DOPAMINERGIC NEURONS IN THE VENTRAL TEGMENTAL AREA:
ROLE OF L-TYPE CALCIUM CHANNELS IN FIRING REGULATION

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Dopaminergic Neurons in the Ventral Tegmental Area: 
Role of L-type Calcium Channels in Firing Regulation

by

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A thesis submitted to the
School of Graduate Studies
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Division of Biomedical Sciences
Faculty of Medicine
Memorial University of Newfoundland

April, 2009

St. John's
Newfoundland
Abstract

Dopaminergic projections from the ventral tegmental area constitute the mesolimbocortical system that underlies drug abuse and schizophrenia, primarily as the result of increased dopamine transmission. Essentially, more intense spiking releases more dopamine at the terminal, such as fast single spike firing or burst firing. L-type calcium channels are expressed in dopaminergic neurons, however their role in regulating firing frequency and modes remains unknown. In this thesis, I combined patch clamp recording, western blotting and L-type calcium channel transgenic mice to examine the effects of L-type calcium channels on the firing behavior of dopaminergic neurons in brain slices. Results revealed that calcium influx through L-type calcium channels following FPL64176 or (S)-(−)-Bay K8644 application induced burst firing independent of dopamine, glutamate or calcium from internal stores. The burst firing induced as such was completely blocked by the substrate site protein kinase C (PKC) inhibitor chelerythrine but not by the diacylglycerol site inhibitor calphostin C. Western blotting analysis showed that FPL64176 and (S)-(−)-Bay K8644 increased the cleavage of PKC to generate protein kinase M (PKM) and the specific calpain inhibitor MDL28170 blocked this increase. Prevention of PKM production by inhibiting calpain or depleting PKC blocked burst firing induction whereas direct loading of purified PKM into cells induced burst firing. Activation of the NMDA type glutamate or cholinergic receptors known to induce burst firing increased PKM expression. These results indicate that calcium influx through L-type calcium channels activates a calcium-dependent protease that cleaves
PKC to generate constitutively active and labile PKM resulting in burst firing of dopaminergic cells (Chapter 2). Next, I examined the role of the L-type calcium channel and PKC in firing responses to carbachol, NMDA or AMPA. All three ligands induced a reversible increase in firing, however, only the carbachol-induced increase was attenuated by the PKC inhibitors chelerythrine and GF 109203X. The L-type calcium channel blocker nifedipine partially blocked carbachol-induced excitation similar to the PKC inhibitors. PKC inhibition and L-type calcium channel blockade did not significantly alter NMDA- or AMPA-induced excitation. Concurrent blockade of PKC and L-type calcium channels with chelerythrine and nifedipine did not additionally suppress carbachol-induced excitation indicating they were sequential events in the same signaling pathway. Furthermore, preincubation with the PKC inhibitor GF 109203X reduced the carbachol-induced increase in nifedipine-sensitive high-voltage gated calcium currents. These results indicate that cholinergic activation enhances PKC activity, which in turn facilitates L-type calcium channel opening to excite dopaminergic cells (Chapter 3). Then, I examined which subtype of L-type calcium channels was involved in firing activities in L-type transgenic mice that have a mutant dihydropyridine (DHP) site. Single spike firing was inhibited to the same extent by the DHP site blocker nifedipine in both Ca_{v}1.2DHP^{(+/-)} and Ca_{v}1.2DHP^{(-/-)} mice. The DHP site opener (S)-(−)-Bay K8644 and the non-DHP opener FPL64176 induced bursting of dopaminergic cells in Ca_{v}1.2DHP^{(+/-)} mice similarly and the DHP blocker nifedipine blocked bursting induced by either agent. Since Cav1.2 and Cav1.3 are the only subtypes expressed in dopaminergic cells, these
results underscore the importance of Ca,1.3 L-type calcium channels in single spike and burst firing of these cells (Chapter 4).
Acknowledgements

I wish to thank my supervisor Dr. Xihua Chen, who has provided me the opportunity to participate in the Neuroscience program in Memorial University and to complete my Ph.D. thesis work in his lab; who has opened a door to me toward a scientific world and guided me patiently and persistently; who has taught me the scientific thinking and how to find a useful way in complex scientific and reality world. St. John’s is my first place to visit far away from my home country. His wife, Jieying Xiong and he, have provided countless valuable help in my life like a mother and a father.

I wish to thank Dr. Jules Dore, who has provided me an opportunity and resources to learn Western blotting and PCR genotyping, with which my thesis work is more complete.

I wish to thank Dr. Gilbert Kirouac, my committee member, who has provided me useful suggestions on my thesis work and my future plans.

I wish to thank my parents Min Wang and Hongmin Liu, who have not only nurtured me but also taught me many things in my life; who always encourage and support me to pursue my dreams in any time; whose endless love has always accompanied me.

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I wish to thank my extended family, my grandpa and grandma, my aunts and uncles, my cousins, especially Xin Chen, and my sister Yongdan Liu, who has provided endless love and countless support to me in my life.

I wish to thank my lovely Chinese friends, Zhili Kang, Dr. Jinguo Wang, Sa Li, Lan Chen, Dr. Yaping Li, Dr. Xuhan Liu, Dr. Ningyuan Feng, and Dr. Chunming Dai, who always has shared laughs and tears with me and held my hands whenever I needed. Without them, I could not survive in the tough Ph.D. study far away from my home country.

I wish to thank my lovely Canadian friends, Heather Moore and Meghan Harding, who has provided me their love and care; who has shared with me the excitement of life; who has always made me laugh; who has taught me a lot of English and things about western life. Without them, my life in Canada could not be this colorful.

I wish to thank the School of Graduate Studies, Faculty of Medicine and my supervisor Dr. Xihua Chen for having provided me the financial support throughout my program, and all the faculty members and students in the Neuroscience program who have given me the help and support.
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List of Abbreviations

ACSF, artificial cerebrospinal fluid;
AChR, acetylcholine receptor;
ACH, acetylcholine;
AHP, afterhyperpolarization potential;
CaMKII, calcium/calmodulin-dependent protein kinase II;
CV, coefficient of variance;
CPA, cyclopiazonic acid;
DA, dopamine;
DAG, diacylglycerol;
DAT, dopamine transporter;
DHP, dihydropyridine;
EGFP, enhanced green fluorescent protein;
GAD, glutamic acid decarboxylase;
GIRK channel, G protein-activated inwardly rectifying K⁺ channel;
HVA, high-voltage-activated;
Ih, hyperpolarization-activated current;
ISIs, interspike intervals;
LDTg, laterodorsal tegmental nuclei;
LSD, least significant difference method;
mGluRs, metabotropic glutamate receptors;
NAc, nucleus accumbens;
PKC, protein kinase C;
PKM, protein kinase M;
PKA, protein kinase A;
PMA, phorbol 12-myristate 13-acetate;
PMSF, phenylmethlysulfonyl fluoride;
PPTg, pedunculopontine tegmental nuclei;
PVDF, polyvinylidene fluoride;
RMP, resting membrane potential;
SK channel, calcium-activated small conductance potassium channel;
SERCA, sarco-endoreticulum Ca$^{2+}$-dependent ATPase;
SEM, standard errors of the mean;
SNpc, substantia nigra pars compacta;
TH, tyrosine hydroxylase;
TRP channel, transient receptor potential channel;
TTX, tetrodotoxin;
VTA, ventral tegmental area;
WT, wild type.
Chapter 1 Introduction and Overview

The central nervous system allows us to process environmental stimulation into conscious percepts and organize our actions in response to a specific task. This calls for two fundamental requirements of neurons as functional units of the central nervous system: they need to be able to receive inputs and they need to transform these inputs into an output. The neuronal output is electrical in nature which is referred to as an action potential. Action potentials are the language of neurons, like the notes of music, the dots of Braille and the signs of a sign language. Action potentials are all-or-none: a neuron either fires an action potential or nothing at all. Because of this feature, an individual action potential has a very limited capacity for information coding. However, a train of action potentials occurring at different frequencies and in different temporal patterns codes for all our different brain functions such as movement, learning and motivation. This is analogous to the huge amount of words formed with a limited number of letters.

Dopaminergic (DA) systems originating from the ventral tegmental area (VTA) play a vital role in reward processing, leading to addiction and psychosis primarily as the result of increased DA transmission. Since the frequency and temporal pattern of action potential trains code for all neuronal information, the regulation of the firing behavior of DA neurons is essential for their roles in reward processing and disease states. This chapter gives a general review on (1) the functional role of the DA system, (2) the modulation of firing activities and its functional importance, (3) the functional
significance of L-type calcium channels in the DA systems and (4) the hypotheses of my projects.

1.1 The central DA systems and their functions

The Swedish pharmacologist Arvid Carlsson performed a series of pioneering studies during the late 1950's, which showed that DA is an important transmitter in the brain (Carlsson, et al., 1957; Carlsson, et al., 1958). It was previously believed that DA was only a precursor of another transmitter, noradrenaline. Arvid Carlsson developed an assay that made it possible to measure tissue levels of DA with high sensitivity. He found that DA was concentrated in specific parts of the brain, which led him to the conclusion that DA is a transmitter in its own right. Since this first discovery that dopamine is an independent transmitter in the brain and further studies and conclusion that DA plays an important role in Parkinson's disease by Arvid Carlsson (Carlsson, 1988), the central DA systems and their roles in physiological functions have intrigued neuroscientists for several decades.

1.1.1 Brief anatomy

It is known that 70-80% of the DA neurons in the brain are mainly located in three distinct areas in the midbrain: the VTA (A10), substantia nigra pars compacta (SNpc, A9) and the retrorubral field (A8). The rest of the DA neurons are primarily located in the
hypothalamus. DA neurons in areas of the midbrain are organized in two major systems: the mesostriatal and mesolimbocortical systems (Bjorklund and Dunnett, 2007). The mesostriatal system mostly originates in the SNpc and projects to the dorsal striatum, such as the putamen and caudate nucleus (Anden, et al., 1964). The mesolimbocortical system (Figure 1.1) originates in the VTA and mostly projects to the prefrontal cortex and ventral striatum such as the nucleus accumbens (NAc) (Anden, et al., 1965; Ungerstedt, 1971). The VTA is an area without clear anatomical boundaries and with neurons scattered diffusely. In general, the level of the caudal tip of the mammillary tract is considered as the rostral border and the medial edge of the medial terminal nucleus of the accessory optic tract as the lateral border. There are some subregions in the VTA, such as the parabrachial pigmental area, which have different neuronal compositions and densities (Nair-Roberts, et al., 2008; Yamaguchi, et al., 2007). With the use of neurochemical markers, for example, the vesicular glutamate transporter 2 for glutamatergic neurons, tyrosine hydroxylase (TH) for DAergic neurons, and glutamic acid decarboxylase (GAD) for GABAergic neurons, three types of neurons have been found in the VTA: 60% are DA neurons; 35% are GABAergic neurons; only 2-3% are glutamatergic neurons highly enriched in medial parts of rostral VTA (Kosaka, et al., 1987; Nair-Roberts, et al., 2008; Yamaguchi, et al., 2007).
1.1.2 Connectivity of the VTA

A variety of transmitter terminals exist in the VTA including GABAergic, glutamatergic, cholinergic, serotonergic and noradrenergic. Other amino acid transmitters such as glycine and neuropeptides such as neurotensin, substance P, opioids and orexin also have effects on DA cells in the VTA. This section only covers the important excitatory and inhibitory transmitters: glutamatergic, GABAergic and cholinergic inputs (Figure 1.2). The glutamatergic afferents arise mainly from the prefrontal cortex, the laterodorsal (LDTg) and pedunculopontine (PPTg) tegmental nuclei (Carr and Sesack, 1999; Georges and Aston-Jones, 2001; Semba and Fibiger, 1992). In the meantime, there is also a possible local glutamatergic input since a small portion of cells utilizing glutamate as a neurotransmitter have been reported to be present in the VTA (Nair-Roberts, et al., 2008; Yamaguchi, et al., 2007). Moreover, it has been suggested that glutamate can also be co-localized in VTA DA neurons and DA neurons can use glutamate as a cotransmitter (Chuhma, et al., 2004; Mendez, et al., 2008; Descarries, et al., 2008; Lavin, et al., 2005). The GABAergic inputs to the VTA DA neurons mostly originate in the ventral striatum and pallidum (Groenewegen, et al., 1993; Smith and Bolam, 1990; Somogyi, et al., 1981) and also from the GABAergic interneuron within the VTA (Bayer and Pickel, 1991; Kosaka, et al., 1987). The cholinergic inputs arise in the LDTg and PPTg (Beninato and Spencer, 1987; Bolam, et al., 1991; Clarke, et al., 1987; Cornwall, et al., 1990; Satoh and Fibiger, 1986). These pontine nuclei thus provide both glutamatergic and cholinergic innervations to the DA neurons (Lavoie and Parent,
1994a;b) and send an extensive projection to the midbrain, where the terminals closely surround the soma and proximal dendrites of the DA neurons (Lavoie and Parent, 1994c).

The DA neurons of the VTA project to limbic and cortical areas along mesolimbic and mesocortical pathways (Figure 1.1). The limbic terminal areas include the ventral striatum, the ventro-medial part of the head of the caudate-putamen, amygdala and hippocampus (Bjorklund and Dunnett, 2007). The cortical DA innervation is largely confined to areas of the frontal, cingulate and entorhinal cortex (Bjorklund and Dunnett, 2007). Based on connectivity and morphological features, the VTA DA neurons can be separated into a dorsal and a ventral tier. The dorsal tier includes cells located in the dorsal aspect of the VTA innervating ventral striatal, limbic and cortical areas as well as the matrix compartment of the dorsal striatum (Bjorklund and Dunnett, 2007; Gerfen, et al., 1987; Lynd-Balta and Haber, 1994). These cells are round or fusiform in shape, are characteristically calbindin-positive, and express relatively low levels of the dopamine transporter (DAT). The ventral tier comprises a sheet of more densely packed, angular cells located in the ventral parts of VTA (Gerfen, et al., 1987; Prensa and Parent, 2001). These cells are calbindin-negative, express higher levels of DAT and are mostly immunopositive for the ion channel protein GIRK2 (G protein-activated inwardly rectifying K⁺ channels). The ventral tier neurons project to the striatum where they innervate the patch compartment.
1.1.3 Function of the VTA

The VTA DA system plays an important role in the regulation of cognitive functions (Le Moal and Simon, 1991), memory (Lisman and Grace, 2005), emotion (Laviolette, 2007; Pezze and Feldon, 2004), and reward-related behaviors (Koob, et al., 1998; Robbins and Everitt, 1999) through their networks with the limbic or cortical areas (Figure 1.1, 1.2, 1.3). Specifically, distorted DA output from the VTA has been implicated in diseases such as schizophrenia (Carlsson, 1988; Laviolette, 2007) and drug dependence (Di Chiara, 1995; Di Chiara and Imperato, 1988).

The prefrontal projections of the DA neurons from the VTA are considered to modulate neuronal activities related to cognitive functions such as working memory, planning and execution of behavior, and maintenance of focused attention (Le Moal and Simon, 1991). Local injection of DA D₁ receptor antagonists, such as SCH 23390, into prefrontal cortex impairs the adequate performance of working memory tasks (Sawaguchi and Goldman-Rakic, 1994), while it also has been shown that activation of DA neurons in the VTA by stimulating the mu-opioid receptor with enkaphalin produces a dose-dependent impairment of working memory tasks, which is restored by blocking the DA D₁ receptor with the antagonist SCH 23390 in prefrontal cortex (Romanides, et al., 1999). Therefore, it has been proposed that working memory function is optimized when DA D₁ occupancy is within a critical range of an inverted U-shaped function (Arnsten and Li, 2005; Floresco and Phillips, 2001; Zahrt, et al., 1997). Increased DA efflux during
working memory tasks has been observed in the primate prefrontal cortex as well (Watanabe, et al., 1997). In addition, reward-related stimuli also increase DA activities in this region (Taber and Fibiger, 1997).

The VTA DA neurons are profoundly implicated in the abuse of many drugs, e.g. cocaine and nicotine, which are known to directly or indirectly cause an increased transmission of DA in terminal areas such as NAc and prefrontal area (Di Chiara, 1995;Di Chiara and Imperato, 1988), inducing euphoria and reinforcement of drug seeking behavior. For example, in animals that are trained to self-administer cocaine, infusion of a DA receptor antagonist into the terminal field NAc dose-dependently decreases the frequency and total amount of the drug self-administration (Bachtell, et al., 2005). Selective destruction of the mesolimbic DA system using the neural toxin 6-hydroxydopamine inhibits self-administration of drugs of abuse in rats (Corrigall, et al., 1992;Roberts, et al., 1980). Rats can also learn to electrically self-stimulate the DA neurons of VTA (Kilpatrick, et al., 2000). Psychostimulants primarily enhance DA transmission through blocking DA reuptake or facilitating reverse transport in the terminal (Bradberry and Roth, 1989), but they also involve the cell bodies by synaptic receptor activation, D₂ autoreceptor-mediated inhibition or other compensatory changes associated with chronic use (Bunney and Aghajanian, 1977;Paladini, et al., 2001;Paladini and Williams, 2004). For instance, in several in vitro studies nicotine has been shown to excite DA neurons (Calabresi, et al., 1989;Picciotto, et al., 1998;Pidoplichko, et al., 1997) whereas opiates have been shown to inhibit the GABAergic inputs to DA neurons.
(Johnson and North, 1992a), leading to a disinhibition of the DA neurons, and therefore both nicotine and opiates excite the DA neurons in the VTA to enhance the DA transmission in terminal fields although by different mechanisms.

Schizophrenia is caused by multiple factors involving multiple transmitter systems, however, it is strongly believed that the central DA system plays a significant role in this disease (Carlsson, 1988; Laviolette, 2007). Drugs which directly or indirectly facilitate DA neurotransmission in the brain, e.g. amphetamine or L-DOPA, can precipitate or aggravate psychosis. All effective neuroleptic drugs used for the treatment of schizophrenia share anti-DA activity and the clinical potency of an antipsychotic drug to suppress positive symptoms of schizophrenia, such as delusions and hallucinations, and the ability of the drug to block D₂ receptors are strongly correlated (Seeman, et al., 1976). However, the atypical antipsychotic drug clozapine, with lower occupancy of D₂ receptors and higher affinity to block D₁ and D₄ receptors, produces a better relief of the negative symptoms of schizophrenia, such as blunted affect and emotion, alogia and avolition (Seeman, 1990). Therefore, schizophrenia has been thought to be associated with a differential dysfunction of the DA systems with hyperactivity in some areas like the NAc inducing the positive symptoms, and hypofunction in others like prefrontal area resulting in the negative symptoms (Goto, et al., 2007). However, a new version of the DA hypothesis of schizophrenia was proposed by Howes and Kapur recently (Howes and Kapur, 2009), who thought that the hypothesis of subcortical hyperdopaminergia with prefrontal hypodopaminergia focused too narrowly on DA itself, conflated psychosis and
schizophrenia, and predated advances in the genetics, molecular biology, and imaging research in schizophrenia. The new hypothesis proposed by Howes and Kapur has 4 distinctive components: "multiple risk factors, such as genes, stress and trauma, drug use, or pregnancy and obstetric complications, interact to result in DA dysregulation – the final common pathway to increase presynaptic striatal DA function to induce the psychosis in schizophrenia; the locus of DA dysregulation moves from being primarily at the D₂ receptor level to being at the presynaptic DA control level; DA dysregulation is linked to 'psychosis' rather than schizophrenia; and the DA dysregulation is hypothesized to alter the appraisal of stimuli, perhaps through a process of aberrant salience." The new hypothesis has one major implication for treatment approaches. Current treatment are acting downstream of the critical neurotransmitter abnormality. Future drug development and research into etiopathogenesis should focus on identifying and manipulating the upstream factors that converge on the DA funnel point.

1.2 The modulation of firing activities and its functional importance

Action potential firing is the sole neuronal output, therefore, the firing behavior of neurons is important for their functions.
1.2.1 Firing activities and DA transmission

The functional importance of the DA system in the VTA is closely related to changes in DA transmission. DA release is a spike-dependent process like that of other transmitters: a 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 efficiently it is reuptaken into the terminals. 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 modes and by responding to terminal as well as somatic autoreceptors (Bunney, et al., 1973; Cubeddu and Hoffmann, 1982).

Firing patterns, as well as firing rates, play an important role in DA release. DA neurons exhibit two common firing patterns: tonic spiking and bursting (Figure 1.4) (Grace and Bunney, 1984a;b). Tonic spiking refers to firing at a regular or irregular frequency that is generally lower than that of burst firing. Burst firing refers to a cluster of spikes appearing on a slow undulation of the membrane potential. The frequency of firing within a burst is irregular and a burst is usually followed by a pronounced post-burst hyperpolarization. Based on the measurement of extracellular DA concentrations in vivo in VTA terminal sites, burst firing is shown to release more DA from the terminal than
the same overall frequency but with a constant inter-stimulus interval (Goron and Buda, 1985; Manley, et al., 1992). This is probably due to Ca\(^{2+}\) accumulation in the pre-synaptic terminal (Grace, 2000; Suaud-Chagny, et al., 1992), or to saturation of the DA reuptake mechanisms during burst firing (Chergui, et al., 1994). The accumulation of DA in the synaptic cleft will have two significant effects: more pronounced postsynaptic effects and stronger autoinhibition of DA cell bodies in the VTA by activation of the D\(_2\) autoreceptors because of the higher dendritic DA release due to an increase firing (Grace, 2000). 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 transmission.

In addition, the importance of firing activities is also reflected in behavior. Freeman and Bunney have shown that burst firing in behaving rats is associated with the presentation of a novel visual or auditory stimulus (Freeman and Bunney, 1987). Studies in the monkey by Schultz et al (Schultz, 1997; 1998; Tobler, et al., 2005) have analyzed the role of midbrain DA neurons in reward-related tasks and demonstrated that the DA neurons are, indeed, activated by a primary reward and especially if it is unexpected. If the reward is coupled to a conditioning signal (visual or auditory), the regular firing of the DA neurons will switch to burst firing or increase their firing rates from the moment of appearance of the actual reward to the instant when the conditioned signal is delivered. Moreover, if the conditioning stimulus is not followed by the expected reward the DA neurons decrease their firing. The DA neurons can thus detect deviations from the
expected reward so that a positive signal is delivered to the brain if the reward is better than expected, no change if the reward is just as expected and negative if less than expected.

In summary, firing activities in the DA neurons signal the occurrence of novel and rewarding stimuli, which implies that highly processed information is being passed to the DA neurons where the changes of firing activities have effects on projecting areas related to differing functions.

1.2.2 Firing regulation

Although it is widely recognized that firing activities of DA neurons in VTA are important for their function, how firing activities of DA neurons are regulated still remains to be fully understood. Up to the present, it has been thought that the regulation of firing activities would result from the interplay of excitatory and inhibitory mechanisms at the synaptic level, as well as from discrete intrinsic membrane properties at the cellular level. Two aspects of firing activities, firing rate and firing pattern, will be discussed below.
1.2.2.1 Extrinsic factors--excitatory and inhibitory synaptic inputs

DA neurons exhibit a highly regular pacemaker-like firing pattern in vitro, while bursting is more frequent in vivo (Grenhoff, et al., 1988), suggesting that the firing activities of these cells are strongly under the control of afferent inputs. DA neurons in the VTA receive intense inputs from a variety of origins. This section will only cover the important excitatory and inhibitory transmitters because they play a major role in modulating DA neuronal activity.

1.2.2.1.1 Glutamatergic input

As mentioned in section 1.1.2, DA neurons in the midbrain majorly receive direct or indirect glutamatergic inputs from different structures such as the prefrontal cortex, PPTg and LDTg (Carr and Sesack, 1999; Georges and Aston-Jones, 2001; Semba and Fibiger, 1992). Glutamatergic input from the prefrontal cortex, one of the major excitatory inputs, was shown to synapse onto DA neurons that loop-back to the prefrontal cortex, but not to those projecting to the NAc (Carr and Sesack, 1999; 2000a;b). It is the input from other structures such as the LDTg that is likely to synapse onto DA neurons projecting to the NAc (Di Loreto, et al., 1992; Forster and Blaha, 2000; Omelchenko and Sesack, 2005; Sesack, et al., 2003).
Electrical stimulation of the medial prefrontal cortex enhances DA neuronal activity and produces burst firing (Gariano and Groves, 1988; Tong, et al., 1996b). Inactivation of this projection by cooling of the cortex or administration of glutamate receptor antagonists (Tong, et al., 1996a) reduces the firing rates and bursting, replacing it with pacemaker-like activity. Activation of the PPTg also enhances VTA firing activity and induces burst firing of DA cells (Lokwan, et al., 1999). Pressure-injected glutamate in the VTA stimulates the firing rate of DA cells and enhances extracellular DA concentration in projecting areas such as the NAc (Suaud-Chagny, et al., 1992). The effects of glutamate are due to three general groups of glutamate receptors that are expressed on DA neurons: AMPA, NMDA ionotropic and metabotropic receptors.

The major effects of glutamatergic input to DA neurons are mediated by AMPA and NMDA ionotropic receptors (Mathon, et al., 2003; Overton and Clark, 1997; Wang and French, 1993a;b). In vivo studies have shown that application of both AMPA and NMDA receptor agonists via microiontophoresis increases the firing rate of spontaneously active DA neurons. Both AMPA and NMDA receptor activation increases bursting activity in the SNpc, but the bursting activity in the VTA appears to be independent of AMPA receptors (Chergui, et al., 1993; Christoffersen and Meltzer, 1995; Overton and Clark, 1991; Zhang, et al., 1994). Studies in brain slices also show the excitatory effect of these receptors on DA neurons. Both application of AMPA and NMDA evokes an inward current and increases neuronal firing in a dose-dependent manner (Mercuri, et al., 1992b; Wang and French, 1993a;b; Wu, et al., 1994; Wu and Johnson, 1996). However, the
effects of a low dose of glutamate (≤ 30 μM) on firing rate and membrane potential are mostly due to its actions at the NMDA receptors as they can be blocked by the noncompetitive NMDA blocker, phencyclidine, and the selective competitive NMDA receptor antagonist CGS 19755 (Wang and French, 1993b). NMDA receptor activation has been shown to induce burst firing under some conditions, particularly in the presence of hyperpolarizing currents or when calcium-activated potassium currents are blocked by apamin (Johnson and Seutin, 1997; Johnson and Wu, 2004; Seutin, et al., 1993). The mechanism underlying NMDA-induced burst firing can involve different ion species. For example, it is reported that Na⁺ influx via NMDA-gated channels and the subsequent extrusion of Na⁺ by the ouabain-sensitive electrogenic Na⁺ pump comprises the full cycle of bursting (Johnson, et al., 1992). In summary, these findings underline the important excitatory role of AMPA and NMDA glutamate receptor activation in DA neuron regulation.

The metabotropic glutamate receptors (mGluRs) play a bidirectional role, either excitatory or inhibitory, on DA neurons depending on how the receptors are activated. Activation of the group 1 mGluRs located on DA neurons via synaptically-released glutamate, produces inhibitory postsynaptic currents and mediates a slow inhibition of DA neurons (Fiorillo and Williams, 1998). These inhibitory currents are a consequence of mobilization of intracellular calcium stores, which in turn activates inhibitory calcium-dependent potassium currents. However, with prolonged activation, the inhibitory response produced by mGluR activation desensitizes. Therefore, continuous
activation of these receptors induces a slowly developing sodium-dependent excitation, which can lead to an increase in impulse activity of DA cells (Fiorillo and Williams, 1998; Meltzer, et al., 1997; Mercuri, et al., 1993; Zheng and Johnson, 2002). It has also been suggested that activation of mGluRs modifies DA neuron activity indirectly by acting on presynaptic glutamatergic terminals so as to modify glutamate release. As for the post-synaptic effects, they can also be either stimulatory or inhibitory (according to the level of activation of the phospholipase C pathway) and produce, respectively, enhanced or reduced NMDA-mediated excitatory currents in post-synaptic cells (Bonci, et al., 1997; Gereau and Conn, 1995; Herrero, et al., 1992). On a similar line, at high doses, bath application of glutamate to isolated DA neurons has been shown to enhance spontaneous firing, but also to temporarily inhibit firing through two distinct calcium-dependent mechanisms: via activation of NMDA and AMPA receptors, or mGluRs (Kim, et al., 2004). Recently, it has also been shown that the action of mGluRs is linked to activation of transient receptor potential (TRP) channels because extracellular application of the non-selective TRP channel blocker SKF96365, flufenamic acid and ruthenium red causes reversible inhibition of mGluR1-activated EPSCs (Bengtson, et al., 2004). The group I mGluRs have been shown to play a role in the regulation of firing mode of midbrain DA neurons, especially in the SNpc (Meltzer, et al., 1997). The group I mGluR agonist increases the frequency of burst in vivo in the SNpc. Selective group I mGluR agonist (s)-3, 5-dihydroxyphenylglycine in the presence of tubocurarine or apamine, both of which are blockers of SK channels, can transform single spike firing to bursting in SNpc DA cells (Prisco, et al., 2002). Furthermore, the mGluR antagonist
a-methyl-4-carboxyphenyl glycine [(s)-MCPG] blocks the post-burst hyperpolarization in bursting induced by repetitive extracellular stimulation or iontophoretic application of aspartate in midbrain DA slices (Morikawa, et al., 2003).

Together, these findings indicate that bursting may be ascribed, at least in part, to a complex combination of glutamate receptors. Activation of these receptors can exert both excitatory and inhibitory roles, either directly, or indirectly, by modifying glutamate release or the activity of other channels.

1.2.2.1.2 GABAergic inputs

GABA, the major inhibitory neurotransmitter, is responsible for most synaptically-induced direct inhibition of DA neuron activity. As described in section 1.1.2, the VTA receives a major GABAergic input originating in the NAc (Figure 1.2, 1.3) (Groenewegen, et al., 1993;Smith and Bolam, 1990;Somogyi, et al., 1981). Another important GABAergic input arises from local GABA interneurons in the midbrain (Bayer and Pickel, 1991;Kosaka, et al., 1987). Picrotoxin injected into the VTA to block GABA_A 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 influences on firing activities of DA cells in the VTA.
GABA afferents from the striatal complex play an important role in certain conditions, such as after the administration of psychostimulant drugs (Einhorn, et al., 1988; Pitts, et al., 1993). Hemitranssection of this pathway fails to modify basal impulse activity in VTA DA neurons (Einhorn, et al., 1988; Pitts, et al., 1993; Pucak and Grace, 1994). After the injection of kainic acid into the dorsal striatum, there is a transient decrease in the percentage of activity of DA cells due to depolarization inactivation (Braszko, et al., 1981). The injection of kynurenic acid, the glutamate receptor antagonist, to the NAc produce a transient decrease in firing rate and burst firing of VTA DA neurons (Floresco, et al., 2001).

The presence of GABA neurons within the VTA (Figure 1.3) increases the complexity of the GABA effects and explains many biphasic effects produced by stimulation of brain structures projecting to the midbrain, or by systemic pharmacological treatments. Thus, stimulation of afferents onto DA neurons can have an initial response that is immediately followed by an opposite effect (Grace and Bunney, 1985; Overton, et al., 1996; Tong, et al., 1996b). This is because afferents can form synapses onto both DA and GABA neurons. Synapses onto DA neurons may produce direct neuronal inhibition, while synapses onto GABA neurons may produce indirect excitation of DA neurons by inhibiting 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 tonically and can be prevented by tetrodotoxin (TTX) that blocks
spontaneous action potential firing of GABA interneurons in the slice (Johnson and North, 1992a). In addition, GABAergic synapses also exist on presynaptic terminals, indirectly affecting the DA neurons’ excitability. For example, activation of GABA$_B$ receptors on presynaptic glutamatergic nerve terminals suppresses excitatory transmission onto GABA interneurons, relieving the tonic inhibition exerted by those neurons on DA cells (Wu, et al., 1999). Due to this anatomical arrangement, systemic administration of GABAergic drugs can have a paradoxical excitatory effect on DA neurons (Grace and Bunney, 1979; Waszczak and Walters, 1980).

The effects of GABA are due to two general groups of receptors that are expressed on DA neurons: GABA$_A$ ionotropic and GABA$_B$ metabotropic receptors. Local microiontophoretic application of the GABA$_A$ receptor agonist muscimol induces a clear-cut inhibition of DA neurons on a fast time scale (Erhardt and Engberg, 2000). On the other hand, the selective GABA$_A$ receptor antagonists bicuculline, gabazine and picrotoxin enhance both firing and bursting activity. Studies show that the GABA$_A$ receptor agonists modulate DA neuron activity by opening ionotropic receptors which directly allow chloride influx, thereby hyperpolarizing the cell membrane (Johnson and North, 1992b; Sugita, et al., 1992).

In contrast to GABA$_A$ receptors, GABA$_B$ receptors mediate inhibition of midbrain DA neurons on a slower time course. Local administration of the GABA$_B$ receptor agonist baclofen reduces firing and burst firing and converts NMDA-induced burst firing
to a single-spike firing pattern in DA neurons (Erhardt, et al., 1998; Erhardt, et al., 2002). Conversely, application of GABA<sub>B</sub> receptor antagonists increases DA firing and bursting, and prevents the effects produced by GABA<sub>B</sub> receptor activation (Chen, et al., 2005; Engberg and Nissbrandt, 1993; Erhardt, et al., 1998; Paladini and Tepper, 1999). Experiments using brain slices show that GABA<sub>B</sub> receptor agonists modulate the activity of DA neurons by activating G<sub>B</sub> proteins, the βγ dimer dissociates from the heterotrimeric protein and binds to GIRK channels, which are differentially expressed by DA and non-DA neurons within the VTA (Cruz, et al., 2004). The consequent opening of these channels allows potassium outflow, which hyperpolarizes the cell membrane thereby inhibiting neuronal activity (Cruz, et al., 2004; Watts, et al., 1996).

1.2.2.1.3 Cholinergic input

The VTA has a high density of cholinergic terminals, indicating that it receives a substantial cholinergic input (Henderson and Sherriff, 1991). As detailed in section 1.1.2, the VTA receives bilateral innervation from PPTg and LDTg (Figure 1.2.1.3), regions rich in cholinergic neurons (Beninato and Spencer, 1987; Bolam, et al., 1991; Clarke, et al., 1987; Cornwall, et al., 1990; Satoh and Fibiger, 1986).

Cholinergic input has been shown to play a vital role in regulating the firing activities of VTA DA neurons. Electrical stimulation of the PPTg produces an increase in the number of neurons firing in a bursting pattern, and this effect is produced only in
neurons that were already firing; PPTg inputs do not appear to be able to recruit inactive neurons or increase the firing rate of active neurons (Floresco, et al., 2003). In addition, electrical stimulation of LDTg results in a prolonged increase in DA release in NAc (Forster, et al., 2002) and chemical stimulation of the LDTg by infusion of a glutamate agonist or a muscarinic antagonist increases the number of active neurons in the VTA which is blocked by local application of the nicotinic receptor antagonist, mecamylamine. Interestingly, the inactivation of the LDTg by the administration of GABA agonists produces a pronounced decrease in the burst firing of DA neurons in the VTA that is not reversed by PPTg stimulation (Lodge and Grace, 2006). During LDTg inactivation, VTA neurons fire tonically in a very regular pattern, resembling the pacemaker firing pattern observed in vitro (Grace and Onn, 1989). The effects of cholinergic input are due to two general groups of receptors: nicotinic and muscarinic acetylcholine receptors (AChR). It has been shown that neurons in the VTA express somatic and dendritic nicotinic (Clarke and Pert, 1985;Clarke, et al., 1985;Sorenson, et al., 1998) and muscarinic (Nastuk and Graybiel, 1991) AChRs.

Nicotinic receptors are crucial in the initial fast response to acetylcholine (ACh) and mediate addiction (Balfour, et al., 2000). Acute systemic administration of nicotine increases intracranial self-stimulation rates (Druhan, et al., 1989) and locomotor activity in rats (Picciotto, 1998), while 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). On the same line, nicotine,
administered systemically in vivo, increases the firing rates and burst firing activity of VTA DA neurons associated with an increased release of DA in the NAc that is blocked by local application of the nicotinic receptor antagonist mecamylamine into the VTA, but not into the NAc (Grenhoff, et al., 1986; Nisell, et al., 1994). Infusion of nicotine into the VTA produces in DA neurons either a decrease in the input resistance and a transient depolarization of the membrane that results in the generation of action potentials, or an increase in the firing rate if the neuron was already firing and is followed by a rapid desensitization (Calabresi, et al., 1989; Pidoplichko, et al., 1997; Sorenson, et al., 1998). Furthermore, nicotinic receptors are shown to mediate presynaptic effects by enhancing glutamate release in experiments which exhibit that nicotine increases evoked and spontaneous excitatory synaptic currents in the VTA DA neurons (Dani, et al., 2001; Mansvelder and McGee, 2000).

The muscarinic receptors are responsible for the prolonged response to ACh in DA cells (Lacey, et al., 1990) and are implicated in reward processing, e.g. eating and drinking. For example, infusion of the non-selective muscarinic receptor antagonist atropine into the VTA can stop self-stimulation and reduce food intake in rats (Rada, et al., 2000). In vitro, activation of postsynaptic M1-like muscarinic receptors evokes a slow depolarization with an increase in firing rate and burst firing (Lacey, et al., 1990; Zhang, et al., 2005). Besides direct excitatory postsynaptic effects through M1 and M5 subtypes, muscarinic agonists can potently increase firing rates by reducing GABAergic transmission through presynaptic M3 receptors (Grillner, et al., 1999). In addition,
muscarine is able to decrease after-hyperpolarizations which would facilitate firing at higher frequencies and possibly bursting (Scroggs, et al., 2001). Muscarinic receptors are shown to be coupled to $G_\text{q}$ or $G_{10}$, activating phospholipase C leading to the hydrolysis of phosphoinositol or inhibiting adenylyl cyclase so as to have effects on different ion channels (Caulfield, 1993; Hulme, et al., 1990).

Several mechanisms have been proposed to explain how the activation by ACh might facilitate burst firing, including an increase in the level of intracellular calcium mediated by influx through NMDA receptors (Kitai, et al., 1999), activation of L-type calcium channels (Zhang, et al., 2005) and the mobilization of calcium from internal stores. All of these support the idea that the switch from single spike firing to burst firing following the activation of AChRs is a calcium-dependent mechanism.

In summary, cholinergic neurons of the brainstem regulate the firing rate of midbrain DA neurons and initiate their burst firing.

### 1.2.2.2 Intrinsic membrane properties

Numerous intrinsic membrane properties regulate the firing activity of DA neurons. This part will consider some of the most important ones, such as the DA autoreceptors, the afterhyperpolarization potential (AHP) and a hyperpolarization-activated current ($I_h$).
Voltage-gated Ca\textsuperscript{2+} channels, especially L-type calcium channels, will be described in 1.3.

1.2.2.2.1 DA autoreceptors

DA autoreceptors play an important role in providing an efficient negative feedback mechanism. These receptors are in the D\textsubscript{2}/D\textsubscript{3} DA receptor family and are located in axon terminals and the somatodendritic region of VTA DA neurons (Clark and Chiodo, 1988; Mercuri, et al., 1992a). They are activated by synaptically and somatodendritically-released DA (Beart, et al., 1979; Cheramy, et al., 1981; Kalivas and Duffy, 1991) to reduce DA neuron activity by activating GIRK channels presumably via G protein \(\beta\gamma\) subunits that are liberated by receptor activation of \(G_{i/o}\) proteins. This produces a hyperpolarization in the cell body to inhibit firing (Figure 1.5) thus causing the terminals to reduce DA release (Davila, et al., 2003; Innis and Aghajanian, 1987; Lacey, et al., 1987; Mercuri, et al., 1992a; Williams and Lacey, 1988). Recent evidence shows that DA release depends on neuronal depolarization and this exocytotic release can directly inhibit neuron excitability (Beckstead, et al., 2004). Furthermore, the sensitivity of autoreceptors affects how the cell fires: fast-firing DA neurons have sub-sensitive impulse-regulating autoreceptors, conversely, slow-firing neurons exhibit greater sensitivity of these receptors (Marinelli and White, 2000; White and Wang, 1984) as revealed by the dose-dependent neuronal inhibition following local administration of D\textsubscript{2}/D\textsubscript{3} receptor agonists into the VTA (Akaoka, et al., 1992; Gariano, et al., 1989; Pucak...
and Grace, 1994). This effect is independent of differences in neuronal inputs because slow-firing cells are more sensitive to autoreceptor-mediated inhibition even when they are driven to a faster rate by local application of glutamate (White and Wang, 1984). Overall, these findings suggest that the functional state of somatodendritic DA autoreceptors mediates the activity of DA neurons.

1.2.2.2 Calcium-dependent potassium channels

Potassium channels come in many forms; both voltage-gated and calcium-activated small conductance potassium (SK) channels are implicated in midbrain DA neuron firing. Intracellular injection of the potassium channel blocker TEA increases the firing rate of DA neurons and produces burst-like activity (Grace and Bunney, 1984a). In addition, it has been shown that voltage-gated A-type potassium channels play a key role in modulating the pacemaker activity of DA neurons by combining real-time single-cell RT-PCR with slice patch clamp electrophysiology (Liss, et al., 2001). This pacemaker activity is related to A-type potassium channel density and the number of subunits that make up the potassium channel, indicating that both the amount and the type of gene expression are important in regulating DA neuron firing.

SK channels are found in DA neurons, where they contribute to the membrane AHP that follows each action potential by inducing an outward current after an individual action potential and thereby suppress repetitive firing at high frequencies. They have also
been proposed as the ionic basis for post-burst hyperpolarization in which a series of individual AHPs after a train of action potentials are summated to terminate the burst. These channels have slow dynamics to allow such a summation and are reported to be largely responsible for modulating burst firing activity (Overton and Clark, 1997). For example, regularly spiking DA cells in the VTA that display small AHPs respond to orexin A with bursting (Korotkova, et al., 2003).

Because SK channels are selectively permeable to potassium, and open when the intracellular calcium concentration is elevated, they are very sensitive to fluctuations of internal calcium concentrations. An increase in intracellular calcium activates SK channels, whereas a reduction in intracellular calcium prevents their activation. Thus, reducing the intracellular concentration of calcium, by intracellular injection of the calcium chelator EGTA, attenuates the AHP that follows a spike train (Grace and Bunney, 1984b) and increases DA neuronal firing (Grace and Bunney, 1984a). However, application of intracellular EGTA does not produce bursting because this manipulation not only inhibits SK channel activation, but also lowers intracellular calcium concentrations and prevents calcium influx from initiating burst firing. Physiologically, SK channels are activated by rises in intracellular calcium that can be produced by changes in calcium release from intracellular stores (Seutin, et al., 2000) such as those occurring after activation of metabotropic glutamate receptors (Morikawa, et al., 2000). Recently, it was shown that SK channels can also be activated by the calcium influx produced by activation of T-type calcium channels (Wolfart and Roeper, 2002). Because
of this, activation of T-type calcium channels can inhibit, rather than increase, DA neuron activity like bursting.

SK family channels that mediate AHP are described as SK1, SK2 and SK3 (Kohler, et al., 1996). 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). SK2 and SK3 channels are very sensitive to the calcium-activated SK channel blocker apamin. Application of very low doses of apamin to DA neurons in vitro has been shown to interrupt pacemaker activity of DA neurons in the SNpc (Wolfart, et al., 2001), suggesting that these channels could play a role in modulating DA neuron pacemaker activity. Most importantly, application of apamin to midbrain DA neurons facilitates bursting activity produced by application of excitatory amino acids (Johnson and Seutin, 1997; Seutin, et al., 1993) and can be sufficient to produce burst firing in the tissue slice preparation (Ping and Shepard, 1996; Shepard and Bunney, 1991). Burst events produced by apamin consist of long bursts, two to tens of spikes per burst, which resemble natural bursting. The transition between the slow irregular firing and bursts is also controlled by fluctuations in membrane potential, which are modulated by SK channels (Amini, et al., 1999; Wilson and Callaway, 2000). Among SK channels, the SK3 subtype is expressed ubiquitously in midbrain DA neurons. In addition, its expression in these cells is either exclusive with respect to the other SK subtypes, or at least fourfold higher (Wolfart, et al., 2001), suggesting that it is the SK3 subtype that plays a major role in modulating burst firing activity in DA neurons.
1.2.2.3 $I_h$ channels

$I_h$ channels are largely expressed by DA neurons (Neuhoff, et al., 2002). As their name implies, these channels are activated when the neuron is hyperpolarized beyond -50 to -70 mV; their opening allows the inward flow of Na$^+$ and K$^+$, which produces membrane depolarization (Frere, et al., 2004). After hyperpolarizing steps, this is visible as a gradual increase in inward current when measured in voltage-clamp mode (Figure 1.5B), and as a typical “sag” when recorded in current-clamp (Figure 1.5C); in fact, the presence of this current or sag is often used as a “marker” for DA neurons recorded in the tissue slice (Grace and Onn, 1989; Johnson and North, 1992b; Mercuri, et al., 1995; Neuhoff, et al., 2002). $I_h$ has been suggested to regulate low frequency pacemaker activity, however only a subset of SNpc neurons show altered pacemaker frequency in the presence of the $I_h$ channel inhibitor ZD-7288; therefore, it is debatable whether the expression of this current regulates the overall firing pattern of most midbrain DA neurons (Mercuri, et al., 1995; Neuhoff, et al., 2002; Seutin, et al., 2001). $I_h$ does however, regulate the amplitude and duration of the AHP that follows action potentials in all subtypes of midbrain DA neurons with a possible function of the integration of inhibitory input to these cells (Neuhoff, et al., 2002).
1.3 Role of L-type calcium channels in DA neurons

L-type calcium channels are one class of the voltage-gated calcium channels which consist of L-, N-, P/Q-, T- and R-type calcium channels, all of which are present in DA neurons (Cardozo and Bean, 1995; Takada, et al., 2001). Influx of calcium through calcium channels depolarizes the neuronal membrane, which can lead to cell firing. In midbrain DA neurons, intracellular injection of calcium in high concentrations in vivo increases burst firing whereas the calcium chelator EGTA reduces the depolarization that initiates bursting and thus also greatly reduces bursting (Grace and Bunney, 1984a). Although it is known that calcium regulates neuronal firing, it is unclear what channel calcium uses to enter the cell to mediate this action. In vitro studies show that calcium channel blockers for N- and P/Q-type channels reduce the membrane depolarization that initiates bursting but their contribution to the total depolarization is far less than the L-type calcium channels and may not play a large role in DA neuron firing (Durante, et al., 2004). In contrast, activation of T-type calcium channels decreases neuronal firing by activating SK channels (Wolfart and Roeper, 2002). In addition, R-type calcium channels have not been shown to affect neuronal activity in any experiments. However, L-type calcium channels have been shown to play an important role in the regulation of firing activities in DA neurons.

L-type calcium channels are responsible for approximately one third of the total Ca$^{2+}$ currents (Cardozo and Bean, 1995; Durante, et al., 2004; Takada, et al., 2001) and
contribute preferentially to whole-cell \( \text{Ca}^{2+} \) currents evoked by small depolarizations (Durante, et al., 2004; Xu and Lipscombe, 2001) in DA cells. *In vitro* studies show that L-type calcium channel agonists produce membrane depolarization and subsequently increase firing rates, while antagonists reduce membrane depolarization and decrease firing rates, in DA neurons (Cardozo and Bean, 1995; Chan, et al., 2007; Durante, et al., 2004; Mercuri, et al., 1994; Zhang, et al., 2005). In addition, it has recently been found that the L-type calcium channel antagonist, nifedipine, prevents burst firing and the underlying membrane potential oscillation induced by cholinergic activation in slice preparations (Zhang, et al., 2005).

The role of L-type calcium channels in regulating DA neurons' firing activities parallels their functional importance in addictive and motivational behaviors. For example, repeated amphetamine injections increase the expression of L-type calcium channel subunits in the VTA (Rajadhyaksha, et al., 2004) and repeated stimulation of L-type calcium channels in the VTA mimics the initiation of behavioral sensitization to cocaine (Licata, et al., 2000), while direct injection of L-type calcium channel antagonists into the VTA attenuates the development of psychostimulant-induced behavioral sensitization (Licata, et al., 2004).

L-type calcium channels have unique properties, compared to other voltage-gated \( \text{Ca}^{2+} \) channels: they are activated by strong depolarizations (high-voltage-activated [HVA]), display a high sensitivity to dihydropyridine (DHP) agonists and antagonists,
have relatively slow activation kinetics, have calcium-dependent inactivation with little voltage-dependent inactivation (long-lasting), and have large single-channel conductance (Lipscombe, et al., 2004). There are four subtypes of L-type calcium channels: \( \text{Ca}_v1.1 \), \( \text{Ca}_v1.2 \) (formerly \( \alpha 1C \)), \( \text{Ca}_v1.3 \) (formerly \( \alpha 1D \)) and \( \text{Ca}_v1.4 \) (Lipscombe, et al., 2004). Only \( \text{Ca}_v1.2 \) and \( \text{Ca}_v1.3 \) L-type calcium channels are expressed in DA neurons, especially in the cell body and proximal dendrites (Hell, et al., 1993; Takada, et al., 2001). Functionally, \( \text{Ca}_v1.3 \) L-type calcium channels display atypical properties for L-type calcium channels: having low activation thresholds and showing less sensitivity to DHP antagonists (Durante, et al., 2004; Xu and Lipscombe, 2001). Pharmacologically, there is no effective way to isolate the contribution of \( \text{Ca}_v1.2 \) and \( \text{Ca}_v1.3 \) subtypes. \( \text{Ca}_v1.3 \) and \( \text{Ca}_v1.2 \) transgenic mice, however, provide a means to study the role of L-type calcium channel subtypes (Platzer, et al., 2000). Recently, a dominant role for \( \text{Ca}_v1.3 \), a subtype of the L-type calcium channel that operates at close to resting membrane potentials, was reported in sustain pacemaking activities of DA cells in adult mice (Chan, et al., 2007).

### 1.4 Rationale and hypotheses for the present thesis

The foregoing overview summarizes the current available information about the functional role and regulation of electrical activities of DA neurons in the VTA, with a special focus on the L-type calcium channels. Up to the present, L-type calcium channels have been shown to play an important role in regulating the firing activity in DA neurons in the midbrain (Cardozo and Bean, 1995; Chan, et al., 2007; Durante, et al., 2004; Mercuri,
et al., 1994; Zhang, et al., 2005), while the signaling pathways preceding and following the L-type calcium channels to mediate this function are still unclear. The work in this thesis is concerned with unraveling the details of the signaling pathway(s) underlying L-type calcium channel regulation of the firing activities of DA neurons.

As previously reported, L-type calcium channels mediate the bursting induced by the general cholinergic activator carbachol (Zhang, et al., 2005). In the first series of experiments (Chapter 2), I hypothesize that L-type calcium channels can directly induce bursting by way of activating calcium-related signaling pathways in DA neurons. In this set of experiments, I asked whether direct activation of L-type calcium channels induces bursting of DA neurons, and if it does, what cellular and synaptic machinery is involved such as activating autoreceptors on the cell body, enhancing neurotransmitter release from afferents, mobilizing internal calcium stores, and activating calcium-activated potassium channels or activating calcium-dependent kinases.

The second series of experiments was derived from the results of the first series that showed calcium influx through L-type calcium channels generates protein kinase M (PKM) to induce bursting of DA neurons. These experiments test the hypothesis that protein kinase C (PKC) might mediate the excitation of carbachol, NMDA or AMPA. In this set of experiments (Chapter 3), I asked whether PKC or L-type calcium channels mediates the firing rate increase induced by application of carbachol, NMDA or AMPA, and if it does, whether they are sequentially coupled in order to modulate firing activity.
In the third series of experiments (Chapter 4), I tested the hypothesis that different subtypes of L-type calcium channels mediate different aspects of the firing activity of DA neurons, which was also derived from the first series of experiments. I asked which subtypes of L-type calcium channels is involved in the spontaneous pacemaker firing and L-channel-induced bursting and whether different subtypes play different roles in the regulation of firing activity by using the Ca,1.2 transgenic mouse strain.
Figure 1.1 The mesolimbocortical system. This simplified diagram shows projections from the ventral tegmental area to the prefrontal cortex and nucleus accumbens (dense lines with arrows).
Figure 1.2 The connectivity of the ventral tegmental area (VTA). This simplified diagram shows the afferent inputs (dense lines with arrows) to the VTA. The red lines represent the major GABAergic inputs from the nucleus accumbens (NAc); the yellow lines represent the major glutamatergic inputs from the prefrontal cortex, the pedunculopontine tegmental nuclei (PPT) and the laterodorsal tegmental nuclei (LDT); and the yellow lines represent the major cholinergic inputs from the PPT and the LDT.
Figure 1.3 A synopsis of the neuronal connections and their interactions with each other in the ventral tegmental area (VTA). DA, DAergic neuron; G, GABAergic neuron; Glu, glutamatergic neuron; ACh, cholinergic neuron; PFC, prefrontal cortex; NAc, nucleus accumbens; PPT, pedunculopontine tegmental nuclei; LDT, laterodorsal tegmental nuclei.
Figure 1.4 Different firing patterns of dopaminergic neurons.

1. irregular firing  2. regular firing  3. burst firing.
Figure 1.5 Two electrophysiological markers that I used to identify DA neurons. A. Continuous current clamp recording from a representative cell showing the inhibitory effects. B. Voltage clamp recording showing a gradual increase in an inward current ($I_h$ current) in response to a voltage step to -110 mV for 1 sec from the holding potential (-45 mV). C. Current clamp recording showing a typical $I_h$ "sag" when current injections were adjusted to result in a peak hyperpolarization of about -110 mV.
Co-authorship Statement

I, Yudan Liu, hold a principle author status for all the manuscript chapters (Chapter 2-4) in my thesis. I also have a second-authored paper which is not included in this thesis. However, each manuscript is co-authored by my supervisor and co-workers, whose contributions have greatly facilitated the development of my hypotheses in the manuscripts, the practical aspects of my experiments and the manuscript writing as described below.

Chapter 2, titled “Calcium Influx through L-type Calcium Channels Generates Protein Kinase M to Induce Burst Firing of Dopaminergic Cells in the Rat Ventral Tegmental Area”, is co-authored by Drs. Xihua Chen and Jules Dore. As the principle author, I participated in the experimental design and accomplished all the experimental work. I accomplished the first draft writing. Dr. Chen contributed greatly to the design of the research in this paper, and the completion and improvement of the paper. Dr. Dore provided substantial technical training and support in the experiments using Western blotting technique, and the editing of the paper.

Chapter 3, titled “Cholinergic Excitation of Dopaminergic Cells Depends on Sequential Activation of Protein Kinase C and the L-type Calcium Channel in Ventral Tegmental Area Slices” is co-authored by Dr. Xihua Chen. As the principle author, I participated in the development of the experimental designs and performed all the
experimental work and finished the first draft of the paper. Dr. Xihua Chen contributed greatly to the design of the research and the completion and improvement of the paper.

Chapter 4, titled “Ca,1.3 L-type Calcium Channels Mediate Single Spike and Burst Firing of VTA DA Neurons in Mice” is co-authored by Dr. Xihua Chen, Dr. Jules Dore and Meghan Harding. As the principle author, I participated in all of the electrophysiological experiments and most of the PCR experiments. I accomplished the first draft writing. Dr. Xihua Chen contributed greatly to the design of the research and the completion and improvement of the manuscript. Dr. Jules Dore provided substantial technical training and support in genotyping DHP knock-in mice using the PCR technique. Meghan Harding co-worked with me on PCR experiments for breeding and genotyping the mice.
Chapter 2. Calcium Influx through L-type Calcium Channels Generates Protein Kinase M to Induce Burst Firing of Dopaminergic Cells in the Rat Ventral Tegmental Area


2.1 Introduction

DA projections from the VTA constitute the mesolimbocortical system that underlies drug abuse and schizophrenia, primarily as the result of increased DA transmission (Kelley, 2004; Koob, 2000; Seamans and Yang, 2004). The strength of DA transmission is regulated by the interplay between spike-dependent DA release, DA reuptake and autoreceptor-mediated negative feedback. Essentially, more intense spiking releases more DA at the terminal, which in turn activates the negative feedback mechanism to shut the system off. For enduring DA transmission at greater intensity, it would require a higher accumulation of DA at the synapse without triggering the negative feedback machinery. Burst firing is one such mode of discharge that has been shown to enhance DA transmission (Floresco, et al., 2003; Garris, et al., 1994; Gonon, 1988; Suaud-Chagny, et al., 1992) by saturating reuptake transporters and reducing autoreceptor inhibition (Chergui, et al., 1994).

DA cells in the VTA are capable of both pacemaker-like and burst firing (Hyland, et al., 2002), the latter being shown to signal novelty and salient stimuli in whole animal
studies (Tobler, et al., 2005). DA cells in slices predominantly display pacemaker-like firing, burst firing can be induced by surrogate synaptic stimulation with bath application of glutamatergic or cholinergic agonists (Johnson, et al., 1992; Zhang, et al., 2005) that mobilize Ca\textsuperscript{2+}. Ca\textsuperscript{2+} entry has been shown to be pivotal in regulating firing patterns of DA neurons. Intracellular administration of Ca\textsuperscript{2+} evokes burst firing while intracellular Ca\textsuperscript{2+} chelators block it (Grace and Bunney, 1984a). Our previous study shows that carbachol, a general cholinergic agonist, induces burst firing primarily by promoting Ca\textsuperscript{2+} influx through L-type calcium channels (Zhang, et al., 2005). These channels are responsible for approximately one third of total Ca\textsuperscript{2+} currents of DA neurons (Cardozo and Bean, 1995; Durante, et al., 2004; Takada, et al., 2001) and contribute preferentially to whole-cell Ca\textsuperscript{2+} currents evoked by small depolarizations (Durante, et al., 2004; Xu and Lipscombe, 2001). In line with this, L-type calcium channels have been shown to be involved in spontaneous and burst firing (Johnson and Wu, 2004; Mercuri, et al., 1994; Nedergaard, et al., 1993; Zhang, et al., 2005).

Activation of L-type calcium channels has been shown to modulate synaptic strength in DA cells (Bonci, et al., 1998). Additionally, L-type calcium channels gate Ca\textsuperscript{2+}-release from internal Ca\textsuperscript{2+} stores, activate plasma membrane Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels, as well as several Ca\textsuperscript{2+}-dependent kinases such as PKC, calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase A (PKA) (Berridge, et al., 2003). These kinases are capable of phosphorylating a variety of ion channels to regulate the excitability of neurons (Levitan, 2006). Finding the mechanism that controls the firing mode of DA cells
would provide a vital means of modulating the system in both normal and disease conditions. Here, I present results that \( \text{Ca}^{2+} \) influx through L-type calcium channels activates a \( \text{Ca}^{2+} \)-dependent protease which in turn cleaves PKC to generate a labile fragment that is constitutively active (termed protein kinase M, PKM) to induce burst firing in DA neurons.

2.2 Methods

All 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. Animals were housed in a temperature-controlled facility under a 12 hr/12 hr light/dark cycle with food and water available \textit{ad libitum}.

2.2.1 Slice preparation

Sprague-Dawley rat pups (9-21 days old) of either sex were deeply anaesthetized with halothane and killed by chest compression. The skull was quickly opened to expose the brain, which was cooled \textit{in situ} with ice-cold, carbogenated artificial cerebrospinal fluid (ACSF, composition: 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgCl\textsubscript{2}, 2.4 mM CaCl\textsubscript{2}, 18 mM NaHCO\textsubscript{3}, and 11 mM glucose, pH 7.4 when bubbled with 95% O\textsubscript{2} and 5% CO\textsubscript{2}). The brain was removed and a block containing the midbrain was cut horizontally on a Leica vibratome (VT 1000, Heidelberger, Germany). Tissue slices
(400 μm thick) were allowed to recover at room temperature (22°C) in carbogenated ACSF for at least 1 h prior to recording. Slices were further trimmed to fit into a recording chamber and continuously perfused with carbogenated ACSF at a rate of 2-3 ml min⁻¹ at room temperature. For PKC depletion experiments, one of the two VTA slices from the same animal was incubated in ACSF with or without 1-2 μM phorbol 12-myristate 13-acetate (PMA) for 20-24 h at room temperature in a partially sealed beaker continuously bubbled with carbogen.

2.2.2 Patch clamp recording

All recordings were made from the VTA identified under a dissecting microscope (Leica MZ6). Patch electrodes were prepared from KG-33 glass micropipettes (OD 1.5 mm, Garner Glass CO., Claremont, CA, USA) on a P-97 Brown-Flaming micropipette puller (Sutter Instruments, Novato, CA, USA). For nystatin-perforated patch clamp recording, glass electrodes were filled to the tip with intracellular solution (120 mM potassium acetate, 40 mM HEPES, 5 mM MgCl₂, and 10 mM EGTA with pH adjusted to 7.35 using 0.1 N KOH) and then back-filled with the same solution containing 450 μg ml⁻¹ nystatin and Pluronic F127, yielding a tip resistance of 4-8 MΩ. For conventional whole-cell recording, electrodes were filled with a solution containing 126 mM potassium gluconate, 10 mM KCl, 0.2 mM EGTA, 10 mM HEPES, 4 mM MgATP and 0.3 mM Na₃GTP with pH adjusted to 7.35 and osmolarity to 295 mOsm, yielding a resistance of 2-4 MΩ. Gigaohm seals were made using a Warner PC-505B (Warner Instruments Inc.,
Hamden, CT, USA) or a MultiClamp 700B (Axon Instruments, Foster City, CA, USA) amplifier. Signals were sampled at 5 kHz and digitized by DigiData 1320A using pCLAMP (versions 8 and 9) software (Axon Instruments).

Selection of nystatin-perforated cell recordings in current clamp mode was determined by the size of the action potential, since many VTA cells were spontaneously active. After adequate partitioning of nystatin into the membrane, action potentials overshot 0 mV and measured at least 50 mV. Quality of conventional whole-cell recordings was assessed by a brief voltage step (-20 mV, 10 ms) from the holding potential (-55 mV). Only cells that had an access resistance of <30 MΩ and an input resistance of >200 MΩ were included. Cells whose access resistance increased significantly during the course of recording (>20%) were discarded. Episodic protocols were used to induce Ih and derive passive characteristics of the cell such as current-voltage relationship and input resistance. Current pulses for Ih induction were 1 s and the intervals between pulses were 8 s to allow complete recovery of Ih channels. Cells that displayed a prominent Ih and an apparent DA-induced hyperpolarization were identified as putative DA cells (Figure 1.5) (Johnson, et al., 1992; Lacey, et al., 1989; Zhang, et al., 2005).

Components of extracellular and intracellular solutions were purchased from bulk distributors Fisher Scientific (Nepean, Ontario, Canada) and VWR International (Mississauga, Ontario, Canada). All other chemicals were obtained from Sigma (St Louis,
MO, USA) and Tocris (Ellisville, MO, USA). Chemicals were dissolved in deionized water or DMSO (0.1% final concentration) as required. Aliquots of stock solutions were kept at -30°C. Prior to application, an aliquot was diluted to working concentration and applied to the ACSF bath. PKM (Sigma) was kept at -80 °C and was diluted to 1 unit ml⁻¹ immediately before use in the internal solution for conventional whole-cell recording. DA solution was made fresh daily with an equimolar concentration of the antioxidant disodium metabisulfite.

2.2.3 Western blotting

Phosphorylated PKC and PKM were detected by a phospho-PKC (pan) antibody (Cell Signaling, CA, USA) that recognizes PKCa, βI, βII, δ, ε, η and θ isoforms only when phosphorylated at a carboxyl-terminal residue homologous to Ser660 of PKCβII. Ser660 is in the catalytic domain of PKC and allows simultaneous visualization of phosphorylated PKC and PKM. VTA slices from the same animal were hemisected to give equivalent halves for control and experimental groups. Slices were trimmed to contain the VTA and part of the substantia nigra and were homogenized in RIPA [1x PBS, 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 0.5% deoxycholate, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 5 mM EDTA, 0.1% sodium orthovanadate, 0.1% SDS, 75 ng/ml phenylmethylsulfanyl fluoride (PMSF) and 1x complete protease inhibitor (Roche Diagnostics, QC, Canada)]. Insoluble materials were removed by centrifugation (15,000xg for 10 min), protein concentration for each sample was
determined by BCA™ assay using BSA standard (Pierce, Rockford, USA). Equivalent mass of total protein from slice homogenates were separated on an 8.5% acrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The blots were blocked with Blotto (5% w/v non-fat milk in TBST; 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween20) for 1 h at room temperature, then incubated with phospho-PKC (pan) antibody (1: 1,000) diluted in Blotto, rocking overnight at 4°C. Blots were washed 5 times in TBST and followed by goat anti-rabbit HRP-conjugated IgG diluted in Blotto (Cell Signaling, 1: 15,000) for 90 min at room temperature. After five washes in TBST, blots were incubated in enhanced SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, USA) to visualize specific immune complexes using hyperfilm X-ray film (Amersham Biosci., NJ, USA). Blots were then stripped in a solution of 2% SDS, 63 mM Tris-HCl (pH 6.8) and 0.1 M β-mercaptoethanol for 30 min at 50°C. Stripping solution was removed by two washes with TBST at room temperature, followed by blocking with Blotto and incubation with anti-β-actin antibody (Sigma, 1: 4,000) to act as loading controls. Quantification was obtained from densitometric measurements of immunoreactive bands using Gel Logic100 imaging system with Gel Logic200 Software (Kodak, USA).

2.2.4 Data analysis

Electrophysiological data were analyzed offline with Mini Analysis (Synaptosoft Inc., Decatur, GA, USA) and pCLAMP software. Basal firing frequencies were averaged
values of at least 5 min stable baseline recording; $I_h$ was measured as the difference in current or voltage between instantaneous and steady-state readings. Analysis of firing behavior was based on interspike intervals (ISIs) measured with the Mini Analysis program. Averaged as well as instantaneous firing frequencies were derived from those intervals. Coefficient of variance (CV) was calculated as the mean of ISIs over a 1-min period divided by their standard deviation. To compare CV values between cell groups, they were normalized against the mean CV value of the first 5 min. Relative density of ISIs in 2-second bins were plotted to reveal the distribution of a given ISI series, and the resulting histogram was fitted to a Lowess function. Burst firing was defined as two spikes or more in each bursting cycle at a frequency higher than non-bursting periods and separated by a post-burst hyperpolarization, which was visually judged and quantified. For western blotting analysis, density of phospho-PKM and PKC bands were normalized against the density of β-actin from the same sample.

Data were expressed as means and standard errors of the mean (SEM). Statistical comparisons of electrophysiological data were performed using two-tailed unpaired Student’s t test. Western blotting data were compared using paired t tests since each control and experimental pair contained hemisected slices from the same animal. Values were considered significant when $P < 0.05$. 
2.3 Results

Except when indicated, experiments were done using the nystatin-perforated whole-cell recording method at room temperature. Dialysis of PKM through the electrode into recorded cells was done using conventional whole-cell recording. Only DA cells in the VTA identified according to criteria outlined in Methods section were included. In nystatin-perforated recording, the average hyperpolarization following a brief application of 50 μM DA (within 90 sec) was $-8.29 \pm 0.57$ mV ($n=71$, excluding cells used for PKC depletion test). Most cells (47 of 71, 66%) were spontaneously active with single spike firing at a low basal firing frequency of $0.41 \pm 0.04$ Hz; the remainder (24 of 71, 34%) were quiescent during baseline recordings.

2.3.1 Opening of L-type calcium channels converts firing patterns

Bath application of 1-4 μM FPL 64176, a benzoylpyrole site L-type calcium channel opener, for 5-10 min converted firing patterns from quiescent state or single spiking to burst firing in 80.3% of treated cells (57 of 71). Application of 1 μM FPL 64176 induced burst firing in 63.5% cells (33 of 52). The responses were spike-dependent. Percentage of converted burst firing in spontaneously firing cells (41 of 47, 87.2%) was much higher than that in quiescent cells (16 of 24, 66.7%), a response that appeared to be related to their different resting membrane potentials ($-49.35 \pm 0.54$ mV for spiking cells vs. $-52.52 \pm 0.92$ mV for quiescent cells, $P<0.05$). The responses were dose-dependent: 2 μM
FPL 64176 induced burst firing in 9 of 17 cells that did not respond to 1 μM and similarly, 4 μM induced burst firing in 4 of 10 cells that did not respond following a dose of 2 μM.

In cells that were spontaneously firing, FPL 64176 first induced a membrane depolarization (2.00±0.38 mV, n=41) accompanied by a 48.8±9.3% increase in firing rate in the first 5 minutes of drug application, burst firing started after a varying period of latency. The lag between the start of drug application and burst firing ranged 5-25 min with an average of 13 min (Figure 2.1A, 2.1F). Within a burst firing cycle, action potentials were fired with increasing frequencies followed by a pronounced post-burst hyperpolarization (Figure 2.1B). The average intra-burst firing frequency (1.12±0.13 Hz, n=41) was much higher than basal tonic frequency (0.43±0.05 Hz). The development of burst firing in quiescent cells (n=16) was slightly different: they responded to FPL 64176 with a membrane depolarization (1.38±0.38 mV) followed by a sudden appearance of burst firing (Figure 2.1D) or irregular single spiking that evolved into burst firing (Figure 2.1E). The L-type calcium channel-mediated burst firing was long lasting even after prolonged washout up to 3 hours, however, it was readily inhibited by the L-type calcium channel blocker nifedipine at a range of doses (1-10 μM, n=29). Density plots of ISIs in 2-second bins showed an ISI distribution that fits to a single Gaussian distribution under control conditions and to a mixed Gaussian distribution following FPL 64176 application (Figure 2.1C). The leftward shift of the main peak indicates higher firing frequencies and
the second peak at much lower frequency represents the long pauses of firing between adjacent bursts.

Bath application of 5 μM (S)-(−)-Bay K8644, a DHP site L-type calcium channel activator, also induced burst firing in 3 of 5 cells tested (Figure 2.1G). The time course of the response was similar to that following FPL 64176, a depolarization and an increase in firing rates followed by a conversion of firing patterns 10-25 minutes after the start of application.

2.3.2 FPL 64176 induces burst firing independent of an intermediate transmitter

L-type calcium channels have been shown to enhance a slow NMDA current in DA neurons (Bonci, et al., 1998), suggesting that L-type calcium channel opening could induce burst firing by promoting glutamate transmission. Because FPL 64176-induced burst firing was long-lasting, I examined the involvement of synaptic mechanisms in two ways: whether induced burst firing persisted in the presence of a cocktail containing 100 μM APV, 10 μM CNQX and 100 μM picrotoxin (blocking NMDA, AMPA and GABA_A receptors, respectively) and whether pretreatment with this cocktail altered the ability of FPL 64176 to induce burst firing. FPL 64176-induced burst firing was still robust following the application of the cocktail for 10-30 min (n=7, Figure 2.2A). Similarly, cells that were treated with the cocktail for 5-10 minutes prior to FPL 64176 (1 μM) application in the presence of the cocktail still responded with burst firing (n=3).
These results suggest that L-type calcium channel opening does not induce burst firing either directly through increased glutamatergic transmission or indirectly by way of GABAergic interneurons in the VTA.

Activation of L-type calcium channels may release DA from the soma and dendrites since this release has been shown to be Ca\(^{2+}\)-dependent (Beckstead, et al., 2004; Chen and Rice, 2001). DA acting at somatodendritic autoreceptors forms the short-loop negative feedback to regulate the excitability of DA cells. I therefore examined whether D\(_2\) receptors were involved in burst firing. The D\(_2\) receptor antagonist sulpiride (1-10 μM), which itself did not change the firing activity, applied for 10-15 min to 5 burst firing cells induced by FPL 64176 had no effect on firing patterns (Figure 2.2B). Two cells to which 10 μM sulpiride was applied for 8 min prior to FPL 64176 application in the presence of sulpiride displayed burst firing similar to that induced by FPL 64176 alone. These data suggest that L-type calcium channel activation does not result in somatodendritic DA release to induce burst firing.

2.3.3 Internal Ca\(^{2+}\) stores are not involved

Ca\(^{2+}\) entry through L-type calcium channels gates ryanodine receptors on internal Ca\(^{2+}\) stores to mediate Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Berridge, et al., 2003). So I tested whether this was involved in burst firing. Cyclopiazonic acid (CPA) and thapsigargin were used to inhibit the sarco-endoreticular Ca\(^{2+}\)-dependent ATPase (SERCA) that
refills internal Ca\(^{2+}\) stores. After burst firing was induced by FPL 64176, CPA (20-30 μM) applied for 20-60 min (n=4) or thapsigargin (1-2 μM) applied for 50-70 min (n=3) did not stop the induced burst firing (Figure 2.2C). In three cells that were pretreated with CPA (20 μM) for 30 min, FPL 64176 (1 μM) was still able to induce strong burst firing. These results indicate that internal Ca\(^{2+}\) stores are not necessary for L-type calcium channel-mediated burst firing.

2.3.4 Ca\(^{2+}\)-dependent protein kinase mediates burst firing

Increased intracellular Ca\(^{2+}\) activates Ca\(^{2+}\)-sensitive protein kinases such as PKC, PKA and CaMKII that can phosphorylate ion channels including L-type calcium channels themselves and regulate the excitability of neurons (Dzhura, et al., 2000; Lee, et al., 2006; Yang, et al., 2005; Young and Yang, 2004). After burst firing was induced by FPL 64176, bath application of the PKA inhibitor H-89 (5 or 10 μM) for 40-130 min (n=4), the CaMKII inhibitor KN-93 (1-10 μM) for 25-100 min (n=6), or both for 60-70 min (n=3) did not block burst firing (Figure 2.3A). The substrate site PKC inhibitor, chelerythrine (40 μM), applied for 15-44 min (n=6) completely blocked FPL 64176-induced burst firing and the accompanying membrane potential oscillation. This blockade was reversible since burst firing reappeared after washing out chelerythrine for 18-33 min (Figure 2.3B). To establish the role of PKC in burst firing, I further used an inhibitor that binds to the diacylglycerol (DAG) site of the regulatory domain. Calphostin C (1-2 μM) applied for 40-180 min (n=4) was largely ineffective except in one cell where
burst firing slowed marginally but persisted (Figure 2.3C). These results suggest that the catalytic subunit of PKC itself or a kinase with a similar substrate site is involved in burst firing.

2.3.5 Proteolytic cleavage of PKC mediates burst firing

To solve the apparent inconsistency between the two PKC inhibitors, I considered other modes of PKC activation. Ca\(^{2+}\) entry has been shown to activate the protease calpain that proteolytically cleaves PKC to form PKM which has been shown to be constitutively active with a very short half-life within the cell (Al and Cohen, 1993; Cressman, et al., 1995; Kishimoto, et al., 1983; Shea, et al., 1994). I therefore tested whether proteolytic cleavage of PKC could explain the inconsistency in response to the two PKC inhibitors. Cells were induced to burst fire by FPL 64176, the calpain inhibitor MDL 28170 (200 μM) was then applied. In all cells tested as such, burst firing reverted to single spike firing in 25-40 min and resumed after washing 20-30 min (n=5, Figure 2.4A). Prior application of MDL 28170 (200 μM) for 30-40 min completely prevented FPL 64176-induced (2-4 μM) burst firing (n=3).

To further validate the role of PKM in L-type calcium channel-induced burst firing, I compared the levels of phospho-PKC and PKM by Western blotting total protein lysates from slices that were treated with (S)-(−)-Bay K8644 or FPL 64176 alone and with MDL 28170 followed by FPL 64176. As shown in Figure 2.4B, phospho-PKC antibody raised
against the catalytic domain visualized three bands: two at approximately 82 kDa corresponding to intact PKC isoforms and another at 45 kDa corresponding to the PKC catalytic unit or PKM. Application of L-type calcium channel opener FPL 64176 (2 μM) or (S)-(-)-Bay K8644 (5 μM) for 30 min caused a considerable increase in phospho-PKM expression (Figure 2.4C). Densitometry of the bands corresponding to phospho-PKM showed that (S)-(-)-Bay K8644 (224±61% relative to untreated values, n=8, P<0.05) or FPL 64176 (244±51% relative to untreated values, n=6, P<0.05) significantly increased phospho-PKM levels (Figure 2.4D). The increase by FPL 64176 could be completely blocked by prior treatment with the calpain inhibitor MDL 28170 (200 μM for 30 min; 8±1% of FPL 64176 alone, n=5, P<0.0001; Figure 2.4C, D). There were no detectable changes in full-length phospho-PKC levels following (S)-(-)-Bay K8644 (102±10% of control values, n=8, P>0.05), FPL 64176 (104±6% of control values, n=6, P>0.05) or MDL 28170 with FPL 64176 (95±2% of FPL 64176 alone, n=5, P>0.05). These data indicate that PKM levels parallel burst firing behavior.

2.3.6 Burst firing can not be induced after PKC depletion

Since PKM is generated by cleavage of PKC (Cressman, et al., 1995; Shea, et al., 1994), it is an obvious test to determine if burst firing can be induced following PKC depletion. Persistent activation by phorbol esters such as PMA, leads to degradation of PKC isoforms that have the diacylglycerol site and is used experimentally to produce a PKC-depleted cell (McArdle and Conn, 1989; Szallasi, et al., 1994). Slices were incubated
with or without PMA (1-2 μM) for 20-24 hours followed by FPL 64176 (4 μM) to induce burst firing. Regardless of incubation conditions, cells from these slices were no longer spontaneously active although they were capable of firing action potentials (Figure 2.5A). There was no difference in resting membrane potential (control: 50.05±1.36 mV, n = 6; PMA-treated: 48.25±1.38 mV, n = 7, P > 0.05) or input resistance (control: 403.24±56.12 MΩ, n = 6; PMA-treated: 293.58±42.93 MΩ, n = 7, P > 0.05) between control and PMA-treated groups. PMA-treated cells had a significantly smaller DA-induced hyperpolarization (control: 12.03±3.43 mV; PMA-treated: 2.99±0.67 mV, P < 0.05).

Western blots showed a disappearance in PMA-treated slices of a band around 82 kDa and the concomitant decrease of the PKM band at 45 kDa following FPL 64176 (Figure 2.5B).

There was a clear difference in FPL 64176-induced firing between the two groups. Of 6 cells from control slices, FPL 64176 (4 μM) induced burst firing in 3 (Figure 2.5C1), large membrane potential oscillations in 2 and no change in one cell. Both burst firing and membrane potential oscillation could be blocked by nifedipine (5 μM). Of 7 cells from PMA-treated slices, FPL 64176 (4 μM) only induced an average membrane depolarization of 0.62±0.26 mV with no burst firing or membrane potential oscillation in any of the cells tested (Figure 2.5C2). These data show depletion of PKC decreased the PKM expression and blocked the bursting production, further indicating that PKM levels parallel bursting firing behavior.
2.3.7 Direct loading of PKM induces burst firing

If PKM links L-type calcium channels and burst firing, it would be expected that direct loading of PKM (1 unit/ml pipette solution) into the cells through the conventional whole-cell recording pipette should induce similar firing mode switching. Out of seven cells, three had single spike firing initially that became irregular and clustered by 3-5 min, with burst firing appearing at 9-12 min. Burst firing was strong and stable for 20-30 min (Figure 2.6A) and was qualitatively similar to burst firing induced by FPL 64176. When burst firing was prevalent, density plots of ISIs showed a typical two-peak ISI distribution (Figure 2.6C2). In another 2 cells, single spike firing became burst-like characterized by clustered spikes followed by a pause without a post-burst hyperpolarization. Only in 2 cells was no response seen. The control experiments were done using the same internal pipette solution without PKM and all 6 cells tested maintained regular firing during the 40 min recording period (Figure 2.6B). Plotting normalized coefficient of variance values against the average value of the first 5 min (Figure 2.6D) revealed that PKM significantly increased variance while it remained unchanged in control cells (unpaired t test on areas-under-the-curve in arbitrary units, control: 3323.38±291.91, PKM: 5001.95±427.84, P<0.05), indicating that PKM disrupts regular spiking and promotes burst firing.
2.3.8 Activation of NMDA and cholinergic receptors increases PKM expression

To test whether PKM is involved in the physiological regulation of burst firing in DA neurons, glutamate or cholinergic agonists known to induce burst firing in DA cells were used to stimulate VTA slices and the amount of PKM was detected by Western blotting. Hemisected slices from the same animal were treated with or without NMDA (20 µM) or carbachol (20 µM) for 20 min to test whether it increased the levels of phosphorylated PKM. As shown in Figure 2.7A, application of either the glutamate agonist NMDA or cholinergic agonist carbachol caused a considerable increase in phospho-PKM expression. Densitometry of the bands corresponding to phospho-PKM of six independent experiments (Figure 2.7B) showed that both NMDA (159±26% relative to untreated values, P<0.05) and carbachol (154±41% relative to untreated values, P<0.05) significantly increased phospho-PKM levels. Since glutamate and cholinergic inputs are major synaptic modulators of DA cells, these data indicate that PKM is involved in the synaptic regulation of firing behavior of DA cells.

2.4 Discussion

Burst firing is an important property of DA cells in the VTA to signal novelty and salience that are associated with normal or abnormal expression of motivation and reward. I have previously reported that the cholinergic agonist carbachol induces a calcium-dependent burst firing of DA cells by promoting Ca^{2+} influx through L-type
calcium channels. This paper studies how L-type calcium channel activation induces burst firing. In this study, I observed that direct activation of L-type calcium channels converted firing patterns of VTA DA cells independent of glutamate, dendritically released DA or Ca\(^{2+}\) released from internal stores. Nor did Ca\(^{2+}\) influx through L-type calcium channels activate PKA or CaMKII to induce burst firing. I have shown that Ca\(^{2+}\) influx through L-type calcium channels activates calpain proteases that in turn cleave PKC, releasing the short lived, active PKM fragment to produce burst firing. Disruption at any point along this chain of events results in an inability to cause burst firing of DA neurons in the VTA. I have also shown that PKM is involved in the regulation of DA cells under physiological stimulations where the NMDA type of glutamate receptors or cholinergic receptors were activated.

### 2.4.1 L-type calcium channel-induced burst firing does not require an intermediate transmitter

L-type calcium channels are primarily expressed in the soma and dendrites of DA cells in the VTA area (Takada, et al., 2001) although they have been shown to modulate synaptic transmission to the VTA (Bonci, et al., 1998). Glutamate acting at the NMDA receptors mediates a slow excitatory synaptic transmission to VTA DA cells (Mercuri, et al., 1996) that can be enhanced by L-type calcium channel activation (Bonci, et al., 1998), suggesting that the effects of L-type calcium channel activation could be secondary to increased glutamate transmission. This is particularly relevant since NMDA is one of the
pharmacological tools used to induce burst firing in DA neurons (Johnson, et al., 1992; Johnson and Wu, 2004). Our results showing that combined synaptic blockade at the NMDA, AMPA and GABA_A sites before or after L-type calcium channel activation did not alter burst firing suggest that glutamate transmission is not involved in L-type calcium channel-induced burst firing. Alternatively, activation of L-type calcium channels increases firing (Mercuri, et al., 1994) and subsequently releases DA from somatodendritic sites in order to terminate firing by D_2-mediated autoinhibition (Beckstead, et al., 2004). Autoinhibition wanes as DA is taken back by DAT and a new cycle starts. This mode of action appears logical since all the proposed mechanisms have been shown to work in DA cells. However, L-type calcium channel activation induced strong burst firing that was not blocked by the D_2 receptor antagonist sulpiride. Blocking the D_2 receptor before application of L-type calcium channel opener did not alter burst firing either, arguing against a role for somatodendritic D_2 receptors in L-type calcium channel-induced burst firing.

It has been shown previously that burst firing of DA cells depends on Ca^{2+} oscillations (Zhang, et al., 2005), a phenomenon that could be supported by so-called Ca^{2+}-induced Ca^{2+} release from internal stores. The L-type calcium channel has been shown to directly gate Ca^{2+} releasing channels on the membrane of sarcoplasmic reticulum (Berridge, et al., 2003). The IP_3 channels have a bell-shape sensitivity relationship with intracellular Ca^{2+} levels (Berridge, et al., 2003) so that their conductance increases initially with higher Ca^{2+} concentrations, further increases in Ca^{2+} reduces their
conductance capabilities. Also, Ca\(^{2+}\) release from internal stores has been found to mediate a slow inhibition in DA cells taking hundreds of milliseconds to develop (Fiorillo and Williams, 1998). This is on a similar time scale to burst firing cycles I observed in slice preparations. These mechanisms can possibly produce a biphasic response that fits the membrane oscillation I observed. In my experiments, burst firing induced by the L-type calcium channel opener FPL 64176 persisted when internal stores were depleted by prolonged inhibition of the refilling enzyme SERCA, suggesting that internal stores do not play an obligatory role in burst firing.

2.4.2 Ca\(^{2+}\) influx generates PKM to induce burst firing

The opening of L-type calcium channels did not induce burst firing within the first 5 minutes following FPL 64176 application although Ca\(^{2+}\) influx had already changed the excitability of the cells in the form of membrane depolarization and increased firing frequency. Burst firing emerged 5-25 min following the start of drug application and lasted for hours. The lag seems to indicate that Ca\(^{2+}\) entry activated a signaling cascade that takes time to develop or a second messenger must migrate between the intracellular compartments. Since protein kinase activation requires multiple stages of phosphorylation and translocation (Liu and Heckman, 1998), the involvement of Ca\(^{2+}\)-dependent protein kinases presents a reasonable mechanism underlying Ca\(^{2+}\)-dependent burst firing. For example, CaMKII has been shown to become persistently active during Ca\(^{2+}\) oscillations to enhance L-type calcium channels (Hudmon, et al., 2005). Similarly, PKA has long
been associated with adrenergic enhancement of L-type calcium channel activity (Hoogland and Saggau, 2004; van der Heyden, et al., 2005). The involvement of these kinases could explain why L-type calcium channels continue operating despite high intracellular Ca\(^{2+}\) levels. Channel phosphorylation has been suggested to reduce the Ca\(^{2+}\)-dependent inactivation of L-type calcium channels (Budde, et al., 2002). The fact that burst firing persisted in the presence PKA and CaMKII blockers supports the concept that they are not necessary for Ca\(^{2+}\)-dependent burst firing.

The striking result that chelerythrine, a PKC catalytic domain inhibitor, reversibly blocked burst firing, whereas calphostin C, a regulatory domain inhibitor did not, raised the possibility that Ca\(^{2+}\) influx through L-type calcium channels activates a PKC-like kinase or PKC operates in an atypical fashion. Elevated intracellular Ca\(^{2+}\) has been shown to activate calpain (Goll, et al., 2003), a protease that cleaves the PKC catalytic domain from the regulatory domain (Al and Cohen, 1993; Cressman, et al., 1995; Kishimoto, et al., 1983) to generate PKM. My results support this mode of PKC action and its regulation of burst firing through: 1) L-type calcium channel openers induced burst firing accompanied by increased levels of phosphorylated PKM; 2) inhibition of the Ca\(^{2+}\)-dependent protease calpain reduced the level of phosphorylated PKM and concomitantly blocked burst firing; 3) PKM is generally thought to be a proteolytic product of PKC (Al and Cohen, 1993; Cressman, et al., 1995; Kishimoto, et al., 1983) and depletion of PKC isoforms following prolonged incubation with PMA prevented L-type calcium channel-induced burst firing; 4) direct loading of the cell with purified PKM through the recording pipette
induced burst firing in a similar manner to $\text{Ca}^{2+}$ influx, with the temporal development of burst firing being consistent with diffusion of PKM into the cell. The evidence collectively indicates that $\text{Ca}^{2+}$ influx through the L-type calcium channel activates proteolytic cleavage of PKC to generate PKM that facilitates the development and maintenance of burst firing of DA cells.

2.4.3 PKC is functionally important for DA-related conditions

PKM was found in the rat brain in the 1970s (Inoue, et al., 1977) but its function remained obscure until recent reports associating it with learning and memory (Osten, et al., 1996; Sacktor, et al., 1993). Several PKC isoforms have been found to give rise to proteolytic PKM counterparts, and it is reasonable to assume that all members of the PKC family are capable of generating their respective PKMs because of their similar structure of the hinge region that joins the regulatory and catalytic domains. My results did not identify the parent PKC isoform that was cleaved to form PKM, it may involve several PKC isoforms, since many are expressed in DA neurons (Yoshihara, et al., 1991). The PKM used for cellular loading is derived from enzymatic digestion of total PKC, but from the PKC depletion experiments, it appears that the PKM involved in burst firing is generated by the isoforms with the diacylglycerol site. PKC has been implicated in addiction and motivation. Injection of PKC inhibitors into the VTA reduces cocaine-induced DA release in the NAc (Steketee, 1993) and delays the onset of behavioral sensitization (Steketee, 1994; 1997). Repeated administration of cocaine
increases overall PKC activity in the VTA which may initiate behavioral sensitization (Steketee, et al., 1998). Being persistently active, PKM could fulfill all of these behaviors involving PKCs. More importantly, PKM could achieve all this by its role in burst firing reported here as burst firing elevates terminal DA more effectively and all of these behaviors involve increased DA transmission. In addition, addiction is a learned behavior and PKM has been shown to facilitate learning and memory in rats (Osten, et al., 1996; Sacktor, et al., 1993). Taken together, PKC and its cleaved product PKM might play a significant role in central DA transmission and its related pathologies such as drug abuse. It is especially encouraging that activation of the NMDA and cholinergic receptors increased PKM because these receptors not only mediate synaptic regulation of DA cells, but they are also known to induce burst firing in DA cells.

2.4.4 Mechanisms of PKM-induced burst firing

There are many factors contributing to the strength of DA transmission. Besides the efficiency of the release machinery, it is usually agreed that DA reuptake and autoreceptor-mediated inhibition are important regulators of DA transmission. Released DA is rapidly taken back to the terminal by DAT, whereas D2 autoreceptors at both terminal and somatodendritic sites respond to increased DA levels by a negative feedback loop that inhibits DA cell activity. PKC has been shown to modulate both processes. Activation of PKC leads to a decrease in DAT capacity (Daniels and Amara, 1999; Melikian and Buckley, 1999) due to accelerated internalization (Holton, et al.,
2005; Sorkina, et al., 2005), reduced recycling (Loder and Melikian, 2003) and
degradation (Miranda, et al., 2005). Additionally, PKC activation enhances
phosphorylation, desensitization and trafficking of D2 receptors (Namkung and Sibley,
2004) resulting in a dampened negative feedback. In PMA treated slices, somatodendritic
autoreceptors’ responses to DA were blunted, it remains to be tested whether these
counterparts in the terminal are similarly affected. Together the effects of PKC on DAT
and autoreceptor inhibitory feedback, with my results that the proteolytic product of PKC
(PKM) induces burst firing, a firing mode that is more effective in increasing DA release,
it is a tantalizing possibility that PKC serves as a signaling hub, integrating different
aspects of DA transmission to boost its functionality over a long period of time. This
postulate further supports the findings that implicate PKC in addiction, a chronic and
debilitating condition that has long been thought to be due to enhanced DA transmission.
Figure 2.1 FPL 64176 induces burst firing of VTA DA cells. A. Continuous current clamp recording from a representative cell showing that FPL 64176 converted regular firing to burst firing which could be blocked by nifedipine. B. Traces on expanded time scales showing regular firing before FPL 64176 application (1), regular firing at higher rates (2) and burst firing after drug application (3) that was brought back to regular firing by nifedipine (4). Note the low frequency of regular firing with a pronounced afterhyperpolarization following each action potential and the clustering of action potentials at increased frequencies followed by a steep post-burst hyperpolarization in burst firing mode. C. Density plot of interspike intervals (ISI) in 2-second bins in control conditions (C1, 76 events) and following FPL 64176 application (C2, 246 events). FPL 64176 dramatically shifted the primary peak to the left and gave rise to a secondary peak corresponding to the frequency of burst firing cycles. D. Continuous current clamp recording from a representative cell showing that FPL 64176 induced sudden burst firing in a quiescent cell. E. Continuous current clamp recording from a representative cell showing that FPL 64176 induced irregular firing that evolved into burst firing in a cell that had no baseline firing. F. Histogram of number of cells (n=57) that started burst firing following the onset of FPL 64176 application. G. Continuous current clamp recording from a representative cell showing that (S)-(−)-Bay K8644 converted regular firing to burst firing.
Figure 2.2 FPL 64176 induces burst firing independent of glutamate and GABA ionotropic receptors, D, DA receptors and internal Ca\(^{2+}\) stores. Continuous current clamp recording from representative cells showing FPL 64176-induced burst firing that persisted in the presence of a cocktail containing blockers at the GABA\(_{A}\), AMPA and NMDA site (A), the D\(_2\) antagonist sulpiride (B) and the SERCA inhibitor thapsigargin (C).
Figure 2.3 PKC mediates FPL 64176-induced burst firing. Continuous current clamp recording from representative cells showing FPL 64176-induced burst firing that persisted in the presence of PKA or CAMKII inhibitors or both (A), was reversibly blocked by the substrate site PKC inhibitor chelerythrine (B), but not by the PKC blocker that binds to the DAG site in the regulatory subunit (C).
Figure 2.4 Proteolytic cleavage of PKC mediates FPL 64176-induced burst firing. Continuous current clamp recording from a representative cell showing that FPL 64176-induced burst firing was reversibly blocked by the calpain inhibitor MDL 28170 (A). Western blots showing phosphorylated PKC isoforms at approximate 82 kDa and a smaller band of PKM at approx 45 kDa (B). PKM was increased by FPL 64176 (2 μM, 30 min) or (S)-(−)-Bay K8644 incubation (5 μM, 30 min) and abolished by prior treatment with MDL 28170 (200 μM, 30 min) followed by FPL 64176 (2 μM, 30 min) with MDL 28170 (C). Levels of phosphorylated PKM in response to FPL 64176 (n=6,) or (S)-(−)-Bay K8644 (n=8) relative to control slices and PKM levels with prior MDL 28170 treatment relative to FPL 64176 alone (n=5) (D). Levels of PKM were normalized against β-actin values obtained from the same blot.
Figure 2.5 FPL 64176 fails to induce burst firing following PKC depletion.
A. Comparable current-voltage relationships between cells that were incubated in normal ACSF or in PMA 2 μM for 20 h. B. Western blots showing a diminished band of phosphorylated PKC isoforms (B1) and a much smaller phosphorylated PKM increase (B2) following FPL 64176 (4 μM, 30 min) in PMA-treated slices (2 μM, 20 h). C. Continuous current clamp recording from representative cells showing FPL 64176 induced burst firing that could be blocked by nifedipine in ACSF-treated slices (C1) but not in PMA-treated slices (C2).
Figure 2.6 Direct loading of purified PKM induces burst firing. A. Continuous current clamp recording from a representative cell showing that PKM loading gradually transformed regular firing into burst firing. Traces on expanded time scales showing regular firing at the beginning (1) and burst firing 30 min after going whole cell (2). B. Control whole cell recording showed regular firing throughout the recording period. Traces on expanded time scales showing regular firing at the beginning (1) and 30 min after going whole cell (2). C. Density plot of interspike intervals (ISI) in 2-second bins for the first 3 min (C1, 136 events) and 22-35 min (C2, 370 events) following PKM loading. Note the single ISI distribution in the first 3 min (C1) and PKM loading generated a second distribution at lower frequencies (C2). D. Normalized coefficient of variance (CV, over 1 min periods) against the average value of the first 5 min in control (n=6) or PKM loading groups (n=7) showing PKM increased CV values.
Figure 2.7 NMDA or carbachol induces PKM expression.
A. Western blots showing phosphorylated PKM was increased by NMDA (20 μM, 20 min) or carbachol (20 μM, 20 min) incubation.
B. Levels of phosphorylated PKM in response to NMDA (n=6) or carbachol (n=6) relative to control slices.
Figure 2.8 Proposed model of PKM-induced bursting.
Schematic representation of the proposed model of PKM-induced bursting, inferred from our results. Calcium entry through L-type channels activates the Ca$^{2+}$-dependent protease calpain which cleaves PKC into cofactor-independent PKM to induce burst firing in dopamine-responsive neurons in ventral tegmental area.
Chapter 3 Cholinergic Excitation of Dopaminergic Cells Depends on Sequential Activation of Protein Kinase C and the L-type Calcium Channel in Ventral Tegmental Area Slices

(published in Brain Res 1245: 41-51, 2008)

3.1 Introduction

DA cells in the VTA project to the NAc and prefrontal cortex, a circuitry that is commonly known as the reward pathway (Cooper, 2002; Ikemoto, 2007). It has been shown that reward processing is initiated in the VTA which codes for novelty and salient valence of a stimulus in the form of burst firing (Cooper, 2002; Tobler, et al., 2005). The cortical analysis of sensory information is relayed to the VTA through a direct projection from the prefrontal cortex or, indirectly, via its connections to midbrain nuclei, the PPTg and the LDTg. Direct projections from the prefrontal cortex are glutamatergic in nature whereas those via the midbrain nuclei are mixed glutamatergic and cholinergic (Mena-Segovia, et al., 2008; Sesack, et al., 2003). Stimulation of these areas induces large changes in the rate and pattern of firing in the VTA (Cooper, 2002; Grillner and Mercuri, 2002; Kitai, et al., 1999; Lodge and Grace, 2006).

PKC phosphorylates specific serine/threonine sites in a protein to alter its functions. Many proteins are substrates of PKC including glutamatergic and cholinergic receptors as
well as ion channels that are involved in maintaining a cell's excitability (Majewski and Iannazzo, 1998). As a result, PKC in the VTA has been implicated in addiction and motivation. Repeated administration of cocaine increases overall PKC activity in the VTA (Stecketee, et al., 1998) whereas injection of PKC inhibitors into the VTA reduces cocaine-induced DA release in the NAc (Stecketee, 1993) and delays the onset of behavioral sensitization (Stecketee, 1994;1997). Glutamate and acetylcholine are primary excitatory neurotransmitters in the VTA, both of which could activate PKC through their ionotrophic (by increasing intracellular Ca\(^{2+}\)) and metabotropic receptors (by activating phospholipase C to generate lipid second messengers). It is therefore logical to examine whether PKC is involved in increased VTA firing following activation of these transmitter systems.

L-type calcium channels are responsible for approximately one third of total Ca\(^{2+}\) currents (Cardozo and Bean, 1995;Durante, et al., 2004;Takada, et al., 2001) and contribute preferentially to whole-cell Ca\(^{2+}\) currents evoked by small depolarization (Durante, et al., 2004;Xu and Lipscombe, 2001) in DA cells. Recently, a dominant role of Ca\(_{\text{v}1.3}\), a subtype of the L-type calcium channel that operates at close to resting membrane potentials, was reported in adult mice to sustain pacemaking activities of DA cells (Chan, et al., 2007). Since L-type calcium channels are substrates of PKC and both glutamatergic and cholinergic receptors are coupled to the PKC cascade, it is possible that synaptic modulation of DA firing is due to L-type calcium channel phosphorylation that increases channel opening and conductance (Kamp and Hell, 2000). This is important not
only because of the number of Ca,1.3 channels expressed in DA cells (Chan. et al., 2007; Takada, et al., 2001), but also because of the voltage range at which these channels operate such that their opening, and the modulation of their opening, makes a dominant contribution to the subthreshold depolarization that precede action potential firing.

Previously, we reported that carbachol induces burst firing in a small percentage (20%) of DA cells, longer period of application or higher concentration of carbachol did not increase the percentage of bursting cells, by increasing L-type calcium channel activity (Zhang, et al., 2005). I further reported that Ca^{2+} influx through L-type calcium channel results in PKC cleavage to generate persistently active PKM and induces burst firing (Liu, et al., 2007; Zhang, et al., 2005). However, brief application of cholinergic and glutamatergic agonists only increases the rate of pacemaker firing without the appearance of burst firing in a much bigger portion of DA cells. I therefore examined whether PKC and L-type calcium channels were recruited in excitation induced this way. Here, I present results showing that carbachol activation enhances PKC activity to regulate L-type calcium channels to excite DA cells, while neither NMDA- nor AMPA-induced excitation is dependent on PKC or L-type calcium channels.
3.2 Materials and methods

3.2.1 Slice preparation

The slice preparation was the same as that described in 2.2.1 except that brain was cooled *in situ* with a different ice-cold, carbogenated (95% O₂ and 5% CO₂) cutting solution, in which all NaCl used in ACSF in section 2.2.1 was replaced by equi-osmotic glycerol during slice preparation (Ye, et al., 2006) (composition: 250 mM glycerol, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.4 mM CaCl₂, 26 mM NaHCO₃, and 11 mM glucose, pH 7.4 when bubbled with carbogen) after the skull was removed and a block containing the midbrain was cut in this cutting solution, and tissue slices were allowed to recover at 31°C in the same ACSF as that in 2.2.1 for 1 hr instead of recovering at room temperature, and then maintained at room temperature until use. This modified glycerol-based ACSF used only during slice preparation is to prevent the possible acute neurotoxic effects of passive chloride entry, subsequent cell swelling and lysis. Examination of some electrophysiological properties of the neurons in the VTA in these preparations reveals similar properties with the traditional ACSF method (Ye, et al., 2006).
3.2.2 Electrophysiological patch clamp recording

The methods of nystatin-perforated patch clamp recording were the same as that described in 2.2.2.

For recording HVA Ca$^{2+}$ currents, standard whole-cell configuration was used. Patch pipettes were filled with a solution containing 120 mM CsCl, 10 mM HEPES, 10 mM EGTA, 4 mM MgATP, 0.3 mM NaGTP. The extracellular solution for HVA Ca$^{2+}$ current recording contained 120 mM TEA-Cl, 2.5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.2 mM MgCl$_2$, 2.4 mM CaCl$_2$, 18 mM NaHCO$_3$, 11 mM glucose, 3 mM CsCl, 0.3 mM Ni$_2$Cl, 1 mM 4-AP, 0.3 μM TTX. This solution blocks voltage-gated K$^+$, Na$^+$, I$_h$ and the low voltage gated T-type Ca$^{2+}$ currents to isolate HVA currents. The holding potential was -60 mV, and the test pulse for HVA Ca$^{2+}$ current recording was 0 mV for 150 ms. No series resistance compensation or leak subtraction was used in these recordings. Cells whose access resistance and capacitance increased significantly during the course of recording (>20%) were discarded.

3.2.3 Drugs

This part was the same as that described in 2.2.2.
3.2.4 Data analysis

The general electrophysiological data analysis was the same as that described in 2.2.4.

Changes in firing relative to baseline were calculated as instantaneous firing rates divided by the average firing rate of the 5 min stable baseline recording and were plotted as 1-min (for experiments using carbachol) or 30-s (for experiments using NMDA or AMPA) bins. Peak response (the highest bin reading following a treatment), response time (duration of the response) and time to peak were also quantified. HVA Ca$^{2+}$ currents were quantified after subtracting residual currents in the presence of the general Ca$^{2+}$ channel blocker Cd$^{2+}$. Statistical comparisons of electrophysiological data were performed using paired or unpaired two-tailed Student's t tests, one-way ANOVA or ANOVA for repeated measures (for comparing time-dependent, multiple points between two treatments) as appropriate. To satisfy the normalization test for small sample sizes, all data entries were cubic root transformed before statistical comparisons. Values were considered significant when $P < 0.05$. 
3.3 Results

3.3.1 PKC mediates carbachol- but not NMDA- and AMPA-induced excitation

First I examined the role of PKC in carbachol-, NMDA- and AMPA-induced excitation of DA cells. In order to compare the response to carbachol, NMDA and AMPA under different conditions, I initially established that repeated bath application of these ligands induced comparable responses (carbachol at 5 μM for 30 s, n=4, 0.403±0.102 Hz following the first application vs. 0.403±0.148 Hz following a repeated application, P>0.05, paired t test; NMDA at 10 μM for 45 s, n=3, 0.485±0.087 Hz vs. 0.507±0.141 Hz, P>0.05; AMPA at 1 μM for 30 s, n=3, 1.263±0.091 Hz vs. 1.216±0.065 Hz, P>0.05). The response to carbachol, NMDA or AMPA was measured. After full recovery, the PKC blocker chelerythrine was applied (20 μM for at least 20 min) and the response to carbachol, NMDA or AMPA was recorded in the presence of chelerythrine. All three ligands caused a significant and reversible increase in firing rates (0.52±0.65 Hz increased to 1.24±0.65 Hz for carbachol, 0.24±0.15 Hz increased to 1.31±0.42 Hz for NMDA, and 0.5±0.26 Hz increased to 1.38±0.38 Hz for AMPA). Chelerythrine itself caused a significant increased firing (n=16, from 0.27±0.09 Hz to 0.42±0.14 Hz, P<0.05, paired t test) and when this response stabilized, the three ligands were still able to further excite DA cells (0.8±0.27 Hz increased to 1.47±0.76 Hz for carbachol, 0.44±0.24 Hz increased to 1.5±0.46 Hz for NMDA, and 0.19±0.97 Hz increased to 1.46±0.51 Hz for AMPA). However, when relative changes before and after chelerythrine treatments were
compared, only excitation induced by carbachol (Figure 3.1A3) was significantly reduced by the PKC blocker chelerythrine (n=6, P<0.05, ANOVA for repeated measures). The effects of NMDA (Figure 3.1B3, n=5, P>0.05) and AMPA (Figure 3.1C3, n=5, P>0.05) were unaffected by PKC inhibition. Peak response to carbachol (Figure 3.1D) was decreased by 42.8±7.5% in the presence of chelerythrine (n=6, P<0.05, paired t test), the decrease was insignificant for NMDA (24.2±9.9%, n=6, P>0.05) and there was an increase in the peak response to AMPA which also did not reach significance (42.9±39.3%, n=5, P>0.05). Chelerythrine did not change the response time (Figure 3.1E) or time to peak for any of the three ligands.

To verify that chelerythrine decreased carbachol’s efficacy by inhibiting PKC, another PKC inhibitor that binds to a different site was used. Similar to chelerythrine, GF 109203X itself caused a significant increase in firing (n=13, from 0.52±0.18 Hz to 0.94±0.36 Hz, P<0.05, paired t test). GF 109203X applied at 2 μM for at least 20 min also reduced carbachol-induced excitation (0.65±0.29 increased to 1.16±0.4 Hz for carbachol alone, and 1.17±0.4 increased to 1.61±0.68 Hz for carbachol in the presence of GF 109203X). GF 109203X reduced both the overall response (n=7, P<0.05, ANOVA for repeated measures) and the peak response to carbachol (decreased by 33.7±0.10% in the presence of GF 109203X, n=7, P<0.05, paired t test).
3.3.2 L-type calcium channels are involved in carbachol- but not NMDA- or AMPA-induced excitation.

The L-type calcium channel is abundantly expressed in DA cells (Takada, et al., 2001) which can be recruited by cholinergic and glutamatergic agonists to increase excitability either by direct depolarization or by PKC-mediated phosphorylation. Similarly to the initial experiments, carbachol (5 μM for 30s), NMDA (10 μM for 45s) or AMPA (1 μM for 30s) was first applied and the response was allowed to recover; then the specific L-type calcium channel blocker nifedipine (5 μM) was applied for 8 min and the response to carbachol, NMDA or AMPA was recorded again in the presence of nifedipine. Nifedipine itself did not change firing rates significantly in the cells tested (n=20, peak response before and after application of nifedipine: 0.37±0.09 Hz vs. 0.44±0.11 Hz, P>0.05, paired t test). Figure 3.2 shows that the carbachol-induced increase (Figure 3.2A3) in firing rates was also significantly reduced by nifedipine (n=5, 0.5±0.23 increased to 1.0±0.35 Hz for carbachol alone vs. 0.52±0.23 increased to 0.76±0.34 Hz for carbachol in the presence of nifedipine, P<0.05, ANOVA for repeated measures), however, increased firing following NMDA (Figure 3.2B3, n=7, P>0.05) or AMPA (Figure 3.2C3, n=7, P>0.05) was unaffected by nifedipine (0.3±0.12 increased to 1.52±0.33 Hz for NMDA alone vs. 0.39±0.19 increased to 1.68±0.49 Hz for NMDA in the presence of nifedipine; 0.34±0.15 increased to 1.61±0.4 Hz for AMPA alone vs. 0.43±0.21 increased to 1.67±0.58 Hz for AMPA in the presence of nifedipine). Peak response (Figure 3.2D) to carbachol was significantly decreased by 34.7±9.4% in the
presence of nifedipine (n=5, P<0.05, paired t test), but peak responses to NMDA (decrease by 1.4±11.0%, n=8, P>0.05) or AMPA (decreased by 19.6±14.3%, n=7, P>0.05) were unchanged. Nifedipine did not change the response time (Figure 3.2E) or time to peak for any of the three ligands.

3.3.3 PKC and L-type calcium channel blockade are not additional in suppressing carbachol-induced excitation

Since both PKC and L-type calcium channels were involved in carbachol-induced excitation, I asked whether the two were related events of the same cascade. If PKC and L-type calcium channels were sequentially activated by carbachol, it would be expected that the combined application of both a PKC inhibitor and L-type calcium channel blocker would produce no more suppression than the same PKC inhibitor or L-type calcium channel blocker alone. Carbachol (5 μM for 30s) was first applied and the response was allowed to recover; then a cocktail containing 5 μM nifedipine and 20 μM chelerythrine was applied for 15 min and the response to carbachol was recorded again in the presence of the cocktail. The cocktail itself did not change the firing rates in the cells (n=6, 0.38±0.08 Hz vs. 0.20±0.08 Hz, P>0.05, paired t test). Carbachol increased firing from 0.38±0.08 Hz to 0.84±0.11 Hz and this increase was smaller in the presence of the cocktail (from 0.2±0.08 Hz to 0.34±0.1 Hz). Figure 3.3 shows that the carbachol-induced fold increase in firing was significantly reduced in the presence of the cocktail (Figure 3.3C, n=6, P<0.05, ANOVA for repeated measures). The peak response (Figure 3.3D)
was also significantly decreased by 41.0±13.2% in the presence of the cocktail (n=6, P<0.05, paired t test). The response time (Figure 3.3E) and time to peak were not significantly changed by combined application of the PKC inhibitor and L-type calcium channel blocker.

Relative increases in firing rates following carbachol combined with chelerythrine or nifedipine, or both as a percentage of increased rates induced by carbachol alone obtained in their respective control groups were plotted to assess whether chelerythrine, nifedipine or their combined application altered carbachol’s actions to the same extent. There were no significant differences among the three treatment groups: carbachol with chelerythrine (n=6), nifedipine (n=5) or both (n=6) (Figure 3.3F, P>0.05, ANOVA for repeated measures). Peak responses to carbachol in these three groups (Figure 3.3G) were not significantly different (P>0.05, one-way ANOVA).

3.3.4 Carbachol increases nifedipine-sensitive L-type calcium currents by activating PKC

Inhibiting PKC and blocking L-type calcium channels reduced carbachol-induced excitation to the same extent and combined application of both did not produce additive effects. This suggests that PKC activation and L-type calcium channel opening are sequential steps in the same cascade. To examine how L-type calcium currents are altered by carbachol and PKC inhibition, I isolated HVA Ca^{2+} currents which were blockable by
Cd$^{2+}$. Three parallel groups were set up to record HVA Ca$^{2+}$ currents continuously at 30 s intervals. The control group consisted of a 5 min baseline recording and 7 min in 10 μM nifedipine followed by 5 min in 100 μM Cd$^{2+}$. The carbachol group consisted of 5 min in 20 μM carbachol, 7 min in 10 μM nifedipine and 5 min in 100 μM Cd$^{2+}$. For PKC inhibition, slices were first incubated in 1 μM GF 109203X for at least 40 min, and were continuously perfused with 1 μM GF 109203X during all recordings which were the same as those of the carbachol group. All currents were normalized by subtracting residual currents after a 5-min Cd$^{2+}$ application. In 7 control cells, total HVA Ca$^{2+}$ currents were decreased by 5.3±2.0% in the first 5 min due to current run-down and nifedipine blocked 13.7±3.1% at the 12th minute. In eight carbachol-treated cells, total currents increased by 0.6±1.9% in the first 5 min and nifedipine blocked 25.3±4.3% of the currents. In the 9 cells with PKC inhibition, carbachol decreased the total currents by 3.5±4.8% in the first 5 min, and nifedipine blocked 9.5±2.3% of the currents. Carbachol induced a minimal increase in the currents just enough to overcome the run-down effects (Figure 3.4B, P>0.05, one-way ANOVA). However, carbachol significantly changed the nifedipine-sensitive portion of the currents (Figure 3.4C, P<0.05, one-way ANOVA). Post hoc tests (least significant difference method, LSD) showed that carbachol significantly increased the percentage of nifedipine-sensitive Ca$^{2+}$ currents (P<0.05) and PKC inhibition significantly blunted the carbachol-induced increase in nifedipine-sensitive Ca$^{2+}$ currents (P<0.05). There was no significant difference between the control and PKC inhibition groups (P>0.05).
3.4 Discussion

Firing behavior of DA cells in the VTA is related to DA transmission associated with normal or abnormal expression of motivation and reward processing. VTA-DA cell firing is modulated by synaptic activity (Kitai, et al., 1999). In this study, I found that carbachol, but not NMDA or AMPA, induced increased firing by activating PKC and L-type calcium channels. I further found that carbachol increased the nifedipine-sensitive portion of the HVA Ca\(^{2+}\) currents, an effect that was also blocked by PKC inhibition. My results indicate that cholinergic, but not glutamatergic, activation leads to a positive modulation of L-type calcium channels by PKC to mediate increased firing of DA cells in the VTA.

3.4.1 Carbachol activates PKC to up-regulate L-type calcium channels to induce excitation

My results demonstrate that carbachol-induced excitation of DA cells was dependent on PKC activation and L-type calcium channel opening. These two events affected carbachol’s effects to the same extent and they were not additive suggesting that they are sequential to one another. Carbachol has the capability of activating PKC and the L-type calcium channels through multiple pathways. It can activate PKC by an increase in intracellular Ca\(^{2+}\) by way of nAChR-mediated Ca\(^{2+}\) influx or mAChR-mediated Ca\(^{2+}\) release from internal Ca\(^{2+}\) stores. Additionally, mAChRs are coupled to phospholipase C
to generate lipid signals such as inositol (1, 4, 5) trisphosphate (IP$_3$) and DAG which activate PKC. At the same time, carbachol depolarizes the membrane, which can activate L-type calcium channels especially the Ca$_v$1.3 subtype that operates near resting membrane potentials. Increased spiking, as the result of this depolarization, will bring the majority of voltage-gated channel's including all types of Ca$^{2+}$ channels into play. The question is how carbachol activates the two events sequentially.

Depolarization and increased firing are unlikely the initial steps of this sequence of events because in my series NMDA and AMPA caused similar depolarization and increased spiking which could not be significantly reduced by PKC inhibition and L-type calcium channel blockade. This strengthens the other possibility that carbachol activates PKC which in turn upregulates the L-type calcium channel to increase spiking activity of the DA cells. PKC does phosphorylate both subtypes of the L-type calcium channel at multiple serine/threonine sites. It has been shown that phosphorylation of the Ca$_v$1.2 by PKC leads to positive modulation of the channel (Yang, et al., 2005) so that it becomes easier to open and conducts more Ca$^{2+}$ ions. PKC phosphorylation of the Ca$_v$1.3 subtype has been less intensely studied. Some report that PKC phosphorylation of the Ca$_v$1.3 subtype results in negative modulation of the channel (Chahine, et al., 2008), while others contend that only phosphorylation of the N-terminal produces this effect (Baroudi, et al., 2006). Positive modulation of L-type calcium channels by PKC has been reported in native tissues. For example, M$_2$ mAChR stimulation requires PKC to increase Ca$_v$1.2 currents in rabbit portal vein myocytes (Callaghan, et al., 2004) and M$_1$ receptor
activation involves PKC to mediate facilitation of L-type calcium channels in dissociated autonomic neurons from the major pelvic ganglion (Sculptoreanu, et al., 2001). PKC-mediated facilitation of L-type calcium channels has not been previously demonstrated in midbrain DA cells, so my results that PKC inhibitors reduced carbachol-induced increases in nifedipine-sensitive Ca\(^{2+}\) currents clearly show that such a modulation exists in DA cells.

3.4.2 NMDA- or AMPA-induced excitation does not activate PKC to excite DA cells.

NMDA receptor and AMPA receptors with GluR2 subunit composition are permeable to Ca\(^{2+}\) and they undoubtedly depolarize the membrane and increase the frequency of spiking which promotes Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. However, our results clearly show that both glutamatergic agonists did not cause a PKC-mediated facilitation of L-type calcium channels to bring about their excitatory actions. My previous study shows that NMDA increases the production of PKM (the persistently active catalytic subunit of PKC) in VTA slices (Liu, et al., 2007), a cascade that is required for converting pacemaking firing to bursting in DA cells. Why did PKC inhibition fail to alter NMDA-mediated excitation? The crucial difference between this paper and our previous results is that in this series I applied NMDA at a lower concentration (10 \(\mu\)M) for only a brief period (45 s) so that the response could be washed out completely within a relatively short time to retest the response in the presence of PKC inhibition. In our previous work, however, I used a much higher dose (20 \(\mu\)M) for a more
prolonged period of time (20 min) to maximize PKM expression. This argues against a role of PKM generation to mediate NMDA-induced firing at this particular concentration, however, it does not explain the lack of PKC participation in this process. I think that the discrepancy in PKC reliance following cholinergic and glutamatergic agonists might arise from the following possibilities.

Firstly, carbachol activates nAChRs and mAChRs with a highly probable signaling convergence on PKC as both receptors are known to increase Ca\(^{2+}\) and lipid signals that are activators of this kinase. While NMDA and to a lesser extent AMPA do increase intracellular Ca\(^{2+}\) concentrations, this might not be enough for maximal PKC activation because of the fact that Ca\(^{2+}\) and lipid second messengers activate PKC synergistically (Hug and Sarre, 1993). Secondly, glutamatergic transmission is mostly synaptic while cholinergic terminals utilize volume transmission (Pickel, et al., 2002). As a result, NMDA and AMPA receptors are clustered at synaptic sites in distal dendrites while cholinoreceptors are more widespread throughout the geometry of the cell mostly in the soma and dendrites (Gahring, et al., 2004; Klein and Yakel, 2006; Lena, et al., 1999; Severance and Cuevas, 2004). Since L-type calcium channels are more heavily expressed in the cell body and proximal dendrites (Hell, et al., 1993; Takada, et al., 2001), cholinoreceptors are physically closer to these channels to allow a more ready interaction. Thirdly, it has been shown that there exists a signaling scaffold complex of NMDA or AMPA receptors, PKC and calcineurin (Gomez, et al., 2002). Calcineurin dephosphorylates serine/threonine and is an important regulator of protein phosphorylation
in DA cells. It is also Ca\textsuperscript{2+}-dependent, requiring a lower Ca\textsuperscript{2+} concentration to activate than that required by PKC (Dash, et al., 2007). The net result following NMDA/AMPA receptor activation might be in favor of calcineurin. However, our results do not exclude the possibility of recruiting PKC by metabotropic glutamate receptors in the real glutamatergic transmission that can activate both ionotropic and metabotropic glutamate receptors. Furthermore, it might be argued that bath application of NMDA/AMPA activates all receptors on a cell whereas in real life, glutamatergic transmission is mostly carried by synaptic NMDA/AMPA receptors. Since PKC phosphorylation of NMDA/AMPA receptors affects their membrane trafficking dynamics by inserting more receptors at the synaptic site (Grosshans, et al., 2002), PKC might affect synaptic transmission more in behaving animals than in slices where drugs in the bath are allowed to diffuse to all receptors.

3.4.3 The significance of cholinocceptor-mediated PKC facilitation of L-type calcium channels

The VTA and its projections to the NAc and the prefrontal cortex are viewed as the final common pathway to all forms of addiction. Nicotine is arguably the most addictive substance abused on a large scale. There is solid evidence that nAChRs on synaptic terminals increase glutamate release, promote the formation of long-term potentiation at glutamatergic synapses and long-term depression at GABAergic synapses in the VTA (Kauer and Malenka, 2007), all of these leading to increased activity of DA cells.
However, it has also been known that the α1β2 nAChRs expressed in postsynaptic DA cells are implicated in regulating the firing activities of DA cells (Sorenson, et al., 1998) and behavioral sensitization (Tapper, et al., 2004). This paper shows that, besides synaptic modulation by nAChRs, nicotine can activate PKC in the postsynaptic cell that facilitates L-type calcium channel opening to underlie addictive behavior. Both PKC and L-type calcium channels have been shown to be implicated in addiction and motivation. For example, repeated administration of cocaine increases overall PKC activity in the VTA (Steketee, et al., 1998), while injection of PKC inhibitors into the VTA reduces cocaine-induced DA release in the NAc (Steketee, 1993) and delays the onset of behavioral sensitization (Steketee, 1994; 1997). Similarly, repeated amphetamine injections increase the expression of α1C L-type calcium channel subunits in the VTA (Rajadhyaksha, et al., 2004) and repeated stimulation of L-type calcium channels in the VTA mimics the initiation of behavioral sensitization to cocaine (Licata, et al., 2000), while direct injection of L-type calcium channel antagonists into the VTA attenuates the development of psychostimulant-induced behavioral sensitization (Licata, et al., 2004). These behavioral observations could possibly be explained by our findings in this paper that cholinergic activation leads to PKC activation and L-type calcium channel facilitation, which could drive up the DA firing that is required for behavioral sensitization.
Figure 3.1 PKC mediates carbachol- but not NMDA- and AMPA-induced excitation.

A1-C1. Current clamp recording from representative cells showing carbachol- (A1, 5 μM), NMDA- (B1, 10 μM) or AMPA-induced (C1, 1 μM) increases firing rates in control conditions and in the presence of the PKC blocker chelerythrine (20 μM). A2-C2. Relative changes in instantaneous firing frequencies following carbachol (A2), NMDA (B2) or AMPA (C2) from the same cells as in A1-C1 with (open) or without (filled) chelerythrine. A3-C3. Percentage changes in firing rates by carbachol (A3, n=6), NMDA (B3, n=5) or AMPA (C3, n=5) with (open) or without (filled) chelerythrine. D. Peak responses to carbachol (Car), NMDA or AMPA with chelerythrine (Che) relative to carbachol, NMDA or AMPA alone. E. Response time to carbachol (Car), NMDA or AMPA with chelerythrine (Che) relative to carbachol, NMDA or AMPA alone.
Figure 3.2 L-type Ca\(^{2+}\) channels are involved in carbachol- but not NMDA- or AMPA-induced excitation. A1-C1. Current clamp recording from representative cells showing carbachol- (A1, 5 μM), NMDA- (B1, 10 μM) or AMPA-induced (C1, 1 μM) increases in firing rates in control conditions and in the presence of the L-type Ca\(^{2+}\) channel blocker nifedipine (5 μM). A2-C2. Relative changes in instantaneous firing frequencies by carbachol (A2), NMDA (B2) or AMPA (C2) from the same cells as in A1-C1 with (open) or without (filled) nifedipine. A3-C3. Percentage changes in firing rates by carbachol (A3, n=5), NMDA (B3, n=7) or AMPA (C3, n=7) with (open) or without (filled) nifedipine. D. Peak responses to carbachol (Car), NMDA or AMPA with nifedipine (Nif) relative to carbachol, NMDA or AMPA alone. E. Response time to carbachol (Car), NMDA or AMPA with nifedipine (Nif) relative to carbachol, NMDA or AMPA alone.
Figure 3.3 PKC and L-type channel blockade are not additional in suppressing carbachol-induced excitation. A. Current clamp recording from a representative cell showing a carbachol-induced (5 μM) increase in firing rates in control conditions and in the presence of a cocktail containing both a PKC inhibitor (chelerythrine, Che, 20 μM) and an L-type Ca²⁺ channel blocker (nifedipine, Nif, 5 μM). B. Relative changes in instantaneous firing frequencies by carbachol from the same cell as in A with (open) or without (filled) the cocktail. C. Percentage changes in firing rates by carbachol (n=6) with (open) or without (filled) the cocktail. D. Peak response to carbachol (Car) with the cocktail relative to carbachol alone. E. Response time to carbachol with the cocktail relative to carbachol alone. F. Relative increases in firing rates following carbachol (Car) combined with chelerythrine (Che, n=6), nifedipine (Nif, n=5) or both (n=6) as a percentage of increased rates induced by carbachol alone obtained in their respective control groups were plotted to compare whether chelerythrine, nifedipine or their combined application altered carbachol’s actions to the same extent. G. Peak responses of the three groups in F.
Figure 3.4 Carbachol increases nifedipine-sensitive L-type Ca\(^{2+}\) currents by activating PKC. A. Currents evoked in a representative neuron by stepping the membrane potential from -60 mV to 0 mV for 150 ms under voltage-clamp conditions. B. Changes in total HVA currents as a percentage of the initial current magnitude in control (n=7), carbachol (Car, 20 µM) alone (n=8) or carbachol in the presence of the PKC inhibitor GF 109203X (Bis, 1 µM, n=9). Insets are HVA current traces at the beginning of recording (grey line) and 5 min after (black line) treatment. C. Percentage of nifedipine-sensitive currents for these three groups. Insets are HVA current traces before (grey line) and after nifedipine (black line, 10 µM) application in treated groups shown at the bottom.
Chapter 4  Ca,1,3 L-type calcium Channels Mediate Single Spike and Burst Firing of VTA DA Neurons in Mice

4.1 Introduction

As detailed in Chapter 1, DA neurons in the VTA play an important role in cognition and reward processing (Cooper, 2002; Ikemoto, 2007), and abnormalities in these processes are implicated in such disease states as schizophrenia and drug addiction (Kelley, 2004; Koob, 2000; Seamans and Yang, 2004). DA neurons display two major firing patterns: single spike and burst firing (Grace and Bunney, 1984a,b; Zhang, et al., 2005). The two firing modes have been shown to be involved in different functions: pace-making spiking encodes expected stimulations while burst firing signals unexpected environmental stimuli with reward value related to reward-based learning (Schultz, 1997; 1998; Tobler, et al., 2005). Therefore, it is crucial to better understand the transition and regulation of the two firing modes in DA neurons.

\[ \text{Ca}^{2+} \text{ influx through L-type calcium channels is an important modulator of neuronal firing activities including DA neurons in the VTA. L-type calcium channels are responsible for approximately one third of total Ca}^{2+} \text{ currents (Cardozo and Bean, 1995; Durante, et al., 2004) and contribute preferentially to whole-cell Ca}^{2+} \text{ currents evoked by small depolarizations (Durante, et al., 2004; Xu and Lipscombe, 2001) in DA cells. They have been shown to be involved in spontaneous firing, spontaneous} \]
oscillatory potentials and cholinergic-driven firing increases that are abolished by DHP antagonists (Mercuri, et al., 1994; Nedergaard, et al., 1993; Zhang, et al., 2005). Also, they are involved in the regulation of burst firing. For example, the cholinergic-driven bursting and associated membrane potential oscillations are blocked by the L-type calcium channel blocker nifedipine (Zhang, et al., 2005). Furthermore, direct activation of L-type calcium channels with two different activators, (S)-(−)-Bay K8644 and FPL 64176, induces burst firing in DA neurons (Liu, et al., 2007; Zhang, et al., 2005).

Four subtypes of L-type calcium channels have been identified: Ca\textsubscript{v}1.1, Ca\textsubscript{v}1.2, Ca\textsubscript{v}1.3 and Ca\textsubscript{v}1.4; only Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subtypes are expressed in the midbrain (Takada, et al., 2001). It has been shown from immunohistochemical experiments that Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 L-type calcium channels are distributed throughout the entire midbrain, including DA neurons (Takada, et al., 2001). Takada et al (Takada, et al., 2001) indicates that DA neurons display more intense immunoreactivity against Ca\textsubscript{v}1.3 than Ca\textsubscript{v}1.2 L-channels, but data from our lab (unpublished by Tara Deemayd), using the same method as what Takada used, did not show a dominant expression of Ca\textsubscript{v}1.3 L-type calcium channels in TH-positive midbrain neurons. As detailed in Chapter 1, Ca\textsubscript{v}1.3 L-type calcium channels display atypical properties of L-type calcium channels compared to the Ca\textsubscript{v}1.2 subtype: They have a low activation threshold and a reduced sensitivity to DHP agonists and antagonists (Durante, et al., 2004; Xu and Lipscombe, 2001). The role of L-type calcium channels has been clearly explored but because current pharmacological agents can not isolate Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subtypes, their specific
contribution to functions mediated by L-type calcium channels is still unknown. This chapter reports work aimed to determine the contribution of the two L-type calcium channel subtypes to the firing activities of DA neurons in the VTA by using a knock-in mouse strain carrying a mutant DHP site in the Ca$_v$1.2 subtype.

4.2 Methods

4.2.1 Animals

Wild-type (WT) mice (C57BL/6) (2-9 months old) and transgenic mice carrying a mutant DHP site in $\alpha$1-subunit of Ca$_v$1.2 L-type calcium channels (Ca$_v$1.2DHP$^{+/}$, with C57BL/6 genetic background) were used for this study. Point mutation of the DHP site dramatically reduces the sensitivity of Ca$_v$1.2 L-type calcium channels to DHP modulators (Striessnig, et al., 2006; Zhang, et al., 2007). Animal housing was the same as that described in Chapter two. Congenic knock-in mice were first bred from Ca$_v$1.2DHP$^{+/}$-Ca$_v$1.2DHP$^{+/}$ pairs and maintained with Ca$_v$1.2DHP$^{+/}$-Ca$_v$1.2DHP$^{+/}$ breeding pairs. Mice were ear-tagged and weaned when they were 21 days old and a segment of tail (about 1 cm) was cut for genotyping. Bleeding was generally minor and could be readily stopped by silver nitrate.
4.2.2 Genotyping of the offspring

Mouse tail tips were placed in 1.5 mL microfuge tubes which were filled with 600 μL Lysis buffer/ Proteinase K solution (composition: 99 mM Tris-HCl pH 8.0, 4.95 mM EDTA pH 8.0, 0.198% SDS, 0.198 M NaCl, 0.02% proteinase K). They were left on a rotator at 50 °C overnight and then centrifuged at 15,000 g for 30 min. The aqueous phase was decanted into a new tube, followed by adding 1 mL 100% ethanol and shaking gently to precipitate DNA. The tube was held at an angle, the supernatant gently removed, and the DNA was air-dried. 100 or 150 μL TE pH 8.0 (including 10 mM Tris-HCl and 1 mM EDTA) was added to the air-dried DNA, which was then dissolved at 70 °C for 3-4 hr.

PCR mix was prepared as follows: 1x Taq Buffer (Invitrogen, USA), 0.2 μM dNTP’s, 0.2 μM Loxup2 primer (5’-TCCTGCACCTTAGGATAAGATGCAAGGC-3’) (Fisher Scientific, PA, USA), 0.2 μM Screen1 primer (5’-GAACATGAACTGCAGAGTGTA-3’) (Fisher Scientific, PA, USA), 0.5 μL Taq polymerase (Invitrogen, USA) and 5 μL DNA in a final total volume of 30 μL. PCR samples were run as follows: first at 94 °C for 5 min, followed by 30 sec at 94 °C, 30 sec at 64 °C and 30 sec at 72 °C for 30 cycles and finally held at 4 °C.
DNA samples from the PCR reaction were prepared by adding 1x DNA loading buffer to each reaction tube. 20 µl of which was loaded into a 2.5% agarose gel and was electrophoresed at 134 mV for 30 min. DNA bands in the gel were photographed under UV light. WT yields a fragment of 390 bp. Ca,1.2DHP<sup>-4</sup> gives a fragment of 475 bp and Ca,1.2DHP<sup>4</sup> has both fragments (Figure 4.1).

4.2.3 Slice preparation

The slice preparation was the same as that described in 3.2.1.

4.2.4 Nystatin-perforated patch clamp recording

This part was the same as that described in 2.2.2.

4.2.5 Drugs

This part was the same as that described in 2.2.2.
4.2.6 Data analysis

This part was the same as that described in 2.2.4 and 3.2.4.

4.3 Results

Only DA cells in the VTA identified according to criteria outlined in the Methods section were included.

4.3.1 DA cells of Ca,1.2DHP<sup>−/−</sup> and WT mice have similar basic electrophysiological properties (Table 1)

In the WT group, most cells (15 of 27, 55.6%) were spontaneously active with single spike firing at a low basal firing frequency of 0.72±0.13 Hz; the remainder (12 of 27, 44.4%) was quiescent during baseline recording. Similarly, the majority of cells (27 of 44, 61.4%) in Ca,1.2DHP<sup>−/−</sup> group showed pacemaker firing during baseline recording with a similar low frequency of 0.76±0.13 Hz (unpaired t test compared to that of WT group, P<0.05); 38.6% of cells (17 of 44) did not spontaneously fire. The resting membrane potentials (RMPs) were similar between the two groups (+53.94±1.07 mV for
WT mice vs. -52.76±1.13 mV for Ca\textsubscript{v}1.2DHP\textsuperscript{(-/-)} mice, unpaired t test, P>0.05). There was an apparent difference in RMPs of spiking and quiescent cells in both strains. In the WT mice, the RMP of spiking cells (-49.65±0.67 mV, n=15) was more depolarized than that of quiescent cells (-56.66±1.88 mV, n=12, unpaired t test, P<0.001). The same pattern was observed in Ca\textsubscript{v}1.2DHP\textsuperscript{(-/-)} mice (-50.67±0.91 mV for spiking cells, n=27, vs. -58.56±1.72 mV for quiescent cells, n=17, unpaired t test, P<0.001). There were no differences in RMPs of spiking cells (P>0.05) or quiescent cells (P>0.05) between wild type and congenic knock-in groups. In terms of spiking cells, there were no significant differences in spike amplitude (58.57±2.64 mV in WT mice vs. 57.75±2.17 mV in Ca\textsubscript{v}1.2DHP\textsuperscript{(-/-)} mice, unpaired t test, P>0.05), threshold (-38.91±0.84 mV in WT mice vs. -39.16±0.97 mV in Ca\textsubscript{v}1.2DHP\textsuperscript{(-/-)} mice, unpaired t test, P>0.05), half-width (2.79±0.14 ms in WT mice vs. 3.05±0.19 ms in Ca\textsubscript{v}1.2DHP\textsuperscript{(-/-)} mice, unpaired t test, P>0.05), or hyperpolarization after-potentials (-13.14±1.66 mV in WT mice vs. -13.77±1.32 mV in Ca\textsubscript{v}1.2DHP\textsuperscript{(-/-)}, unpaired t test, P>0.05).

In addition, the average hyperpolarization following a brief application of 50 μM DA (within 90 sec) between the WT (-7.66±1.03 mV) and Ca\textsubscript{v}1.2DHP\textsuperscript{(-/-)} mice (-9.68±1.00 mV) was not significantly different (unpaired t test, P>0.05). When current injections were adjusted in each cell to result in a peak hyperpolarization of about -110 mV (Figure 1.5), the I\textsubscript{h} sags between the WT mice (21.15±1.17 mV) and Ca\textsubscript{v}1.2DHP\textsuperscript{(-/-)} (19.29±0.92 mV) were not significantly different (unpaired t test, P>0.05).
4.3.2 The DHP site L-type calcium channel antagonist reduces single spike firing in both WT and Ca,1.2DHP<sup>−/−</sup> mice

As mentioned in section 4.1, only Ca,1.2 and Ca,1.3 subtypes are expressed in VTA DA neurons, both of which are responsive to DHP site agonists and antagonists although Ca,1.3 channels show a relative low responsiveness. As a result, it is difficult to identify the specific contribution of these L-type calcium channel subtypes to physiological functions. Ca,1.2DHP<sup>−/−</sup> has a mutant DHP site on the α1 subunit of Ca,1.2 L-type calcium channels, which renders DHP modulators practically ineffective thereby providing a good tool to study the role of specific subtypes.

Bath application of 10 μM nifedipine, a DHP site L-type calcium channel antagonist, for 10 min in WT mice decreased baseline firing rate by 20.3 ± 8.1% from 0.85 ± 0.17 Hz to 0.70 ± 0.21 Hz (n = 5, paired t test, P < 0.05). Nifedipine is a lipophilic drug which is not easy to wash out, so the firing rate kept dropping by 39.1 ± 6.3% to 0.58 ± 0.18 Hz after washing out nifedipine for 7 min (paired t test, P < 0.05) (Figure 4.2.1). Nifedipine blocks both Ca,1.2 and Ca,1.3 subtypes in the wild type, this result only underscores the importance of L-type calcium channels in basal firing without any clue as to the individual contributions from the two subtypes.

Determining which of the subtypes drives DA basal firing could be tested in Ca,1.2DHP<sup>−/−</sup> mice because both are expressed in DA cells and the DHP site is not
functional in Ca$_v$1.2DHP$^{(-/-)}$ mice. Therefore, basal firing blocked by nifedipine represents the contribution from the Ca$_v$1.3 subtype. In Ca$_v$1.2DHP$^{(-/-)}$ mice, bath application of 10 µM nifedipine for 10 min reduced firing rates by 24.9±7.7% from 0.77±0.21 Hz to 0.63±0.20 Hz (n=5, paired t test, P<0.05) (Figure 4.2.2). A peak reduction of 34.0±9.2% to 0.57±0.18 Hz was recorded after washing out nifedipine for 7 min (paired t test, P<0.05). There was no significant difference in the reduction of basal firing by nifedipine between WT and Ca$_v$1.2DHP$^{(-/-)}$ mice (unpaired t test, P>0.05) (Figure 4.2.3), clearly indicating that the Ca$_v$1.3 subtype drives basal firing in VTA DA cells.

4.3.3 L-type calcium channel agonists convert single spiking to bursting in the mice

Burst firing is an important firing property of DA cells as detailed in Chapter 1. It has been shown that L-type calcium channel openers can induce firing pattern conversion in rats, so first of all, I would need to confirm that this happens in the WT mice as well before I could use the L-type transgenic strains. Bath application of 5 µM (S)-(−)-Bay K8644, a DHP site L-type calcium channel activator, for 7-11 min converted firing patterns from the quiescent state or single spiking to burst firing in 4 of 6 treated cells in WT mice (Figure 4.3.1), the same that I reported in rats. The percentage of converted burst firing in spontaneously firing cells (3 of 3) was much higher than that in quiescent cells (1 of 3).
In cells that were spontaneously firing, (S)-(−)-Bay K8644 first induced a membrane depolarization accompanied by an increase in firing rate. Burst firing started after a varying period of latency. The lag between the start of drug application and burst firing ranged 9-33 min with an average time of 16 min. Within a burst firing cycle, action potentials fired with increasing frequencies followed by a pronounced post-burst hyperpolarization. The average intra-burst firing frequency was much higher than basal tonic frequency. The development of burst firing in quiescent cells was slightly different: they responded to (S)-(−)-Bay K8644 with a membrane depolarization followed by irregular single spiking that evolved into burst firing. The L-type calcium channel-mediated burst firing was long lasting even after prolonged washout (>30 min), however, it was readily inhibited by the L-type calcium channel blocker nifedipine (10 μM for 10 min, n=3, Figure 4.3.1). Density plots of ISIs in 2-second bins (Figure 4.3.2) showed an ISI distribution: the leftward shift of the main peak indicates higher firing frequencies and the second peak at much lower frequency represents the long pauses of firing between adjacent bursts.

Similarly, bath application of another L-channel opener FPL 64176 1-4 μM, a benzoylpyrole but not DHP site L-channel opener, for 8-24 min converted firing patterns from quiescent state or single spiking to burst firing in 7 of 11 treated cells (Figure 4.3.3), the same as in rats. Differently, however, application of 1 μM FPL 64176 did not induce any burst firing in 2 treated cells and 2 μM only induced bursting in 1 of 7 treated cells. The responses were dose-dependent: 4 μM FPL 64176 induced burst firing in 5 of 6 cells.
that did not respond to 1 or 2 μM. And the percentage of burst firing in spontaneously firing cells (5 of 6) was much larger than that in quiescent cells (2 of 5). The time course was similar to that following (S)-(−)-Bay K8644, a depolarization and an increase in firing rates followed by a conversion of firing patterns 8-42 minutes (average: 25 min) after the start of application. The burst firing was long lasting, which could be transferred to single spike firing or totally blocked to no firing by the DHP site L-type calcium channel antagonist nifedipine (10 μM, 5-10 min, n=3).

4.3.4 The DHP site L-type calcium channel agonist converts firing patterns in Ca₄1.2DHP⁽⁻⁾ mice

For identifying the role of different subtypes of L-channels in burst firing, Ca₄1.2DHP⁽⁻⁾ mice were used, in which only Ca₄1.3 but not Ca₄1.2 L-type calcium channels are responsive to DHP modulators. Bath application of 5 μM (S)-(−)-Bay K8644 for 8-20 min induced burst firing in 4 of 9 cells tested in nine Ca₄1.2DHP⁽⁻⁾ mice (Figure 4.4.1, 4.4.2), similar to what it did in WT mice. The time course of the response was also similar to that in WT mice, a depolarization and an increase in firing rates followed by a conversion of firing patterns, except that the lag between the start of drug application and burst firing was longer (range 27-40 min with the average time of 31 min). Percentage of converted burst firing in spontaneously firing cells (3 of 6) was also much higher than that in quiescent cells (1 of 3). Burst firing in 3 spontaneously firing cells was long lasting, the same as in WT mice, but only existed for 6 min in the quiescent cell.
Nifedipine (10 μM for 10 min), just as it did in WT mice, converted burst firing induced by the DHP site L-type calcium channel opener into single spike firing (n=3) (Figure 4.3.3).

**4.3.5 DHP site L-type calcium channel antagonist inhibits burst firing induced by a non-DHP site L-type calcium channel agonist in Ca$_v$1.2DHP$^{(-/-)}$ mice**

Similarly to what it did in WT mice, bath application of 2-4 μM FPL 64176 for 13-24 min also converted firing patterns from the quiescent state or single spiking to burst firing in 7 of 14 treated cells in Ca$_v$1.2DHP$^{(-/-)}$ mice (Figure 4.5). The responses were dose-dependent as well: 4 μM FPL 64176 induced burst firing in 3 of 6 cells that did not respond to 2 μM, and the percentage of burst firing in spontaneously firing cells (6 of 10) was much higher than that in quiescent cells (1 of 4). The time course was similar to that in WT mice, a depolarization and an increase in firing rates followed by a conversion of firing patterns 13-42 minutes (average: 21 min) after the start of application. The induced burst firing was long lasting except one in the quiescent cell which lasted for only 9 min. Nifedipine (10 μM for 5-13 min), a DHP site L-channel antagonist, also inhibited the burst firing: being converted back into single spike firing (n=4) or into protracted and slow bursting resembling irregular firing (n=2).
4.4 Discussion

The firing behavior of DA cells in the VTA is related to DA transmission associated with normal or abnormal expression of motivation and reward processing. L-type calcium channels play important roles in regulating VTA-DA cell firing, however, which subtype is involved is unclear. In this study, I used a transgenic mouse strain that lacks DHP functionality to differentiate L-type calcium channel subtypes in regulating burst firing of DA cells in the VTA. Our results indicate that Ca\textsubscript{v}1.3 L-type calcium channels are crucial to regulating single spiking and burst firing in DA cells.

4.4.1 Ca\textsubscript{v}1.3 L-type calcium channels play a major role in regulating firing properties

There are four subtypes of L-type calcium channels, although only Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subtypes are expressed in DA cells (Chan, et al., 2007; Takada, et al., 2001). L-type calcium channels are often assumed to be high voltage-activated (e.g. activate between -40\text{--}20 \text{mV} membrane potentials) (Ertel, et al., 2000) and Ca\textsubscript{v}1.2 L-type calcium channels display this typical voltage dependency (Mori, et al., 1993). However, Ca\textsubscript{v}1.3 L-type calcium channels activate at relatively hyperpolarized membrane potentials (between -60\text{--}40 \text{mV}) and are incompletely inhibited by DHP antagonists (Xu and Lipscombe, 2001). The large difference in activation thresholds between these two subtypes implies that they may be coupled to different molecular signaling pathways.
(Zhang, et al., 2006) so as to mediate different physiological roles in neuronal electrical tasks. For example, Ca,1.3 L-type calcium channels activate at relatively hyperpolarized membrane potentials (around -50 or -55 mV) (Xu and Lipscombe, 2001), which is near resting membrane potential, so Ca,1.3 channels are likely to be important in mediating Ca\(^{2+}\) influx in response to relatively small membrane depolarization. Such properties may be important for sustaining spontaneous rhythmic firing in neurons, traditionally attributed to the activities of low-threshold T-type Ca\(^{2+}\) channels (Bean, 1989; Ertel, et al., 2000). Consistent with this, the DHP antagonist nimodipine partially suppresses spontaneous intracellular calcium oscillations and slows rhythmic firing in postnatal cerebellar Purkinje cells (Liljelund, et al., 2000). More direct evidence for the involvement of Ca,1.3 channels in driving rhythmic activities in excitable cells comes from a study of Ca,1.3-deficient mice whose phenotype includes compromised sinoatrial node function (Platzer, et al., 2000). In line with this, our study shows that Ca,1.3 channels mediated rhythmic firing in DA neurons in the VTA because compared with wild type mice, the DHP antagonist nifedipine slowed spontaneous rhythmic firing to a similar extent in Ca,1.2 knock-in mice, in which Ca,1.2 channels were not sensitive to DHP antagonists and only Ca,1.3 channels were inhibited by the DHP antagonist nifedipine. It is consistent with the finding that Ca,1.3 channels mediate the spontaneous firing activities of DA neurons in the substantia nigra of adult mice (Chan, et al., 2007).

Different from rhythmic firing, burst firing is a combination of subthreshold depolarization and clustered action potential firing, which is likely to be mediated by both
Ca, 1.3 (activated at relatively hyperpolarized membrane potentials and assumed to mediate the underlying subthreshold depolarization) (Xu and Lipscombe, 2001) and Ca, 1.2 (high-voltage activated and hypothesized to be involved in the clustered fast firing) (Mori, et al., 1993). It is reasonable to presume that Ca, 1.3 L-type calcium channels mediate the subthreshold depolarization that raises the membrane potential to activate sodium channels initiating firing and then Ca, 1.2 L-type calcium channels take over to maintain sequential firing. Calcium influx precipitates a yet unknown calcium-dependent process to generate a post-burst hyperpolarization which completes a bursting cycle. However, my results support the hypothesis that only Ca, 1.3 L-type calcium channels are involved in bursting because: 1) activating both Ca, 1.2 and Ca, 1.3 subtypes ((S)-(−)-Bay K8644 in wild type slices) induced burst firing similarly to activating Ca, 1.3 subtype alone ((S)-(−)-Bay K8644 in Ca, 1.2DHP(−/−) slices); 2) Bay K-induced burst firing could be blocked by nifedipine in both wild type slices (effective for both subtypes) and Ca, 1.2DHP(−/−) slices (effective only for Ca, 1.3); 3) activating both subtypes with FPL 64176 induced similar burst firing which could be blocked to the same extent with nifedipine (which blocks Ca, 1.2 and Ca, 1.3 in wild type slices and Ca, 1.3 only in Ca, 1.2DHP(−/+) slices). These results collectively demonstrate that Ca, 1.3 L-type calcium channels are obligatory for burst firing. Ca, 1.2 L-type calcium channels are not necessary for burst firing but might aid it in some way because our data showed that the latency to bursting (lag time) was slightly longer in Ca, 1.2DHP(−/+) slices than in WT ones, and in a few cases induced bursting did not last as long. The mechanism for this might be related to contributions to the hump potential, which is already shown to be
mediated by L-type calcium channels (Zhang, et al., 2005), similar to the plateau potential underlying bi-stable membrane behavior in motoneurons in which Ca,1.3 L-type calcium channels are involved (Carlin, et al., 2000; Hsiao, et al., 1998).

4.4.2 Ca,1.3 L-type calcium channels are functionally important

Ca,1.3 L-type calcium channels are one of the four subtypes of L-type calcium channels, but due to technical difficulties in pharmacologically isolating Ca,1.3 from Ca,1.2 L-type calcium channels, both of which are abundantly expressed in neurons, the specific functional roles of Ca,1.3 are still obscure. With the development of transgenic animals, Ca,1.3 L-type calcium channels have been found to be implicated in addiction and other diseases. For example, Ca,1.3-deficient mice lack amphetamine-sensitized locomotor activity (Giordano, et al., 2006) and exhibit an antidepressant-like phenotype (Striessnig, et al., 2006). In addition, the expression of Ca,1.3 L-type calcium channels is decreased by chronic morphine treatment in midbrain regions of mice (Haller, et al., 2008). More importantly, Ca,1.3 L-channels can achieve all these behaviors by its role in regulating pacemaker and burst firing as reported here because fast pacemaker and burst firing elevate terminal DA levels more efficiently and all of these behaviors involve increased DA transmission. Ca,1.3 L-type calcium channels play a crucial role in maintaining rhythmic pacemaking firing in adult substantial nigra neurons, and blocking Ca,1.3 L-type calcium channels in adult neurons reverts them to a juvenile form of pacemaking firing dependent on Na+ channels and protects these neurons in a model of
Parkinson's disease (Chan, et al., 2007). Moreover, addiction is a learned behavior and Ca,1.3 L-channels are also shown to facilitate learning and memory such as in mediating the consolidation of contextually conditioned fear in mice (McKinney and Murphy, 2006). Taken together, Ca,1.3 L-type calcium channels might play a significant role in central DA transmission and its related pathologies such as drug abuse. Further experiments on the role of L-type calcium channel subtypes are now possible through the use of these L-type specific transgenic mouse strains.
Figure 4.1. Genotyping PCRs show that Ca,1.2DHP\textsuperscript{+/+} mice yield a band of 390 bp and Ca,1.2DHP\textsuperscript{-/-} has a band of 475 bp.
Figure 4.2.1 The DHP site L-type calcium channel antagonist nifedipine decreases basal firing rate in Ca,1.2DHP<sup>(m)</sup> mice. A. Current clamp recording from a representative cell showing firing rates in control, nifedipine (10 μM for 10 min) and after washing out nifedipine for 7 min. B. Changes in instantaneous firing frequencies following nifedipine from the same cells as in A.
Figure 4.2.2 The DHP site L-type calcium channel antagonist nifedipine decreases basal firing rate in Ca_{1.2}DHP^{(-)} mice. A. Current clamp recording from a representative cell showing firing rates in control, nifedipine (10 μM for 10 min) and after washing out nifedipine for 7 min. B. Changes in instantaneous firing frequencies following nifedipine from the same cells as in A.
Figure 4.2.3 Percentage changes in firing rate reductions following nifedipine (10μM for 10 min, gray) and 7 min after washing out nifedipine (white) compared with control (black) in Ca,1.2DHP<sup>(−/−)</sup> and Ca,1.2DHP<sup>(+/+)</sup> mice. (* means P<0.05)
Figure 4.3.1 Continuous current clamp recording from a representative cell showing that (S)-(-)-Bay K8644 (5 μM) converted regular firing to burst firing which could be blocked by nifedipine (10 μM) in Ca,1.2DHP<sup>(−)</sup> mice.
Figure 4.3.2 Density plot of interspike intervals (ISI) in 2-second bins in control conditions (210 events) and following (S)-(−)-Bay K8644 application (547 events) in Ca,1.2DHP<sup>(+/−)</sup> mice. (S)-(−)-Bay K8644 dramatically shifted the peak to the left and gave rise to a secondary peak corresponding to the frequency of burst firing cycles.
Figure 4.3.3 Continuous current clamp recording from a representative cell showing that FPL 64176 (4 µM) converted regular firing to burst firing which could be blocked by nifedipine (10 µM) in Ca,1.2DHP<sup>(+/−)</sup> mice.
Figure 4.4.1 Continuous current clamp recording from a representative cell showing that (S)-(−)-Bay K8644 (5 μM) converted regular firing to burst firing which could be blocked by nifedipine (10 μM) in Ca_{1.2}DHP^{(−)} mice.
Fig 4.4.2 Density plot of interspike intervals (ISI) in 0.5- (<2 sec) or 2-second (≥2 sec) bins in control conditions (859 events) and following (S)-(−)-Bay K8644 application (585 events) in Ca_1.2DHP<sup>+/−</sup> mice. (S)-(−)-Bay K8644 dramatically shifted the primary peak to the left and gave rise to a secondary peak corresponding to the frequency of burst firing cycles.
Figure 4.5 Continuous current clamp recording from a representative cell showing that FPL 64176 (4 μM) converted regular firing to burst firing which could be blocked by nifedipine (10 μM) in Ca,1.2DHP<sup>64</sup> mice.
Table 1
the basic electrophysiological properties in dopaminergic cells between Ca\textsubscript{v}1.2DHP\textsuperscript{+/+} and Ca\textsubscript{v}1.2DHP\textsuperscript{/-} mice

<table>
<thead>
<tr>
<th>Property</th>
<th>Ca\textsubscript{v}1.2DHP\textsuperscript{+/+} (WT)</th>
<th>Ca\textsubscript{v}1.2DHP\textsuperscript{/-}</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of spontaneously active cells</td>
<td>15 of 27, 55.6%</td>
<td>27 of 44, 61.4%</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>basal firing frequencies (Hz)</td>
<td>0.72±0.13</td>
<td>0.76±0.13</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>resting membrane potentials (mV)</td>
<td>-53.94±1.07</td>
<td>-52.76±1.13</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>spiking amplitude (mV)</td>
<td>58.57±2.64</td>
<td>57.75±2.17</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>spiking threshold (mV)</td>
<td>-38.91±0.84</td>
<td>-39.16±0.97</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>spiking half-width (ms)</td>
<td>2.79±0.14</td>
<td>3.05±0.19</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>hyperpolarization after-potentials (mV)</td>
<td>-13.14±1.66</td>
<td>-13.77±1.32</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>hyperpolarization following an application of 50 μM dopamine (mV)</td>
<td>-7.66±1.03</td>
<td>-9.68±1.00</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>$I_h$ sag (mV)</td>
<td>21.15±1.17</td>
<td>19.29±0.92</td>
<td>$&gt;0.05$</td>
</tr>
</tbody>
</table>
5.1 Technical considerations

Work presented in this thesis was designed to study the electrophysiological properties of DA neurons, especially their firing activities including bursting and pacemaker single spiking. Neuronal firing in slice preparations can be affected by a number of technical factors, such as the age of animals and the temperature at which all patch clamp recordings were made. This part summarizes the technical factors related to my thesis work.

5.1.1 Age of animals and DA firing activity

The age of animals has been considered as an important factor involving molecular signals and electrical activities. During development, the DA system undergoes important changes. For example, DA innervations to the forebrain and DA receptors in the NAc and striatum increase rapidly from birth to reach a peak around 40 days old in rodents, and decrease gradually thereafter (Andersen, et al., 1997; Tarazi and Baldessarini, 2000). Similarly, DA levels in the striatum also increase from birth to adolescence in rodents.
Concerning L-type calcium channels, age effects exist as well. For instance, after the second postnatal week in mice, the contribution of Ca\(_{v1.3}\) L-channels to somatodendritic L-channel currents increases and at P28, the percentage of Ca\(_{v1.3}\) L-channel currents increases by about 10\% compared with that at P14-17 (Chan, et al., 2007). There is no reference indicating at what age these developing channels stabilized. Whereas currents attributable to Ca\(_{v1.2}\) L-channel remain largely stable (Chan, et al., 2007). Given that this thesis work is mostly concerned with the firing behavior of DA cells in acute brain slices, it would be important to explore whether these age-related changes in the development of the central DA system as well as in the expression of L-type calcium channels have any significant influence on DA firing.

DA neurons have been shown in an in vivo study to display progressive increases in single spiking rates and in burst firing occurrence from birth to early adolescence (28-35 days old) (Pitts, et al., 1990; Lepper, et al., 1990), when autoreceptors in the midbrain appear to be functionally mature (Wang and Pitts, 1995). Firing activities are higher during mid adolescence (35-42 days old) and decline progressively during adulthood (75-90 days old) (Freeman, et al., 1989; Lavin and Drucker-Colin, 1991). As introduced in Chapter 1, DA neurons in acute slice preparations have very different firing patterns in that they barely display burst firing spontaneously even with chemical stimulations. However, Mereu reported that DA neurons in brain slices from immature rats (15-21 days
old) exhibited not only pacemaker firing but also irregular and burst firing pattern (28.3 and 18.3% respectively) (Mereu, et al., 1997), an effect that is attributable to NMDA receptor activation as they stop bursting when NMDA receptor sensitivity declines with age. NMDA does induce burst firing in adult animals, although a hyperpolarizing current is needed for this induction (Overton and Clark, 1997). In my view, the age of the animal does not confound the results in this thesis primarily because animals used in a given set of experiments did not span major developmental stages (9-21 days old). As a result, L-type calcium channel-induced burst firing used as a test model in rats is quite stable and predictable, 80.3% of DA neurons in brain slices harvested from pre-weaning rats displayed a burst firing pattern following L-type calcium channel activation. I also tested this model in adult mice (> 2 month old) presented in Chapter 4 to specifically examine the L-type calcium channel subtypes in transgenic mice, 63.6% of DA neurons could be induced to burst fire. Age does not appear to have a significant role in the expression of burst firing specifically induced in DA cells from acute brain slices. However, the percentage of induced bursting is higher in juvenile rats than in mature mice, but whether this is related to age is not known. Since mice (39°C) have higher body temperatures than rats (37°C), the difference between body temperature and our recording temperature may also contribute to this.
5.1.2 The effects of temperature on neuronal firing

All patch clamp recordings in this thesis work were conducted at room temperature (22°C). Temperature regulates the rate of enzyme-catalyzed reactions and has effects on the function of ion channel proteins. For instance, I_h is found to be sensitive to temperature being more conductive at higher temperatures (Vargas and Lucero, 1999). In addition, L-type calcium channels display increased conductance, more hyperpolarized activation voltages and accelerated recovery from inactivation over a given time at higher temperatures of 36-37°C compared to room temperature at 21-23°C (Huneke, et al., 2004; Peloquin, et al., 2008). Therefore, it is certain that I_h and L-type calcium channels are less efficient at room temperature, which may be reflected in the fact that the basal firing frequency of DA neurons in our experiments (0.41±0.04 Hz in rats) is lower than that reported at higher temperatures (spontaneous spike frequency is >1 Hz) (Johnson and North, 1992b; Lacey, et al., 1989). It is also possible that temperature contributes to the discrepancy in induced bursting rates in rats and mice because mice (39°C) have higher body temperature than rats (37°C) giving rise to a bigger differential temperature at 22°C at which the recording was conducted. This may account for the lower percentage of burst firing following the application of L-type calcium channel activators in mice (63.6%) relative to that in rats (80.3%).
5.1.3 Recording methods used in my thesis work

The perforated patch clamp recording method was used in almost all experiments with only two exceptions: dialysis of PKM into the recorded cell and isolation of Ca²⁺ currents, both of which required conventional whole-cell patch clamp recording for intracellular delivery of the chemical tools. Perforated patch clamp recordings have some advantages over conventional whole-cell recordings: the channels formed by nystatin used in my perforated recordings are smaller and are impermeable to molecules larger than glucose, which means that only small ions such as Na⁺, K⁺, Cl⁻ can pass through while Ca²⁺ and second messengers can not. Therefore, recordings can be conducted without dialyzing important substances from the cell’s cytoplasm. Currents run down significantly more slowly and second-messenger cascades and mechanisms important to cell signaling and channel regulation are kept operative (Fu, et al., 2003). The reason that I observed L-type calcium channel bursting could be due to the perforated recording I used in these experiments. As detailed in previous chapters, L-type calcium channel-induced bursting was dependent on Ca²⁺ and intracellular signaling molecules such as PKM, which are easily dialyzed in conventional whole-cell recording but remain perfectly intact in perforated recording.
5.1.4 Animal species used in my thesis work

It has been shown that there are differences in anatomy and physiological functions among different animal species. For example, the total number of TH-positive (TH, tyrosine hydroxylase) cells and the expansion of their innervation territory, especially in the neocortex, are much less in rodents than in monkeys and humans (Chu, et al., 2002; Emborg, et al., 1998; German and Manaye, 1993; Lewis, et al., 1998; Nelson, et al., 1996). As described in previous chapters, animal species used in my thesis work are rats and mice, which don’t show many differences in anatomy and functions. In terms of L-type calcium channel-induced bursting, there was almost no qualitative difference in bursting induction and the characteristics of bursting itself except a small difference in drug concentration and lag time between the start of drug application and the onset of bursting. Therefore, both of the species can be used in the study of L-type calcium channel-induced bursting. However, mice were used in my later projects because there are transgenic models available to further explore the mechanism of L-type calcium channel-induced bursting. As indicated in previous chapters, the L-type calcium channel and PKC are two important signaling steps involved in the regulation of firing activities but there are no available pharmacological drugs to selectively activate or inhibit the specific subtypes of L-type calcium channels or PKC isoforms to isolate or identify their specific roles. Transgenic mouse models aid in this line of exploration. For instance, two mouse models are available to dissect the physiological role of Ca,1.2 and Ca,1.3 subtypes of L-type calcium channels: in Ca,1.3(−/−) mice, the Ca,1.3 α1-gene is knocked
out thereby Ca$_v$1.3-dependent cellular functions and Ca$_v$1.3-mediated DHP binding are lost (Platzer, et al., 2000); in Ca$_v$1.2DHP$^{(-/-)}$ mice, which were used in my thesis work, Ca$_v$1.2 α1-gene is point-mutated thereby Ca$_v$1.2 channels are normally expressed but Ca$_v$1.2-mediated DHP binding and effects are lost, thus, DHP modulators are Ca$_v$1.3-selective in these animals (Sinnegger-Brauns, et al., 2004). Similarly, individual PKC isoform knockouts generated over the past few years provide the possibility of further investigating PKC isoforms in the regulation of DA firing activities. Cross breeding of PKC and L-type calcium channel transgenic mice can produce double knockouts and provide defining information on the relationship between PKC isoforms and L-type calcium channel subtypes in neuronal firing activities, which will be interesting projects in the future.

5.1.5 Criteria for identifying DA neurons used in my thesis work

The criteria for identifying DA neurons in my thesis work were indirect electrophysiological characterizations: a prominent $I_h$ and an apparent DA-induced hyperpolarization, which are commonly used by investigators because both can be easily obtained during perforated patch clamp recordings without any other effects on neurons’ activities (Johnson, et al., 1992; Lacey, et al., 1989; Zhang, et al., 2005). This has been used in many reports, especially earlier ones, as a reliable electrophysiological fingerprinting. However, recent evidence suggests that it is not as reliable as earlier claims because these general electrophysiological properties used for identifying DA
neurons, including $I_h$ and DA-induced hyperpolarization, are found in a large proportion of TH-negative VTA neurons. Moreover, some TH-positive VTA neurons don't display these typical electrophysiological properties (Margolis, et al., 2006). For example, Lammel et al (Lammel, et al., 2008) showed that the TH-positive mesocortical VTA neurons do not have DA-induced inhibition and $I_h$. It implies that there were some non-DA neurons in the putative DA neurons recorded in my thesis work. The more accurate method is neurochemical identification of TH in VTA neurons. Therefore, loading biocytin into the pipette and breaking in to let biocytin flow into the cell after finishing perforated patch recordings would allow post-hoc identification of DA neurons accurately with double fluorescence immunocytochemistry for TH and biocytin. An alternative method is the use of TH-EGFP transgenic mice (Jomphe, et al., 2005), in which midbrain DA neurons can be directly visualized due to the expression of the enhanced green fluorescent protein (EGFP) under the control of the TH promoter.

5.2 Summary of main findings

5.2.1 The L-type calcium channel-induced burst firing as an in vitro model

One fundamental question of my thesis work is to identify cellular mechanisms regulating burst firing in DA neurons. Compared to in vivo studies, in vitro studies provide ways to manipulate specific steps of the signaling pathways involved. However, spontaneous (also called natural) burst firing barely exists in in vitro brain slices
It has been reported that some neurotransmitters or their analogs such as carbachol or NMDA are capable of inducing burst firing in acute brain slices, but there are technical problems associated with these in vitro burst firing models. For example, the percentage of burst firing induced by carbachol is very low (<20%) which limits its use as an effective model (Zhang, et al., 2005). The proportion of NMDA-induced bursting is higher (around 40-50%) but it needs a hyperpolarizing current to induce bursting. It has been debated whether NMDA-induced bursting is relevant to bursting that occurs in behaving animals (Overton and Clark, 1997). The major point in this debate is that NMDA-induced bursts are sodium-dependent by not calcium-dependent, whereas natural bursts in DA neurons are critically dependent on intracellular calcium. Also, there are technical difficulties in studying the Ca$^{2+}$ dependency of NMDA-induced bursting in that divalent cations used as Ca$^{2+}$ channel blockers, such as NiCl and others, react with NMDA in solution making it difficult to know the effective dose of the ligand as well as the blockers (Gavazzo, et al., 2006; Fayyazuddin, et al., 2000). By contrast, L-type calcium channel-induced bursting is a simple and effective model to further explore the underlying signaling pathways, especially the role of Ca$^{2+}$, at the cellular level.

Firstly, L-type calcium channel-induced bursting is very reliable: it can be induced in a high percentage of cells tested in different animal species. For example, the L-type calcium channel opener FPL 64176 at a commonly used concentration (1 µM) could
induce bursting in 63.5% of putative DA cells in rats, and at a higher concentration (4 μM) this percentage could reach 80.3%. For mice, 4 μM could induce bursting in 63.6% of cells at room temperature. This is very important for a model to be used in further studies. Secondly, the properties of L-type calcium channel-induced bursting are similar to natural bursting. Just as described in sections 2.3 and 4.3, within a bursting cycle induced by L-type calcium channel activation, action potentials were fired with increasing frequencies followed by a pronounced post-burst hyperpolarization, a pattern that fits well with natural bursting. Thirdly, there are established chemicals to manipulate the L-type calcium channel and there are no adverse reactions with common modulators of other Ca^{2+} channels. Bath application of L-type calcium channel openers alone is sufficient for burst induction, and no other interventions are needed for burst induction or maintenance.

The subtype of the L-type calcium channel involved in this model is restricted to only Ca_{1.2} and Ca_{1.3} since they are the only subtypes expressed in DA neurons. In addition, L-type calcium channels are sensitive to DHP modulators therefore it is easy to rule out the involvement of other Ca^{2+} channels. Moreover, L-type calcium channel transgenic mouse strains exist to study the role of individual subtypes (Platzer, et al., 2000; Sinnegger-Brauns, et al., 2004), such as the Ca_{1.2}DHP^{(5)} strain. Ca_{1.3} L-type calcium channels are ideally positioned to regulate firing modes because they have a low activation potential threshold (Durante, et al., 2004; Xu and Lipscombe, 2001) and it has been found that pacemaking and burst firing are Ca^{2+}-dependent. I verified this
hypothesis and found that Ca,1.3 1-type calcium channels mediated regular firing and played a crucial role in burst firing. In mice whose Ca,1.2 1-type calcium channels have a dramatically reduced sensitivity to DHP, the DHP site 1-type calcium channel opener (S)-(-)-Bay K8644 induced burst firing. Similarly, firing induced by a non-DHP site 1-type calcium channel opener could be blocked by the DHP site 1-channel inhibitor nifedipine. All of these results strongly suggest a dominant role for Ca,1.3 in mediating burst firing of DA neurons in brain slices.

To establish 1-type calcium channel-induced bursting as a valid in vitro model, one would need to examine functional correlations of the induced burst firing and DA-related pathologies. While the notion that 1-type calcium channel-induced bursting as a basis of enhanced DA transmission in diseases is yet to be explored, it is known, however, that 1-type calcium channels are implicated in addiction and other diseases. For example, repeated amphetamine injections increase the expression of α1C 1-type calcium channel subunits in the VTA (Rajadhyaksha, et al., 2004) and repeated stimulation of 1-type calcium channels in the VTA mimics the initiation of behavioral sensitization to cocaine (Licata, et al., 2000), while direct injection of 1-type calcium channel antagonists into the VTA attenuates the development of psychostimulant-induced behavioral sensitization (Licata, et al., 2004). 1-type calcium channels can mediate all of these behaviors by their role in regulating the pacemaker and burst firing reported in my thesis work because burst firing elevates terminal field DA levels more efficiently and all of these behaviors arise...
from increased DA transmission. Therefore, this model can serve as an electrophysiological testing platform to screen cellular signaling pathways which underlie affective behaviors such as addiction. In summary, the aforementioned arguments demonstrate that L-type calcium channel-induced bursting is a simple effective bursting model in vitro which has characteristics of natural bursting.

It is noteworthy that a feature of this model is that a lag exists between the start of drug application and the appearance of burst firing, which calls for stable recordings for a relatively long period of time. For instance, in my hands, the lag ranged from 5-25 min with an average of 13 min following a 5-min application of FPL 64176 in rats and 8-42 min with an average of 25 min following 8-24 min application of FPL 64176 in C57BL/6 mice. This means that the cell being recorded needs to be stable for at least 1 hour for a full expression of burst firing. Modifications to the procedure such as increasing the concentration of FPL 64176 or warming up the slices to 35°C, may speed up the onset of burst firing. However, it is highly unlikely that this lag is due to randomness because L-type calcium channel openers cause increased firing and depolarization within a few minutes, it is only that burst firing takes much longer to be induced. It could be that in different cells L-type calcium channel couplings are slightly different and the resulting signaling cascade takes different amounts of time to develop, or the temperature used for slice perfusion is not optimal for PKC-PKM enzymatic conversion.
5.2.2 PKC (PKM) signaling pathway in regulation of DA firing activities

The key to L-type calcium channel-induced burst firing is Ca\textsuperscript{2+} influx. While there are many routes of Ca\textsuperscript{2+} entry into the cell, many of which have specific couplings in DA neurons, it is not known what signaling pathway is coupled to Ca\textsuperscript{2+} influx through L-type calcium channels. The exploration of this issue has led to the discovery of the PKC (PKM) signaling pathway in the regulation of firing activities of putative DA neurons of the VTA.

PKC includes three classes: conventional, atypical and novel PKCs, many of which are shown to be expressed in DA neurons (Yoshihara, et al., 1991). PKC can phosphorylate many different substrates including L-type calcium channels at multiple serine/threonine sites. For example, it has been shown that phosphorylation of the Ca\textsubscript{1.2} by PKC leads to positive modulation of the channel (Yang, et al., 2005) so that it becomes easier to open and conducts more Ca\textsuperscript{2+} ions. In addition, positive modulation of L-type calcium channels by PKC has also been reported in native tissues. For example, M\textsubscript{2} mACHR stimulation requires PKC to increase Ca\textsubscript{1.2} currents in rabbit portal vein myocytes (Callaghan, et al., 2004) and M\textsubscript{1} receptor activation involves PKC to mediate facilitation of L-type calcium channels in dissociated autonomic neurons from the major pelvic ganglion (Sculptoreanu, et al., 2001). In our hands, PKC inhibitors reduced
carbachol-induced increases in nifedipine-sensitive Ca\(^{2+}\) currents, clearly showing that PKC regulates L-type calcium channels to affect firing activities of midbrain DA neurons. PKC has been shown to play a role in drug abuse. For instance, repeated administration of cocaine increases overall PKC activity in the VTA (Steketee, et al., 1998), while injection of PKC inhibitors into the VTA reduces cocaine-induced DA release in the NAc (Steketee, 1993) and delays the onset of behavioral sensitization (Steketee, 1994;1997). The PKC signaling pathway regulating firing activities of DA neurons might underlie drug abuse, an idea that is worth further exploration in the future.

PKM is a persistently active catalytic subunit of PKC. Elevated intracellular Ca\(^{2+}\) has been shown to activate calpain (Goll, et al., 2003), a protease that cleaves the PKC catalytic domain from the regulatory domain (Al and Cohen, 1993;Cressman, et al., 1995;Kishimoto, et al., 1983) to generate PKM. Since its discovery in the 1970s (Inoue, et al., 1977), its function remained obscure until recent reports associating it with learning and memory (Osten, et al., 1996;Sacktor, et al., 1993). This thesis work reports for the first time that PKM is involved in regulating DA firing activities.

It is reasonable to propose that PKM, the persistently active catalytic subunit of PKC, is a critical step in L-type calcium channel-induced burst firing. The evidence is as follows (Figure 2.8): (1) when burst firing was induced by L-type calcium channel
activation, chelerythrine, a PKC catalytic domain inhibitor, reversibly blocked burst firing, whereas calphostin C, a regulatory domain inhibitor did not: (2) L-type calcium channel openers induced burst firing accompanied by increased levels of phosphorylated PKM; (3) inhibition of the Ca\textsuperscript{2+}-dependent protease calpain reduced the level of phosphorylated PKM and concomitantly blocked burst firing; (4) depletion of PKC isoforms following prolonged incubation with PMA prevented L-type calcium channel-induced burst firing; (5) direct loading of the cell with purified PKM through the recording pipette induced burst firing in a similar manner to Ca\textsuperscript{2+} influx. Two conclusions are apparent from the collective evidence: (a) PKM is a critical coupling to L-type calcium channels; (b) PKM is required in burst firing. The need for PKM generation to induce burst firing also helps explain why it takes time for bursting to develop following L-type calcium channel opening. In addition, the persistent active PKM may sustain an enhanced activity of a signaling loop that keeps DA cells in bursting mode. One scenario is that Ca\textsuperscript{2+} influx through the L-type calcium channel activates a Ca\textsuperscript{2+}-dependent protease cleaving PKC to liberate PKM, which in turn phosphorylates L-type calcium channels to allow more Ca\textsuperscript{2+} influx. If proven, this could lend major support for this signaling loop to serve as a molecular substrate for behavioral sensitization and addiction, both of which call for positive reinforcement. This is supported by the results in Chapter 3 that carbachol-induced increases in nifedipine-sensitive Ca\textsuperscript{2+} currents could be blocked by PKC inhibitors. Functionally, being persistently active, PKM could fulfill all PKC-mediated processes reported to occur in addiction and motivation (Steketee, 1993;1994;1997;Steketee. et al., 1998). In addition, PKM could mediate addictive
behaviors by its role in inducing burst firing reported here as burst firing elevates terminal
DA more effectively and all of these behaviors involve increased DA transmission.
Furthermore, addiction is a learned behavior and PKM has been shown to facilitate
learning and memory in rats (Osten, et al., 1996;Sacktor, et al., 1993). Taken together,
PKM appears to be ideally positioned to affect many aspects of rewarding processing and
addiction.

5.3 Future directions

As discussed in previous chapters, the L-type calcium channel-PKC (PKM)
signaling pathway as a mediator of DA bursting is the major finding of this thesis work.
Although our results provide fairly strong support for its role in regulating DA firing
activities, there are still details left to unravel in the future.

Firstly, results in this thesis show that Ca,1.3 L-type calcium channels mediate burst
firing induced by L-type calcium channels. although it remains unclear whether Ca,1.3
L-type calcium channels also mediate the activation and cleavage of PKCs described in
Chapters 2 and 3. This can be tested using Western blotting and L-type calcium channel
transgenic mice to see whether activation or inhibition of Ca,1.3 L-type calcium channels
affects the expression of PKM and carbachol-induced excitation.
Secondly, since the L-type calcium channel-PKC (PKM) signaling pathway is implicated in burst firing, it would be of great interest to identify the PKC substrates that lead to this firing regulation. L-type calcium channels themselves could be one candidate substrate of PKC in this signaling pathway because PKC does phosphorylate both subtypes of the L-type calcium channel at multiple serine/threonine sites. It is reasonable to examine whether L-type calcium channels themselves are the substrate of PKC (or PKM).

In addition, the M channel could be another possible candidate. The M current is a voltage-dependent, slow delayed rectifier $K^+$ current that is activated at subthreshold potentials and contributes to regulation of repetitive firing and excitability (Aiken, et al., 1995; Brown and Adams, 1980). M current is mediated by KCNQ-type potassium channels (Wang, et al., 1998) and immunohistochemical studies have shown that the KCNQ2 and KCNQ4 channel proteins are present in VTA neurons (Cooper, et al., 2001; Kharkovets, et al., 2000). Consistent with the presence of KCNQ channel proteins found in these anatomical studies, M current has been shown to be present in midbrain DA neurons with intracellular recording in brain slices (Lacey, et al., 1990) and clamp recording in isolated DA neurons (Koyama and Appel, 2006). Preliminary results from our lab indicate that blockade of the M current induces increased firing frequencies and even burst firing in some DA cells (unpublished data from our lab). Furthermore, M
currents have been reported to be suppressed by PKC phosphorylation (Higashida, et al., 2005). Therefore, increased PKC/PKM activity following L-type calcium channel opening could lead to phosphorylation and closing of KCNQ channels to aid burst firing.

Thirdly, the upstream events of the L-type calcium channel-PKC (PKM) signaling pathway are still unclear. Chapter 3 presents results that cholinergic activation can sequentially activate PKC and L-type calcium channels to mediate increased firing, which suggests cholinoreceptors might be upstream of this L-type calcium channel-PKC signaling pathway. Whether PKC is involved in bursting induced by cholinergic activation is unclear even though this possibility clearly exists because L-type calcium channels have been shown to mediate carbachol-induced bursting (Zhang, et al., 2005).

Additionally, orexin receptors are another promising candidate. The VTA receives input from the lateral hypothalamic area, including projections from neurons containing the neurotransmitters orexin A and B (Fadel and Deutch, 2002), some of which are passing fibers and some directly synapse onto VTA neurons (Bailey-Pedicino and Sesack, 2007). Also mRNAs for orexin A and B receptors are expressed in the VTA (Korotkova, et al., 2003). In addition, the activity of VTA DA neurons has been shown to be regulated by orexin transmission. For example, activation of orexin A or B receptors in the VTA has been shown to induce DA neuron excitation, displaying increased firing.
frequencies and in some cells, burst firing (Korotkova, et al., 2003). In addition, it has been reported that orexin A activates PKC to increase internal \( \text{Ca}^{2+} \) concentration in DA neurons of the VTA (Uramura, et al., 2001) and orexin B augments L-type calcium currents via a PKC signaling pathway (Xu, et al., 2003). It will be interesting to know whether an orexin-PKC-L-type calcium channel signaling pathway is involved in the regulation of DA firing activities.
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