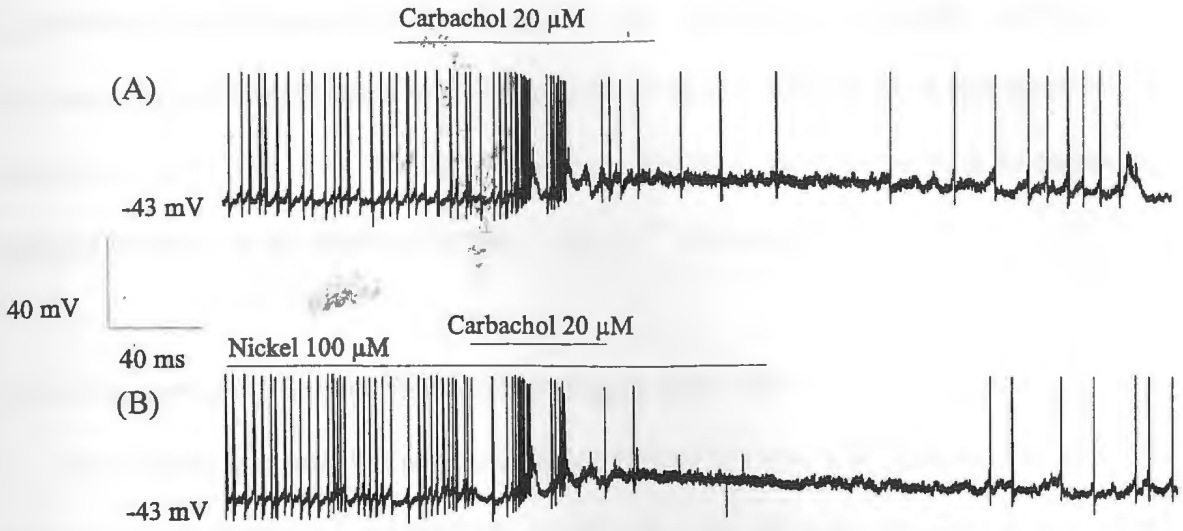


Fig. 3.9 T-type Ca^{2+} channels do not mediate bursting induced by carbachol. These are current clamp recordings showing (A) A regularly firing cell that responded to carbachol (20 μM) with depolarization and bursting. After washout, the cell resumed regular spiking. (B) The same cell was pre-treated with the T-type Ca^{2+} channels blocker, nickel (100 μM), for 5 min, carbachol still depolarized the membrane and induced bursting in the presence of nickel, without any change in bursting pattern, frequency and depolarization as compared to that under control conditions.

carbachol-induced bursting, and contrary to our hypothesis, it slightly increased the frequency of burst oscillations by 27% without changing the duration and amplitude of bursts in 2 cells (Fig. 3.9). The results suggest that spontaneous, as well as carbachol-induced bursting, is not mediated by the T-type Ca^{2+} channels.

3.6.3. L–type Ca^{2+} Channel Mediate Bursting in DA Cells

It has been reported that repeated stimulation of L-type Ca^{2+} channels in the VTA mimics the initiation of behavioural sensitization to cocaine (Licata et al., 2000) implicating L-type Ca^{2+} channels in DA cell output. We have shown that Ca^{2+} influx underlies burst firing. In order to test if the L–type Ca^{2+} channels were involved, we first applied the L-type Ca^{2+} channel blocker, nifedipine (10 μM), on 3 spontaneously bursting cells. After application of nifedipine, there was no change in resting membrane potential (-44.6±3.3 mV), but the firing pattern changed completely. The firing pattern of these cells changed from bursting to pacemaker-like spiking with an average frequency of 0.20±0.14 Hz (n=3). Nifedipine blocked all spontaneous bursting in 4 min and the accompanying membrane potential oscillation in two bursting cells, which were not blocked by 100 μM nickel. This suggests that the L-type, but not the T-type, Ca^{2+} channels mediate bursting.

To examine whether L-type Ca^{2+} channels support carbachol-induced bursting, we applied nifedipine (10 μM) on cells that responded to 20 μM carbachol with burst firing. In the presence of 10 μM nifedipine, carbachol only depolarized the cells, but bursting could no longer be induced (Fig. 3.10) (n=2). These results suggest that bursting,

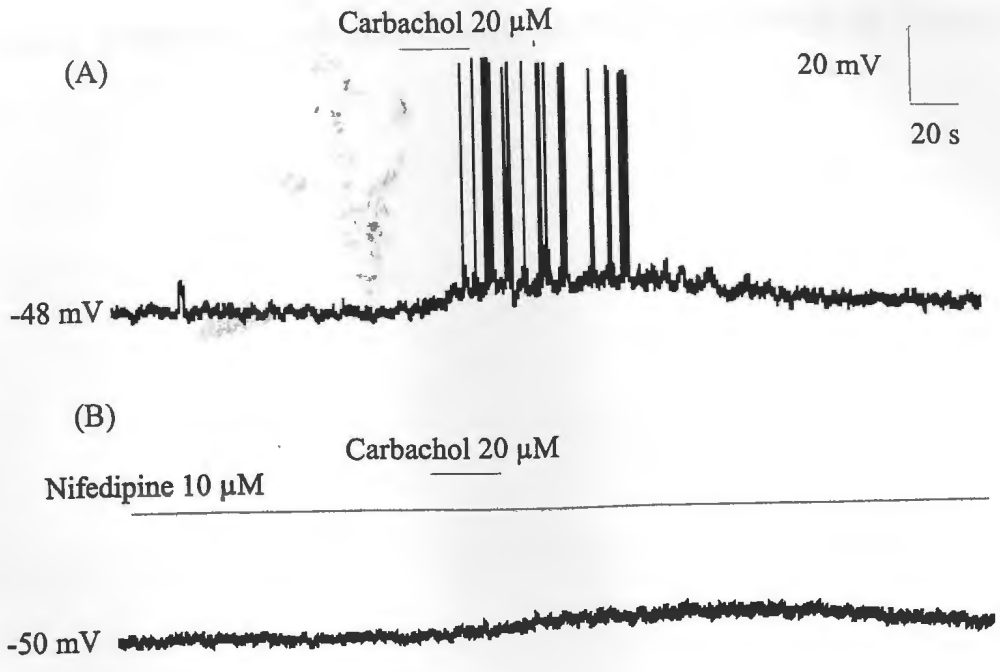


Fig. 3.10 L-type Ca^{2+} channels mediate bursting induced by carbachol. These are current clamp recordings showing: (A) Carbachol (20 µM) induced burst firing in a quiescent cell. (B) In the presence of the L-type Ca^{2+} channel blocker nifedipine (10 µM), reapplication of carbachol (20 µM) only depolarized the membrane but no bursting was induced.

spontaneously or caused by carbachol depends on Ca^{2+} entry through the L-type Ca^{2+} channel.

Chapter 4 DISCUSSION

In this thesis, we observed that DA cells in the VTA were capable of both tonic and burst firing *in vitro*. Activation of cholinoceptors excited most cells in the VTA and induced burst firing in 13% of the cells. Nicotinic and muscarinic receptors appeared to be equally important in mediating carbachol-induced excitation. Burst firing, either spontaneously occurring, or induced by carbachol, was dependent on Ca^{2+} entry into the cell through the L-type, but not the T-type, Ca^{2+} channels. Presynaptic cholinoceptors appeared to be playing a minimal role in burst firing *in vitro*.

Pacemaker-like firing at low frequencies is often seen as a predominant feature of DA cells in slice preparations. This could be because DA cells in slice are deprived of synaptic inputs and are allowed to be governed by their intrinsic membrane mechanisms. In other words, irregular firing and bursting are primarily controlled by synaptic inputs to DA cells. However, we found in this work that a small portion of DA cells in the slice are natural bursters, and more importantly, some DA cells respond to carbachol, a nonselective cholinergic agonist used to mimic the actions of the native transmitter ACh, with strong bursting. We will, therefore, discuss burst firing and the neuronal properties that support burst firing, followed by a discussion of the mechanism of carbachol-induced effects and on the particular role Ca^{2+} plays in cholinergic activation of DA cells in the VTA.

4.1 Factors Related To Burst Firing

Results presented here show that in VTA slices harvested from pre-weaning rats (8-20 days old), 13% of the cells, most likely DA cells according to electrophysiological features, display a burst firing pattern following carbachol application. Although a large proportion of recorded cells in this work showed regular, pacemaker-like firing typical of DA cells in slices, the existence of natural bursters in the slices and of cells whose firing patterns could be switched by cholinergic activation are worth noting. This could arise from a number of factors: the perforated patch configuration used in this work, the age of the animals, and the temperature at which all patch recordings were made.

The perforated-patch recording technique used in this work has a number of advantages over conventional whole cell intracellular recording used in earlier experiments. The channels formed by nystain are smaller and impermeable to molecules larger than glucose; typically only small ions such as Na^+, K^+ and Cl^- can pass through. Therefore, recordings can be done without dialyzing important substances of small molecular weight from the cell's cytoplasm. Currents run down significantly more slowly and second-messenger cascades and mechanisms important to cell signalling and channel regulation are kept operative (Fu et al., 2003).

The reason that we observed spontaneous bursting as well as bursting induced by carbachol could be due to the perforated patch clamp configuration we used in these experiments. As presented in the results section, both spontaneous and carbachol-induced burst firing rely on Ca^{2+} entry into the cell, and carbachol's effects mediated by muscarinic receptors involve soluble second messengers, molecules that are easily

dialyzed in conventional whole cell recordings, but are retained perfectly in the perforated patch recording conditions. This explanation appears plausible, since burst firing in slices is very rarely seen in intracellular and conventional whole cell recording conditions. However, the exclusion of burst firing under those conditions is not complete. Using the conventional patch clamp method, it has been reported that DA cells display small AHPs in response to orexin A (Korotkova et al., 2003) and to low concentration of nicotine (0.5 μM) with bursting *in vitro* (Pidoplichko et al., 1997). Carbachol activates specific signaling cascades giving rise to a number of soluble messenger molecules that can be effectively retained inside the cell using perforated patch recording, it remains a clear possibility that this is the reason for the more frequent occurrence of burst firing in this work.

Another factor is the temperature in our experiments (22°C). Temperature regulates the rates of enzyme-catalyzed reactions and has effects on the function of ion channel proteins. For example, Ca^{2+} influx through the L-type Ca^{2+} channels was significantly more sensitive to adrenergic stimulation at 7°C (Shiels et al., 2003). I_h is also found to be sensitive to temperature being more conductive at higher temperatures (Vargas and Lucero, 1999). It is possible that I_h is less effective at room temperature, which may be reflected by the fact that the firing frequency of DA cells in our experiments is lower than that reported at higher temperatures (spontaneous spike frequency >1 Hz compared to 0.28±0.03 Hz in our experiments) (Johnson and North, 1992b; Lacey et al., 1989). We did observe a decrease in basal firing when I_h channels were blocked by ZD7288, however, blockade of these channels did not seem to alter carbachol-induced responses,

arguing against their role in the cholinergic excitation of DA cells (preliminary observations, not shown in this thesis).

The possibility that burst firing *in vitro* could be related to age must also be considerd because the age of rats used in this thesis work fall between 8-20 postnatal days, which has been associated with *in vitro* bursting. Mereu reported that DA neurons in slices from immature rats (15-21 days old) exhibited, not only pacemaker-like firing, but also irregular and bursting patterns (28.3 and 18.3%, respectively) (Mereu et al., 1997). This is thought to result from NMDA activity and, as the aged-related decline in NMDA receptor sensitivity sets in, the cells stop bursting in slice. However, it does not appear to be the mechanism here, since spontaneous bursting cells could keep bursting in the presence of the NMDA receptor blocker APV and we did not find any relationship between age (8 to 20 days) and the firing pattern. Whether this pattern can be extended to include mature rats remains to be established. There are indeed also age-related changes in the cholinergic system that may be related to carbachol-induced bursting. ChAT-positive neurons already exist in the pedunculopontine tegmental nuclei at birth, ChAT-positive fibers in the lateralis medialis-suprageniculate nuclear complex were observed only after 7 postnatal days. These ChAT-positive fibers gradually increase in number, and almost reach the adult level by postnatal day 28 (Kaiya et al., 2003). This implies that cholinergic input is still developing between the ages of 8 to 20 days, which were these used in this work. At earlier ages, while presynaptic terminals are immature, the firing pattern may mainly depend on intrinsic factors and extrinsic factors, such as presynaptic

input and terminal feedback, may play a more important role in the adult when those connections become complete.

4.2 Carbachol-Induces Depolarization and Bursting

In our experiments, most DA cells were excited following carbachol application and 13% of them were induced to burst. This provides direct evidence at the cellular level for the experimental findings that carbachol injected into the VTA increases the firing rate of DA cells (Redgrave and Horrell, 1976; Yeomans et al., 1985) and that enhanced cholinergic output to the VTA increases burst firing (Floresco et al., 2003).

Carbachol's effects are mediated by both nicotinic and muscarinic receptors because in our experiments, nicotinic and muscarinic blockers, each reduced by nearly half carbachol-induced membrane depolarization. However, these receptors differ in their involvement in different stages of carbachol's response, in that nicotinic receptors seem to be more important in the initial fast response, while muscarinic receptors appear to be responsible for the prolonged response. There is a considerable overlap of responses mediated by the two types of cholinoceptor because blocking either one reduced the peak depolarization induced by carbachol. This is consistent with a previous report showing that depolarization caused by ACh was typically biphasic; only the slower component was blocked by the muscarinic antagonist scopolamine (Calabresi et al., 1989). In cultured hippocampal neurons, ACh also produces two types of response. One class of ACh current exhibits rapid and profound desensitization and is sensitive to inhibition by

nAChR antagonist, α-bungarotoxin (α-BTXN), the other class activates slowly and exhibits no desensitization during prolonged agonist applications (Zorumski et al., 1992).

nAChRs have a high Ca^{2+} permeability. In addition, activation of nicotinic receptors on DA cells produces direct excitation by allowing Na^{+} and Ca^{2+} influx (Yin and French, 2000; Calabresi et al., 1989). Low doses of nicotine application cause midbrain DA neurons to depolarize and burst (Pidoplichko et al., 1997). In our experiments, after application of the Na^{+} channel blocker, TTX, and the Ca^{2+} channel blocker, cadmium, carbachol still caused a small depolarization in DA cells, which implies a complicated excitation mechanism. The remaining depolarization may be due to positive charge entering the cell through nAChRs or non-selective cantionic channels such as TRP channels which are known to be activated by mAChRs (Lee et al., 2003).

Nicotine stimulates DA release in the ventral striatum of wild-type mice, but not in the β_2-mutant mice. Using patch-clamp recording, mesencephalic DA neurons from mice without the β_2 subunit no longer respond to nicotine. This implies the nicotinic β_2 receptor plays an important role in this excitation (Picciotto et al., 1998). nAChRs containing the β_2 subunit are located on the cell body of DA cells (Charpantier et al., 1998; Picciotto et al., 1998). If nicotinic agonists excite DA cells through their actions on these receptors, it would represent a direct axosomatic coupling rather than a modulation through presynaptic mechanisms. Our results support this mode of activation. A selective $\alpha_4\beta_2$ agonist, anatoxin A, excited DA cells in slice in our preliminary results. Carbachol-induced excitation was unaltered in the presence of the synaptic blocker cocktail that

disabled glutamatergic and GABAergic transmission (to be discussed in more detail in the next section).

Injection of the glutamate receptor antagonist, APV, into the VTA *in vivo* largely prevents the stimulatory effect of nicotine (Schilstrom et al., 1998), suggesting that nicotinic modulation of glutamatergic transmission contributes significantly to the enhancement of VTA DA output. Focal injection of methyllycaconitine (MLA), an inhibitor of α_7-containing nAChRs, into the VTA also prevents nicotine-induced increases in DA release (Seguela et al., 1993). These findings have led to the hypothesis that nicotine may influence DA release by presynaptic modulation of excitatory input to these neurons. This mode of action has been reported to exist in slices (Dani et al., 2001) when synaptic currents are evoked by electrical stimulation. In our setup where afferents were left unstimulated, carbachol did not seem to excite DA cells through modulating glutamatergic terminals.

Activating the muscarinic receptors induce G-protein coupled second messenger pathways resulting in modulation of synaptic transmission and neuronal excitability (Forster et al., 2002; Grillner et al., 2000). Intrategmental infusion of the muscarinic agonist oxotremorine M, increases extracellular levels of DA and serotonin in the VTA to a maximum of 200% over baseline in both urethane-anaesthetized and unanaesthetized rats (Gronier et al., 2000). Muscarine increases the rate of spontaneous action potentials and causes a membrane depolarization and an inward current in VTA neurons *in vitro*. The depolarizations, or inward current, can be reduced reversibly or abolished, by a low calcium (0.25 mM)/high magnesium (10 mM) solution (Lacey et al., 1990).

Administration of two phospholipase C inhibitors alters the magnitude of the muscarinic activation of DA cells suggesting that this activation process involves the breakdown of phosphatidylinositol, indicative of $G_{q/11}$ protein coupling. Furthermore, the phospholipase C inhibitors reduced significantly the activation of DA cells induced by the selective muscarinic agonist oxotremorine M. This result constitutes additional evidence that the muscarinic activation of DA cells is mainly mediated by the M_1 receptor subfamily (Gronier and Rasmussen, 1999).

In urethane-anesthetized mice, electrical stimulation of the laterodorsal tegmental nuclei results in a rapid, stimulus-time-locked increase in DA release in the nucleus accumbens, followed several minutes later by a prolonged increase in DA release. In mutant mice with truncated M_5 receptors, the prolonged phase of DA release is absent (Forster et al., 2002). In addition, the rewarding effects of morphine are also substantially reduced in M_5 mutant mice, as measured in the conditioned place preference paradigm (Yamada et al., 2003). These findings indicate that M_5 muscarinic receptors are needed for slow activation of DA neurons and for rewarding brain stimulation (Yeomans et al., 2001). However, it is also reported that activation of presynaptic muscarinic M_3 receptors reduces excitatory synaptic potentials through blocking the NMDA component of glutamatergic transmission and depresses the evoked GABAergic synaptic transmission (Grillner et al., 1999; Zheng and Johnson, 2003). Therefore, the net effect of muscarinic activation depends on how much the involved synaptic machinery contributes to the cell's excitability.

An intermediate molecule that could mediate the effect of carbachol reported in this thesis is ATP, which is co-released with GABA, glutamate and acetycholine, transmitters that are known to be released by presynaptic nAChRs. In addition, purinergic agonists infused into the VTA have been found to increase DA release *in vivo* (Jo and Schlichter, 1999; Mori et al., 2001; Nörenberg and Illes, 2000; Silinsky and Redman, 1996). It is possible that ATP co-released with other transmitters by carbachol can activate the P_{2x} receptor and cause excitation or Ca^{2+} influx. Ca^{2+} influx increases intracellular Ca^{2+} levels through Ca^{2+} -induced Ca^{2+} -release from internal stores and triggers a cascade of cellular events leading to channel modulation (Berridge, 1998). Increased Ca^{2+} levels also regulates Ca^{2+}-activated K^{+}-channels (Morikawa et al., 2000) which participate in shaping up firing patterns (Bennett et al., 2000). Our preliminary results indicate that P_{2X} blockade promotes recovery of DA cells from carbachol-induced excitation.

4.3 Presynaptic Effects

Since glutamatergic and GABAergic afferents are the main excitatory and inhibitory synaptic inputs to the VTA and both are enhanced by nAChRs, synaptic antagonists blocking AMPA, NMDA and $GABA_A$ receptors were used. They did not alter carbachol-induced depolarization or increase firing frequency, nor did they block carbachol-induced or spontaneous bursting. These results indicate that in slice preparations, carbachol-induced excitation and burst firing are not dependent on presynaptic mechanisms.

However, it has been reported that application of nicotine increases the peak amplititude of evoked excitatory synaptic currents and enhances spontaneous excitatory

postsynaptic current frequency in VTA DA neurons in slices. These effects could be inhibited by both the α_7 nAChRs blocker MLA and the NMDA receptors blocker APV (Mansvelder and McGehee, 2000), suggesting nicotinic effects are indirectly through promoting glutamate release. Indeed, activation of nicotinic receptors has been found to increase the release of a variety of neurotransmitters including glutamate, GABA, noradrenaline and acetylcholine (Barazangi and Role, 2001; Rao et al., 2003; Sershen et al., 1997; Mansvelder et al., 2002; Clarke and Reuben, 1996). The intermediate role for NMDA receptors is further strengthened by *in vivo* testing showing that the NMDA receptor antagonist APV infused into the VTA largely prevents the stimulatory effects of nicotine (Schilstrom et al., 1998). The fact that carbachol-induced excitation independent of synaptic intermediaries is observed could arise from a number of possibilities. Firstly, carbachol is a mixed agonist that activates both nicotinic and muscarinic receptors. The M_3 receptor has been found to suppress excitatory synaptic transmission to the VTA. As muscarinic receptors account for nearly half of carbachol's response, it is therefore very likely that presynaptic nicotinic excitatory effects are offset by the inhibition mediated by muscarinic receptors. Pure muscarinic agonists also excite DA cells, but this is mediated, by and large, by postsynaptic M_1 and M_5 subtypes.

Secondly, *in vitro* findings implicating presynaptic nAChRs in enhancing glutamate release are demonstrated either *in vivo* or by evoked synaptic responses. It is highly conceivable that in the whole animal, these synaptic afferents carry active signals at the time of nicotine testing. While it is acknowledged that nAChRs have strong presynaptic effects, which was not observed in carbachol-induced excitations in our experimental

setup, it is likely that the inconsistent results arise from the fact that presynaptic nicotinic effects can only manifest themselves when there are active signals passing through afferent fibers on which nAChRs are located, as is the case *in vivo,* or in slices, where the afferents are electrically stimulated. If afferents were left unstimulated, there would be far less likehood of the nAChRs modulating the flow of signals because there are no signals actively going through these afferents. This is supported by the preliminary results in our laboratory that a preferential α_7 agonist did not increase the firing rate of DA neurons whereas the agonist for the $\alpha_4\beta_2$ receptor, a subtype that is located on the presynaptic cell, strongly excited DA cells.

Thirdly, presynaptic nAChRs enhance both glutamatergic and GABAergic transmission (Schilstrom et al., 1998; Barazangi and Role, 2001) and consequently, the net effect on DA cell excitability through presynaptic mechanisms could be minimal. Although it is reported that nicotine induces long-term potentiation preferentially at the glutamatergic synapses (Mansvelder and McGehee, 2000), in a single bath application setting such as ours, it might be assumed that presynaptic nAChRs would enhance the release of both glutamate and GABA, which would cancel out each other's effects at the postsynaptic cell.

In summary, while nAChRs have been shown to excite axons and their activation results in enhanced release of transmitters, in slice preparations where afferents are not driven by the cell bodies or by electrical stimulation, carbachol-induced excitation is mostly brought about on the postsynaptic cell. This is consistent with Grillner's finding that nicotine directly excites DA cells (Grillner and Mercuri, 2002).

4.4 Carbachol-Induced Bursting Depends on the L-type Ca^{2+} Channel

Carbachol-induced bursting could be blocked by the Ca^{2+} channel antagonist cadmium. This is consistent with numerous studies showing that pacemaker-like slow depolarizations, spontaneous oscillatory potentials, and slow afterhyperpolarizations involved in the regulation of DA neuron firing activity have all been shown to be Ca^{2+} dependent (Grace and Bunney, 1984; Grace and Onn, 1989; Fujimura and Matsuda, 1989; Kang and Kitai, 1993b). A previous study suggested that DA neurons express L-, N-, P/Q-, and R-type Ca^{2+} channels (Cardozo and Bean, 1995). The question is which voltage gated Ca^{2+} channels play an important role in carbachol-induced burst firing? Since in our setup, carbachol does not employ presynaptic machinery to induce excitation and burst firing, we examined the role for the T- and L- type channels in bursting, leaving out those channels primarily involved in transmitter release from presynaptic terminals.

Thalamic neurons undergo a shift from tonic to burst firing upon hyperpolarization. This state transition results from deinactivation of a regenerative depolarizing event referred to as low-threshold spikes that are sensitive to nickel, but not to nimodipine, implying that the T-type Ca^{2+} channels underlies these spikes (Suzuki and Rogawski, 1989). A transient low-threshold current underlying the afterdepolarizing potential that triggers burst firing in a subpopulation of dorsal root ganglionic neurons is also sensitive to nickel blockade (White et al., 1989). The low threshold T-type Ca^{2+} channels are, therefore, thought to play an important role in burst firing. However in our experiments, the T-type Ca^{2+} channels blocker, nickel, did not have any effect on spontaneous bursting

or bursting caused by carbachol. The T-type channels on DA cells have been proposed as a new target for antipsychotic chemotherapy because of their role in inducing burst firing and the finding that current antipsychotics reduce T currents in DA cells (Santi et al., 2002). We didn't observe an apparent involvement of the T-type channels in burst firing. Even more contradictory is the finding that blocking T-type channels induces burst firing in DA cells (Wolfart and Roeper, 2002) because the blockade reduces the AHP which is one of the limiting factors for firing at high frequencies.

The L-type Ca^{2+} channels are also involved in burst firing. In subthalamic neurons, the TTX-resistant rhythmic membrane oscillations underlie burst firing. The duration of such bursts can be irreversibly decreased by the L-type blocker, nifedipine (Beurrier et al., 1999). In DA neurons, spontaneous oscillatory potentials, and spontaneous firing, are also abolished by L-type Ca^{2+} channel blockers (Nedergaard et al., 1993; Mercuri et al., 1994). In fact, previous studies in several models have implicated L-type Ca^{2+} channels in activating DA cells. In the animal model, systemic injections of L-type Ca^{2+} channel antagonists (Karler et al. 1991; Reimer and Martin-Iverson 1994) block the initiation of behavioral sensitization to cocaine or amphetamine. L-type Ca^{2+} channels are reported to control Ca^{2+} influx (de Erausquin et al., 1992) and the amount of DA released from dopaminergic dendrites and synaptosomes (Woodward and Leslie, 1986; Chaudieu et al., 1992). *In vitro*, nifedipine decreases the number of action potentials in the burst (Mercuri et al., 1994) and blocks apamin-induced burst activity in DA neurons (Shepard and Stump, 1999). Orexin A which also induces DA cells to burst, (Korotkova et al., 2003) enhances the L-type Ca^{2+} current (Xu et al., 2002). Our results are consistent with a role

Chapter 5 CONCLUSIONS

In our experiments, bath application of carbachol (20 μM), a cholinergic agonist, excites most of the DA cells and causes 13% of the DA cells to burst *in vitro*. Muscarinic and nicotinic receptors contribute equally to carbachol-induced excitation. The excitation induced by carbachol is mainly through postsynaptic mechanisms as the synaptic blocker cocktail containing 100 μM APV, 10 μM CNQX and 100 μM picotoxin could not prevent carbachol's effects. Spontaneous and induced bursting by carbachol are Ca^{2+}dependent and the route of Ca^{2+} entry is probably through the L-type Ca^{2+} channels.

These results demonstrate that cholinergic inputs to the VTA constitute a synaptic trigger for burst firing of DA cells. Since bursting is one of the strategies employed by DA cells to enhance their terminal output, the underlying L-type Ca^{2+} channel activation in carbachol-induced burst firing implies that the L-type Ca^{2+} channel blockers may be used as potential drugs in the treatment of addiction and psychosis. It has recently been reported that nifedipine is effective for the in-patient management of withdrawal and craving in a broad spectrum of chronic drug addicts (Shulman et al., 1998).The cellular mechanism for nifedipine's efficacy may arise from the involvement of the L-type Ca^{2+} channels in burst firing as reported in this thesis. This hypothesis looks more promising since recent results from our laboratory show that direct L-type Ca^{2+} channel activators increase the excitability of DA cells and promote, in some cases, burst firing in slices.

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