KCNQ/M CHANNELS IN THE MIDBRAIN DOPAMINERGIC SYSTEM IN THE RAT NEONATAL HIPPOCAMPAL MODEL OF SCHIZOPHRENIA

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KCNQ/M channels in the midbrain dopaminergic system in the rat neonatal hippocampal lesion model of schizophrenia

By

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Abbreviations:

ACSF: Artificial cerebrospinal fluid

AHP: Afterhyperpolarization

DA: Dopamine

DAB: 3, 3'-diaminobenzidine

fAHP: Fast afterhyperpolarization

I_h: Hyperpolarization-activated cationic current

IHC: Immunohistochemistry

IFD: Immunoflorescence double labeling

I_M : M current

mAHP: Medium afterhyperpolarization

nVH: Neonatal ventral hippocampus

VTA: Ventral tegmental area

ON: Oculomotor nucleolus

PND: Post natal day

PPI: Prepulse inhibition

RMP: Resting membrane potential

RN: red nucleolus

sAHP: Slow afterhyperpolarization

TH: Tyrosine hydroxylase

Abstract:

Increase in dopaminergic (DA) neurotransmission in the brain has been implicated in schizophrenia. One of the known mechanisms for promoting dopamine release is burst firing of DA cells. However, it is unclear whether alterations in firing patterns of DA cells are associated with the disease. In the present study at first I hypothesized that M current contributes to burst firing of DA cells in slices .I therefore studied the effect of M channel blockade on the firing behavior of DA cells in the ventral tegmental area. Then I hypothesized KCNQ channel expression in VTA DA cells in nVH lesion rats is reduced to promote the excitability of these cells. Therefore I examined the expression of KCNQ3, a subunit that forms heterodimeric channels with other subunits to carry M currents with much greater conductance, in rats that underwent ventral hippocampal lesion early in life which has been widely used as a rat model of schizophrenia.

First, the effect of blocking M channels on the firing behavior was studies in horizontal brain slices that contained the ventral tegmental area using nystatin perforated patch clamp recording. Blocking the channel with linopirdine or XE-991 resulted in excitation of DA, but not GABA cells in the ventral tegmental area. This increase firing was accompanied by a reduction of the medium hyperpolarizing afterpotential and a mild depolarization. In the majority of DA cells tested, blocking these channels also resulted in burst firing.

Next, the expression of KCNQ3 channel in the ventral midbrain was studies in neonatal ventral hippocampal lesioned rats. Bilateral injection of ibotenic acid in the ventral hippocampus at postnatal 7 resulted in cell loss that was confirmed with Nissl staining at the end of the experiment. The lesioned group had a gradual increase in the deficit in prepulse inhibition tested at 35, 49 and 56 postnatal days, a hallmark of this model. The brains were processed immunohistochemically for the expression of KCNQ3 subunits in the midbrain. I found that the number of cells expressing KCNQ3 channels was decreased in the ventral tegmental area of the lesion group as compared with the sham group. Double immunofluorescence labeling with tyrosine hydroxylase and KCNQ3 showed a high percentage of colocalization in dorsal tier of ventral tegmental area, suggesting the reduction in KCNQ3 channel expression occurs in DA cells. In contrast, in the lesion group, the number of KCNQ3 positive cells was significantly increased in the red nucleus and the oculomotor nucleus.

In conclusion, the results in this thesis suggest that neonatal hippocampal lesion leads to decreased expression of KCNQ3 channels in DA cells that may result in increased excitability and/or increased burst firing to enhance DA transmission. This maybe one of the mechanisms by which the DA system becomes overactive in schizophrenic brain.

Chapter 1

Introduction

1.1. Schizophrenia and the central dopaminergic system

Schizophrenia affects about 1% of the general population (Hautecouverture *et al.*, 2006; Shastr y, 2002) with a chronic disability in perception or expression of reality. Diagnosis of the disorder is based on the presence of two groups of symptoms, positive and negative, which usually appear in late adolescence or early adulthood. Positive symptoms include irrational beliefs (delusions), perceptual abnormalities such as hallucinations and thought disorders. These symptoms are typically regarded as manifestations of psychosis. Negative symptoms refer to deficits in normal mental activities creating a "flat, blunted or constricted affection and emotion, poverty of speech and lack of motivation" (Harvey *et al.*, 2002; Oliveria and Juruena, 2006; Philip *et al.*, 2004).

What causes the disease is by and large unknown. There is a well-documented body of literature using quantitative voxel-based morphometry depicting the anatomical abnormality in the schizophrenic brain especially in the limbic structures studies. Some of the abnormalities, for example the reduction in hippocampal size, have been consistently found in schizophrenic brains and are used to assess the severity of the disease (Rametti et al., 2007; Spencer et al., 2007; McClure et al., 2006). Despite this, earlier work on twins suggests that none of the anatomical deficits are diagnostic, nor can

they predict the outcome of the disease in a particular person (Singh *et al.,* 2004; Gottesman, 1991). With advanced technology, more detailed description of neurochemical abnormalities has been documented including a wide range of changes in neurotransmitters, receptors and the interactions between them, as well as genes that are differentially regulated in the brain. One of the more viable hypotheses involves the activity of the central dopaminergic (DA) system. This hypothesis is more than half a century old, implicating a hyperactive DA system in the disease with support primarily stemming from that fact that manipulation of the DA system produces anticipated results. Thus, drugs such as amphetamines that release DA and block its reuptake (i.e., increase DA levels at the synapse) induce psychotic symptoms in healthy volunteers (Angrist and Gershon, 1970). Similarly, all antipsychotic drugs currently in use block DA receptors and their clinical efficacies are positively correlated with their antagonism on DA receptors. Both conventional and atypical antipsychotics relieve positive symptoms (De Oliveria and Juruena, 2006) by blocking D2 receptors (Thompson *et al.,* 2004, Kapur and Seeman, 2001, Zipursky *et al.,* 2007, Stone and Pilowsky, 2006). In other words, although serotonergic (Bruins Slot et al., 2007, De Oleveira and Juruena, 2006), adrenergic (Swanson and Schoepp 2003, Bymaster and Felder, 2002), cholinergic (Sanger, 2004), and histaminergic (Ito, 2004) receptors are modulated by atypical antipsychotic drugs, the antagonism of D2 receptors is necessary for their antipsychotic activity. Moreover, radioenzymatic measurements in post-mortem schizophrenic brains showed increased DA concentrations in the nucleus accumbens (Mackay *et al.,* 1982; Bird *et al.,* 1979), a brain region that receives massive DA innervation and generates positive symptoms as a result of excessive DA output (Farley et al., 1977).

However, the DA hypothesis does not explain why negative and cognitive symptoms of the disease remain relatively resistant to antipsychotic treatment (Buckley and Stahl, 2007; Rummel *et al.,* 2006). This can be accounted for by a modified DA hypothesis, which suggests an imbalance between the mesocortical and mesolimbic DA systems (Weinberger, 1987). According to this hypothesis, the hyperactive mesolimbic system involving D2 receptors in particular underlies positive symptoms, whereas the hypofunctionality of the mesocortical system due to reduced D1 receptor-mediated transmission results in negative symptoms and cognitive impairment (Abi-Dargham and Moore, 2003). The notion that antipsychotic drugs ameliorate positive symptoms by antagonizing DA receptors in the nucleus accumbens has been examined by way of cFos expression, a marker of neuronal activation (Mikkelsen, 2004). In an immunohistochemical study on adult male rats, cFos protein is detected in the dorsal and ventral striatum in response to intraperitoneal injection of amphetamine, an agent that competitively inhibits DA transporters and increases DA levels at the synapse (Graybiel *et al.,* 1990). Whereas single photon emission tomographic techniques have shown that there is correlation between DA levels in the nucleus accumbens and appearance of positive symptoms in schizophrenic patients (Laruelle et al., 1999), the increased cFos expression in the shell region of the nucleus accumbens after treatment with typical and atypical antipsychotic drugs is accompanied by an improvement in positive symptoms. However, cFos expression in the prefrontal cortex is only correlated with improved negative symptoms following treatment with atypical antipsychotics (Ananth *et al.,* 2001).

Alternatively, the DA system is only one part of the problem with other transmitter systems involved. In the last two decades, the glutamatergic and GABAergic systems have been seen as possible underpinnings of schizophrenia (Raynolds and Harte, 2007; Ohnuma *et al.,* 2005; Raynolds *et al.,* 2002). For example, the NMDA receptor antagonist phencycyclidine induces hallucinations in healthy individuals (Tong *et al.,* 1975). However, these two systems are mainly found to be involved in negative and cognitive symptoms (Kondziella *et al.,* 2007; O'grada and Dinan, 2007; Sur and Kinney, 2004). Agents which directly or indirectly activate the glycine modulatory site of the NMDA type glutamate receptor reduce negative symptoms in chronic schizophrenic patients (Duncan et al., 2004). Postmortem studies indicate that there is a reduction in GABAergic interneurons in the prefrontal cortex and hippocampus which has been proposed to cause negative symptoms (Coyle JT *et al.,* 2004). Other systems such as histaminergic, cholinergic and opioid may also be involved as many atypical antipsychotics bind and block these receptors.

The involvement of other systems does not diminish the importance of the DA system in schizophrenia. Rather, it may signify the interaction between DA and other transmitters. The best-studied example of this interaction is the interaction between the D1 DA receptors and the NDMA type glutamate receptors in the prefrontal cortex (Laruelle et al., 2003). The D1 and NMDA receptors are physically close on the dendrites of pyramidal neurons in the same layer of the prefrontal cortex. Interaction between them results in trapping the D1 receptors to dendritic spines and consequently a hypofunctionality of these receptors leading to negative symptoms (Cepeda and Levine, 2006; Yang and Chen, 2005). Also, DA increases NMDA currents (Komendantov and

Canavier, 2002) and working memory (Levin and Rose, 1995), a function that is if primarily executed in the prefrontal cortex. Taken together, in spite of involvement of multiple transmitter systems and possible interactions between them, it is generally accepted that disruptions in the central DA system serve as the final common pathway in establishing psychotic symptoms.

1.2. VTA

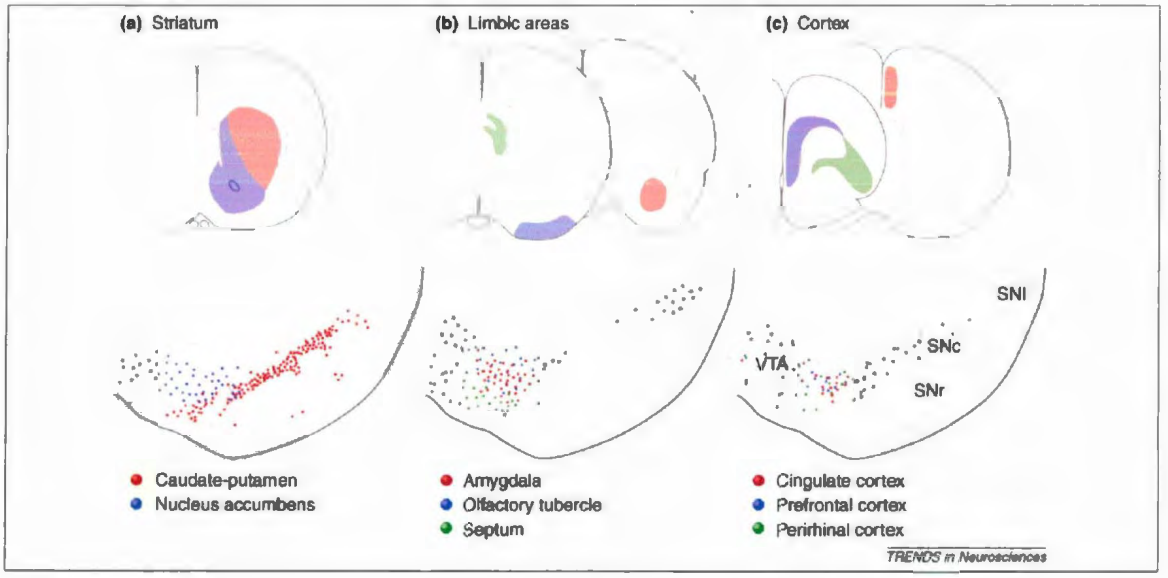
There are 4 major DA cell groups in the brain, one of which resides in the hypothalamus and the remaining 3 are located in the midbrain. DA cells in the arcuate nucleus, located in mediobasal hypothalamus, project to the median eminence and release DA into hypophysial portal blood to regulate prolactin release. The midbrain DA neurons are organized into three major nuclei: A8, A9 and A10 which are located in the retrorubral field, the substantial nigra and the ventral tegmental area (VTA), respectively. There are no clear anatomical boundaries between neurons of the different cell groups in the midbrain. DA neurons in the A8 group besides their projection to forebrain (Williams and Goldman-Rakic, 1998), project to mesotelencephalic dopamine system, amygdala, olfactory tubercle and nucleus accumbens (Deutch *et al.,* 1988) innervate hypothalamic median eminence, an area concerned with neuroendocrine regulation (Kizer *et al.,* 1976) and modulate function of A9 and A10 group (Deutch *et al.,* 1988). A9 DA cells predominantly project to the dorsal striatum as the nigrostriatal pathway to play a role in controlling voluntary movements and postural reflexes. Degeneration of these cells leads to the development of Parkinson's disease where initiation and coordination of

movements are impaired. The A10 cell group in the VTA has massive projections to the nucleus accumbens and the prefrontal cortex forming the mesolimbic and mesocortical projections that are involved in reward and cognitive behaviors (Parkash and Wurst, 2006). Dysfunction of these pathways has been implicated in schizophrenia (Steffensen *et al.,* 1998; Glenthoj 1995; Glenthoj and Hemmingsen, 1997) and drug abuse (Kiatkin, 2002; Wise and Hoffman, 1992)

The VTA (A10) consists of several groups of cells near the midline lies in the ventral midbrain. This area is lying medial to the substantial nigra, which also contains DA cells, and ventral to the red nucleus. The anatomical properties of the VTA are generally similar in different species from rodents to human beings (Oades and Halliday, 1987; Bogerts *et al.* 1983). It is estimated in the rat that the VTA contains 30 pg of DA in 27000-29000 DA cells (Halliday and Tork, 1984; Halliday and Tork 1986). DA cells constitute approximately 70% of the neuronal population of the VTA (Johnson and North 1992).

The VTA DA projection is confined to the limbic and cortical areas, whereas nigral DA neurons project to the dorsal striatum (Oades and Halliday, 1987; van Domburg and Donkelaar, 1991; Beckstead, Domesick & Nauta, 1979). Recent anatomical studies indicate that DA neurons in the VTA also project to the ventro-medial part of the caudate-putamen, ventral striatum (Bjorlkund and Dunnet, 2007, also see Fig 1.1 and 1.2). Because of this, DA cells in the VTA are also divided into two tiers based on their projections: the ventral tier with a sheet of angular cells that project to the sensory motor part of the striatum, internal or external segments of the globus pallidus, and the dorsal tier with scattered cells that mainly project to the ventral striatum

(olfactory tubercle and the nucleus accumbens), cortical areas and matrix compartment of the dorsal striatum (Ikemoto, 2007). As the result, excitation of the dorsal tier results in increased DA release in the nucleus accumbens and other limbic structures (Kiatkin 2002, also see Fig 1.1, 1.2 and 1.3).



Addapted from: Fallon, J.H. and Loughlin, S.E. (1995) Substantia Nigra. In The Rat Nervous System (2nd edn) (Paxinos, G., ed.), pp. 215–237, Academic Press

Fig.1.1) Cells of origin of the mesostriatal, mesolimbic and mesocortical pathways in the rat.

The DA neurons projecting to (a) striatal, (b) limbic and (c) cortical areas are partly intermixed: The cells located in the ventral tier of the SNc [red dots in (a)] innervate, probably exclusively, the sensorimotor part of the caudate-putamen [red area in the inset in (a)], whereas the cells of the dorsal tier comprise neurons that project widely to both limbic and cortical forebrain regions, as illustrated in (b) and (c), respectively. Abbreviations: SNc, substantia nigra pars compacta; SNl, substantia nigra pars lateralis; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area.

I believe that the people who find the unknowns are eternal.

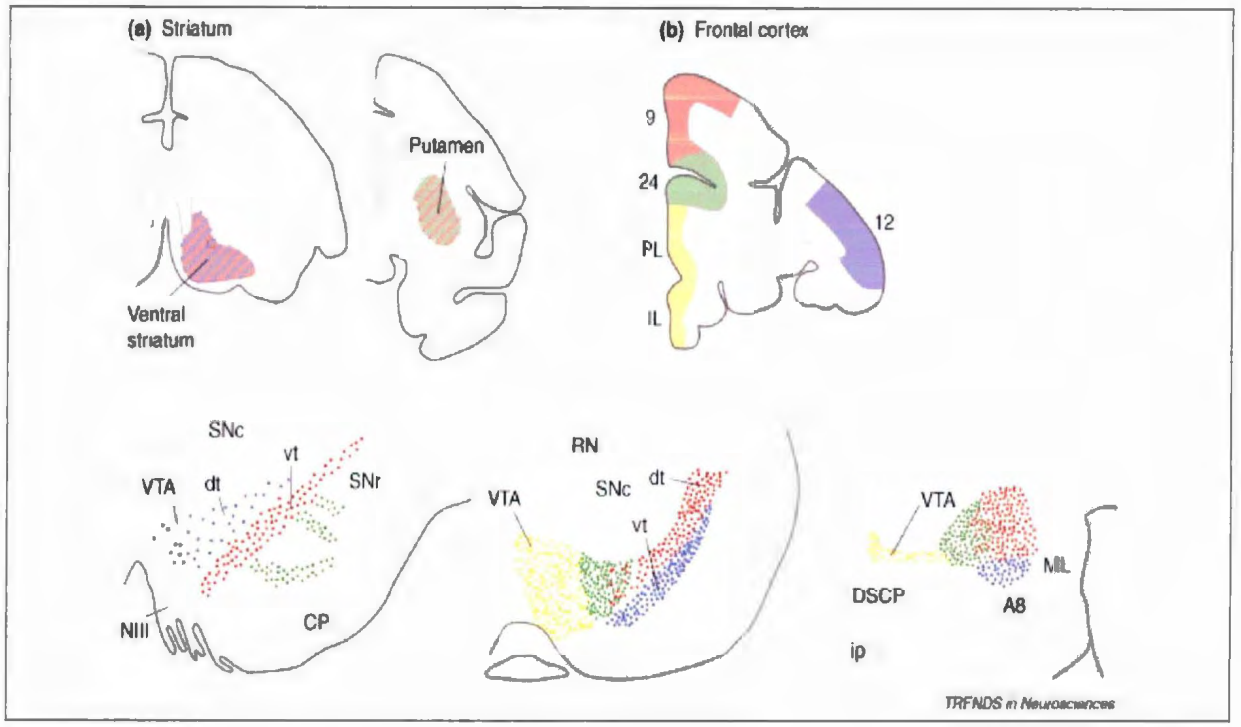
I dedicated this thesis to:

My father...

A great scientist who taught me alphabets of loving science

My Mother...

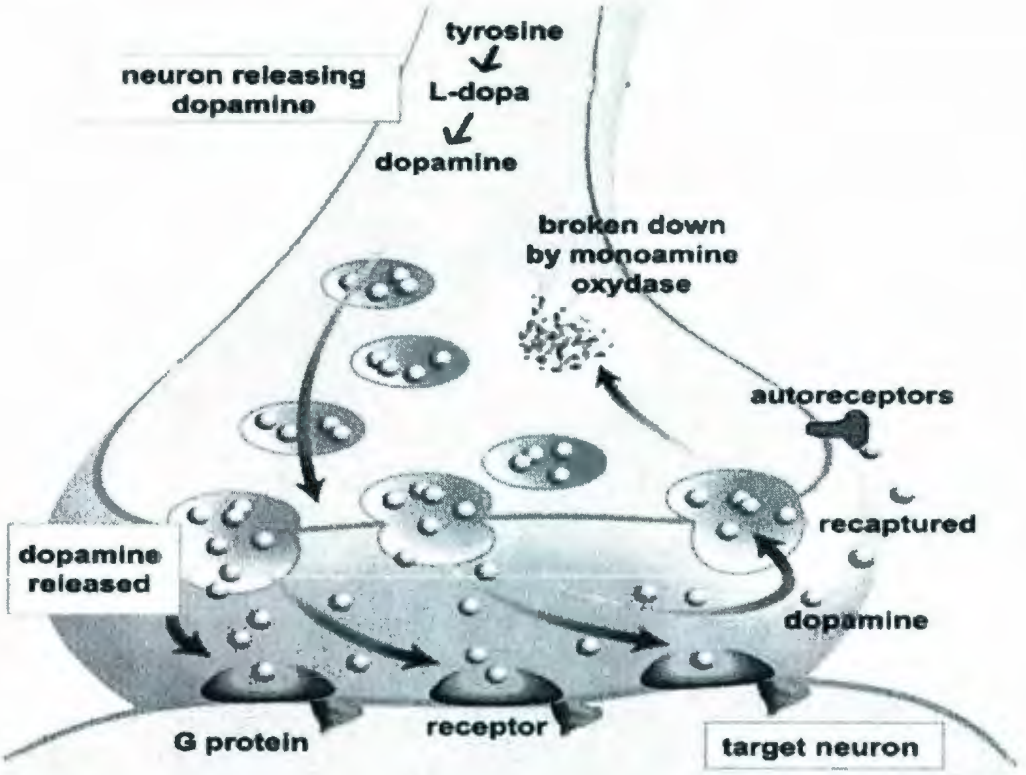
A thinker lady who showed me how to be strong



Adapted from: TRENDS in Neurosciences Vol.30 No.5

Fig. 1.2) Distribution of midbrain DA neurons projecting to striatal, limbic and cortical areas in the primate.

The intermingling of the cells of origin of the mesostriatal,mesolimbic and mesocortical pathways in primates is even more pronounced than that in the rat (Compare with Fig I). The extensive cortical projection, in particular, is derived from all parts of the mesencephalic DA neuron complex, forming a continuous sheet of cells distributed in the dorsal aspect of the A8, A9 and A10 cell groups, and the increase in number of nigral DA neurons projecting to the cortex is particularly striking. Abbreviations: CP, cerebral peduncle; DSCP, decussation of the superior cerebellar peduncle; dt, dorsal tier; IL, infralimbic area of the frontal cortex; ip, interpeduncular nucleus; ML, medial lemniscus; NIII, occulomotor nerve exit; PL, prelinbic area of the frontal cortex; RN, red nucleus; vt, ventral tier.



Adapted from: http://thebrain.mcgill.ca/flash/i/i_03/i_03_m/i_03_m_que/i_03_m_que.html

Fig. 1.3.) Dopamine release *in the* dopaminergic nerve terminal and synapse.

The diagram shows the major steps in the action of the neurotransmitter dopamine. First, dopamine is synthesized from the amino acid tyrosine. The dopamine is then stored in the synaptic vesicles of the presynaptic neuron until it receives action potentials that cause it to release the dopamine into the synaptic gap by a process called exocytosis.

On the post-synaptic neuron, the dopamine then binds to specific receptors that have five different sub-types, designated D1 to D5 , and that are paired with GTP-dependent proteins inside this neuron.

The dopamine is subsequently reabsorbed by transporters on the terminal button of the dopaminergic presynaptic neuron. There the dopamine is either stored again in vesicles or broken down by a mitochondrial enzyme called monoamine oxydase.

Dopamine can also bind to "autoreceptors" in the membrane of the dopaminergic neuron itself. The stimulation of these receptors generally results in negative feedback to the dopamine neurons.

1.3. DA cell firing and DA transmission

The strength of DA transmission is dictated by the amount of DA at the synapse and the number and efficiency of its receptors at the postsynaptic cell. We will now consider factors that contribute to DA release and its accumulation at the synapse. Both the cell body and terminal sites of DA neurons play an essential role in synaptic DA accumulation. At the terminal site, released DA is taken up into the terminal very rapidly by DA transporters. Drugs that affect this mechanism, for example psychostimulants that bind and block the transporters, or saturation of the transporter following intense release, lead to dramatic increase in synaptic DA levels. Moreover, DA autoreceptors located on the terminal mediate a negative feedback that suppresses further DA release (Fig 1.3). At the somatic site, the amount of DA released at the terminal will be reflected by the frequency and/or mode of firing. Of particular significance is the burst firing mode which is characterized by clustered action potentials followed by a hyperpolarization. This is because burst firing is more effective in increasing synaptic DA levels due to Ca^{2+} accumulation inside the terminal during intense spiking (Suaud- Chagny *et al.,* 1992; Grace, 2000), as well as reduction in the inhibitory mechanism of autoreceptors and saturation of DA reuptake mechanisms (Chergui, 1994). Burst firing of VTA DA neurons signifies novel and behaviorally salient stimuli in awake animals with a brief increase in DA release (Schultz, 1998; Schultz and Romo, 1990; Anstrom and Woodward, 2005; Horvitz *et al.,* 1997; Diana *et al.,* 1989).

Dopamine neurons *in vivo* exhibit both tonic firing and burst firing, whereas in *in-vitro* slice preparations they only exhibit tonic firing (Shi *et al.,* 2004; Paladini *et al.,*

1999; Beckstead *et al.,* 2004). This is because burst firing depends on both intrinsic properties of the neuron and its excitatory and inhibitory synaptic input. Synaptic input is interrupted in VTA slice preparations and as the result, spontaneous burst firing is no longer seen (Shi *et al.,* 2004). It is not known how intrinsic ion channels of DA cells contribute to their burst firing behavior *in vivo*. In slice preparations, a number of ion channels have been shown to be involved in generating burst firing of DA cells. For example, blocking Ca^{2+}-activated potassium channels by apamin facilitates burst firing (Seutin *et al.,* 1993; Waroux *et al.,* 2005; Wolfart and Roeper, 2002; Wolfart *et al.,* 2001). Similarly, this pattern can be induced by application of NMDA if a small amount of hyperpolarizing current is simultaneously applied (Johnson *et al.,* 1992).

Since DA cells have a firing threshold of -33±1.5 mV (Grace and Onn 1989), a slow oscillation in membrane potential from resting membrane potential (-55±2.9 mV) is required to initiate burst firing in these cells (Amini et al., 1999). This slow oscillation ranges from –60 to –40 mV pushing a greater number of spontaneous depolarizations beyond the firing threshold at the peak of an oscillation. Meanwhile it has been shown that slow oscillation is dependent on Ca^{2+} channels (Kitai and Surmeier, 1993, Zhang et al., 2005). The involvement of other channels in this voltage range has not been examined in relation to burst firing in DA cells. Channels such as the H and M channels are good candidates because they operate in this range and are expressed in DA cells (Hu *et al.,* 2002).

Although studies consistently indicate that increased firing frequency and bursting of DA cells boost DA levels in the synaptic cleft, there is no direct evidence that this is

the case in schizophrenia. In slices, antipsychotic drugs are found to inhibit T-type currents of DA cells (Santi et al., 2002). T-type currents account for about one-third of the total Ca^{2+} currents in DA cells (Amini et al., 1999) and operate in subthreshold range to modulate their excitability (Uebachs et al., 2006). Taken together, these studies suggest that antipsychotic drugs may regulate the DA system by their actions on the firing behavior of DA cells.

1.4. Prepulse inhibition: a measure of sensorimotor gating deficit in schizophrenia

The startle reflex is a response to sudden intense sound stimuli and is observed as a blink reflex in humans (Duley *et al.,* 2007; Meincke *et al.,* 2001) and a whole-body flinching in rats (Green and Braff, 2001; Duncan *et al.,* 2006). This response can be decreased in magnitude by weaker prepulses presented less than 30-500 ms before the test pulse (Perry *et al.,* 2001), a timeframe required for the brain to assess the initial sensory event for its worth. The decrease in startle response by prepulses is called prepulse inhibition (PPI). In schizophrenic patients, this inhibition is dramatically reduced. Since schizophrenia is regarded to have its underpinnings in excessive DA in the brain, the influence of DA manipulations on PPI has been studied. Numerous studies have shown that apomorphine, a DA agonist, induces a significant deficit in PPI (Yee *et al.,* 2004; Russing *et al.,* 2004; Swerdlow *et al.,* 1991). Moreover, this deficit can be corrected by antipsychotic drug treatment in schizophrenia patients and DA-based animal models of schizophrenia (Zhang et al., 2007; Feifel and Reza 1999). In keeping with this, deficit in startle reflex is considered as a physiological measure in psychological disorders (Swerdlow et al., 2001). Most symptoms in schizophrenic patients are assessed

by self-report; the importance of this independent measure is obvious. It is even more important in animal models of schizophrenia where abnormalities in perception, thought, and cognition are impossible to obtain. Because of the parallel changes between PPI and the functionality of the DA system, it can be used to validate animal models of schizophrenia, in particular the models which have an underlying DA malfunctioning.

1.5. Neonatal ventral hippocampal lesion in the rat as a model of schizophrenia

Schizophrenic studies on human subjects are ideal in a way that the outcome of these studies is directly beneficial to the management of this disease. However, there are also obvious difficulties in such studies in that findings obtained from postmortem schizophrenic brains are not easy to interpret because of long-term antipsychotic treatment that may have induced secondary changes in the brain. Additionally, there is a variable period of time between the time of death and tissue collection, which may affect the results (Harvey *et al.,* 2002; Rodda *et al.,* 2006). Furthermore, experimental manipulation of the disease is in many cases unethical in human studies. Due to these limitations in human studies, investigators have developed some animal models of schizophrenia to test various aspects of the disease such as the DA hypothesis. Over the years different schizophrenic models have been developed by directly manipulating the DA system to mimic the disease (Lipska and Weinberger, 2000). An example for this group of animal models is apomorphine treatment. Apomorphine is a DA agonist; it effectively boosts signal transduction through DA receptors. Similarly, the psychostimulant amphetamine is used to block DA transporters to induce higher levels of DA at the synapse. These models usually limit their behavioral changes to a narrow range

of positive symptoms (Tenn *et al.,* 2005) but they do validate increased DA transmission as the cause of positive symptoms.

A model that is widely used for studying schizophrenia in rodents (Lipska *et al.,* 1993) and monkeys (Bachevalier *et al.,* 1999) is the neonatal ventral hippocampal lesion model (nVH model), which mimics a spectrum of neurobiological and behavioral features of schizophrenia. In contrast to other animal models of schizophrenia that directly manipulate the DA system, this model shows an indirect neurodevelopemental effect on the DA system (Lipska et al., 1993). In this model, neonatal lesioning of the ventral hippocampus leads to emergence of abnormalities in a number of DA-related behaviors. This is in agreement with the hypothesis of hyperactive DA system (Waroux *et al.,* 2005; Lipska *et al.,* 2003; Schroder et al., 1999 and Alquicer *et al.,* 2004). Behavioral abnormalities which are found in the nVH model and also found in schizophrenic patients include deficit in PPI, hyperactivity and hyperlocomotion to stress, and deficit in social activity (Lipska and Weinberger, 2000; Rueter *et al.,* 2004, also see Table 1). These three alterations reflect deficit in information processing, positive symptoms and negative symptoms respectively (Rueter *et al.,* 2004). Importantly, some of these changes, such as PPI deficit and psychomotor agitation in this model can be normalized by treatment with antipsychotic drugs (Lipska and Weinberger, 1994). For instance PPI deficit can be reversed by clozapine similar to its effect on human PPI deficit (Le Pen and Moreau, 2002). However, the animals are apparently normal until past puberty when abnormal behavioral changes become detectable, a feature that is similar to the onset of schizophrenic symptoms in human patients (Stevens, 2002)

On the other hand, antipsychotic drugs in this model (Rueter et al., 2004) do not improve negative symptoms such as social interaction (Levinson, 1991) similar to schizophrenic patients. Other studies have shown that stimulation of the VTA results in a more dramatic increase in DA levels in the nucleus accumbens in this model than sham-operated rats. This effect can also be suppressed by haloperidol treatment (Goto and O'Donnell 2002a; O'Donnell *et al* .2002).

The hyperactivity can result from an increase in DA levels or in the number of DA receptors. However, in the nVH model there is no significant alteration in D2 receptor density (Lillrank *et al.,* 1999). On the other hand, microdialysis of brain in nVH lesion rats has shown that more pronounced hyperactivity in response to amphetamine administration is related to an exaggerated DA output in nucleus accumbens (Corda *et al.,* 2006). It is, therefore, expected that there would be an increase in DA release in this model although disruption of medial prefrontal cortical DA-glutamate interactions has also been proposed (Al-amin *et al.,* 2000; Kato *et al.,* 2001).

Other studies have provided evidence that the function of the prefrontal cortex is compromised in rats with a neonatal ventral hippocampus lesion. For instance, firing patterns of pyramidal neurons in response to VTA stimulation are altered, i.e. stimulation of the VTA in nVH lesion animals induces increased firing of pyramidal cells after puberty, in contrary to reduced firing before it (O'Donnell *et al.,* 2002).

Electromagnetic resonance imaging (MRI) consistently shows abnormalities in limbic structures including the hippocampus and the amygdala of schizophrenic patients

(Oliveria and Juruena, 2006) which can be mimicked by lesioning the ventral hippocampus in this model.

Taken together, the nVH neurodevelopmental model mimics a broad range of abnormalities of the disease including positive and negative symptoms and its dependency on the DA system (Lipska and Weinberger, 2000). Therefore, nVH lesion rats can appropriately model different features of schizophrenia to study the neurodevelopemental hypothesis of this disorder.

	Neonatal VH Lesion Model	Schizophrenia
Behavioral changes:		
	Hyperlocomotion to stress	Stress vulnerability
	PPI deficits	PPI deficits
	LI deficits	LI deficits
	Deficits in delayed alternation tests Reduced social contacts	Working memory deficits
		Social withdrawal
Pharmacological responses:		
	Amphetamine-induced hyperactivity Apomorphine-induced stereotypies Reduced catalepsy to haloperidol MK-801 and PCP-induced hyperactivity	Enhanced symptomatic response to dopamimetics Neuroleptic tolerance Enhanced symptomatic response to ketamine
Molecular changes in the prefrontal cortex:		
	NAA levels↓	NAA levels ↓
	GAD67 mRNA↓	GAD67 mRNA↓
	BDNF mRNA↓	BDNF mRNA↓

Abbreviations: BDNF, Brain derived neurotrophic factor; GAD-67, Glutamate decarboxylase-67; LI, Latent inhibition; NAA, N-acetylaspartate; PCP, phencyclidine; PPI, prepulse inhibition of startle; ↓ reduced vs. controls.

Adapted from: Barbara K Lipska Ph.D and Daniel R Weinberger MD. Neuropsychopharmacology (2000)

Table.1) The Neonatal Ventral Hippocampal Lesion Model: Schizophrenia-like Phenomena.

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There are many people that I would like to thank for their help and encouragement in doing the research discussed in this thesis.

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Prof. Richard Neuman, a wonderful teacher. I am proud of being his student.

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And …

My home of neuroscience …

Memorial University of Newfoundland

Tara - St. John's Summer, 2007.

1.6. KCNQ/M channels

1.6.1. Physiology and Pharmacology

KCNQ genes encode 5 subunits, KCNQ1-5, which form homo- and heterodimeric voltage gated potassium channels (Nakajo and Kubo, 2005). KCNQ1 channels are mainly expressed in cardiac muscle (Tristani-Firouzi and Sanguinetti, 2003), KCNQ5 channels are found in central neurons and skeletal muscles (Lerche *et al.,* 2000). KCNQ4 channels are found in cochlear hair cells (Wangemann, 2002; Beisel *et al.,* 2000). KCNQ2 & KCNQ3 subunits are widely expressed in peripheral and central neurons (Smith *et al.,* 2001; Weber *et al.,* 2006) where they give rise to a muscarinic-sensitive, subthreshold and non-inactivating K^+ current (M current/I_M). Mutation in these channels is known to result in benign familial neonatal convulsion and progressive hearing loss (Lerche *et al.,* 2001; Jentsch, 2000).

Immunohistochemical data indicate that KCNQ3 channels are expressed in axons, somata and dendrites of CA1 pyramidal cell (Yue and Yaari 2006). However, this expression is affected by maturation and accelerated by neurodevelopment (Geiger *et al.,* 2006; Tinel *et al.,* 1998). In the mouse brain, this subunit is detectable at PND 3 and begins to increase at PND 7. This gradual increase continues into adulthood. It is believed that KCNQ2/3 subunits coassemble to form heteromeric channels. KCNQ3 expressed alone in Xenopus oocytes does not generate detectable M currents. The homomeric KCNQ2 channel does conduct M currents; however, coexpression of KCNQ2 and KCNQ3 subunits resulting in heteromeric channels that conduct M current 11-15 fold

higher than homomeric KCNQ2 channels (Wang *et al.,* 1998; Yang *et al.,* 1998). Consequently, expression of KCNQ3 channels has a functional importance as it dramatically increases the conductance of M channels that will in turn exert more influence on the neuron's excitability.

KCNQ3 mRNA has been detected in several parts of the midbrain including the VTA, red nucleus and oculomotor nucleus (Saganich et al., 2001). Nevertheless, in the only immunohistochemistrical study on KCNQ3, no expression has been observed in the VTA and oculomotor nucleus. However in that study, KCNQ2 and more extensively KCNQ4 subunits were detected by double-labeling immunohistochemistry in DA cell rich area, i.e. VTA, substantial nigra and rubral field. Between these two subunits, KCNQ4 has shown an overlap with DA VTA cells (Hansen *et al.,* 2006).

1.6.2. KCNQ/M channels and firing patterns

Being potassium channels that operate at depolarized potentials, M channels function as a 'brake' on repetitive action potential discharges and therefore, has a key role in regulation of the excitability of various central and peripheral neurons (Cox *et al.,* 1998; Lacey *et al.,* 1990), including sympathetic, hippocampal pyramidal and striatal neurons (Shen *et al.,* 2005). Importantly, M current is the only noninactivating current active at a voltage close to action potential threshold. Studies show that M channels control the firing pattern of principal hippocampal neurons so that M channels are activated during spike afterdepolarization that in turn limit the duration of depolarization, thereby precluding an escalation to bursting (Yue and Yaari, 2004). It has also been found in the turtle that a current mediated by M channels increases firing adaptation and

decreases the slope of the frequency-current relation in spinal cord motoneurons (Alaburada *et al.*, 2002). The M channel blocker DuP 996, or linopirdine that is a known cognitive enhancer, and its analogue XE-991 are used as pharmacological tools to isolate M currents. These two blockers show a 10-20 fold greater selectivity to block M currents than other types of potassium currents (Robbins, 2001). VTA DA cells seem to be under the influence of M currents as well. Opening M channels by retigabine dramatically reduces VTA DA cells excitability *in vivo* and *in vitro* (Hansen *et al.*, 2006). Blocking M channels by XE-991 effectively increases the rate of firing in VTA DA cells (Koyama and Appel, 2006). However, the identity of channel subtypes that contribute to this M current remains unknown. Because blocking M currents is known to induce burst firing in the hippocampus (Yue and Yaari, 2004), it is possible that these currents can generate bursting in VTA DA neurons.

M channels could modulate pattern of firing in neurons: M current activation during sustained depolarizations tends to hyperpolarize the neuron and reduce its firing rate (Hensen et al., 2006; Mikkelsen, 2004). Studies have shown that M current is active near the firing threshold in VTA DA neurons and therefore may contribute to the decrease in the excitability of these neurons (Marrion, 1997). Action potentials in many neurons are followed by a hyperpolarizing afterpotential negative to resting membrane potential. Afterhyperpolarizing potential (AHP) has three components based on their time constants: slow (sAHP), medium (mAHP) and fast (fAHP), mediated by different ion channels. For instance, in hippocampal CA1 neurons, time constants for fast, medium and slow components are ~50, 250, and 500 ms ((Maylie et al., 2003), whereas in DA cells are ~ <5, 5-100 and 100-300 ms respectively (Koyama and Appel, 2006). In

hippocampal CA1 neurons the fast component is a voltage and Ca^{2+}-dependent K^+ current carried by the BK and M channels (Maylie et al., 2003) whereas SK channel underlies this component in DA cells (Nedergaards, 2004). In general, since sAHP and mAHP last for a longer time, they are more effective in regulating firing patterns. Whereas the mAHP regulates interspike intervals and is important in tonic firing frequency, the sAHP underlies spike frequency adaptation and thereby play a role in shaping phasic firing pattern (Stocker et al., 1999). Blocking M current reduces the fast and slow components of AHP without any effect on the medium component in dissociated DA neurons which results in shortening of inter-spike intervals and consequently an increase in firing frequency (Koyama and Appel, 2006). Since AHPs play a fundamental role in shaping burst firing *in vitro* (Alger and Williamson, 1988; Overton and Clark, 1997), it seems natural that M channels participate in generating burst firing of DA cells. This is further supported by reports that muscarinic agonists induce burst firing of DA cells (Zhang et al., 2005) and elevated DA release (Martire *et al.*, 2007). While muscarinic agonists activate multiple signaling pathways, they all lead to closing of M channels (Delmas and Brown, 2005) that operate in the range of membrane potential oscillation observed in bursting DA cells.

1.7. Summary, hypothesis and objectives

In summary, an increase in extracellular DA levels has been proposed as a biological basis for schizophrenia. This increase in DA transmission among other factors is regulated by the firing activity of DA cells with burst firing as the most effective firing

mode to increase DA accumulation at the synapse. The M current makes up components of the AHP resulting in increased interspike intervals that prevent clustering of action potentials to form burst firing. Therefore neurons fire in pacemaker-like pattern rather than in bursts. We therefore hypothesized that blocking M current contributes to burst firing of DA cells in slices.

The KCNQ2/3 heterodimeric channel has much higher potassium conductance than KCNQ2 homomeric channel. The existence of KCNQ3 subunit, even in small amounts, will alter the size of current carried by these channels. We therefore chose to study the KCNQ3 expression in the VTA.

KCNQ3 channel expression follows a neurodevelopmental pattern up to adulthood and disruption in this expression has been proposed in pathophysiology of some disease such as familial neonatal convulsion caused by alterations in the excitability of neurons. Similarly schizophrenic manifestations appear after puberty which is basis of neurodevelopmental hypotheses of the disorder. Our next question was whether the distribution of the channel changes in a rat neurodevelopemental model of schizophrenia. We hypothesized a reduction of KCNQ3 channel expression in VTA DA cells in nVH lesion rats to increase the excitability of theses cells.

To achieve these goals, two preparations were used: horizontal VTA slices and nVH lesioned rats. The slices were used to generate patch clamp recordings using the nystatin-perforated patch technique to examine whether blocking M channels altered firing rates and/or patterns of DA cells. Secondly nVH model was histologically and behaviorally validated. Then KCNQ3 expression was investigated using 3,3'-

diaminobenzidine (DAB) immunohistochemistry. Also colocalization of KCNQ3 channel with tyrosine hydroxylase was visualized with immunoflurescence double labeling in two tiers of VTA , ventral and dorsal that project to different parts of the forebrain.

Chapter 2

Materials and Methods

2.1. Effect of blocking M channels on firing patterns of VTA neurons: Electrophysiological study

2.1.1. Animals

Sprague-Dawley rats of both sexes 13-21 days of age were used for slice preparation. These pups were housed with their mother in the same cage. Animals were on a 12/12 h light/dark cycle in a temperature-controlled environment (20-24°C) and access to food and water *ad libitum* for the nursing mother. All experimental protocols were approved by the Institutional Animal Care Committee at the Memorial University of Newfoundland and conform to the standards set by the Canadian Council on Animal Care.

2.1.2. Slice preparation

Pups were anesthetized by halothane and were killed by a strong compression of the chest. The brain was removed quickly and cooled in ice-cold artificial cerebrospinal fluid (ACSF), which was gassed with carbogen (5% CO_2 / 95% O_2). Horizontal sections through the VTA region were cut at 400 μm on a Leica vibratome. These slices were

incubated in carbogenated artificial cerebrospinal fluid (ACSF) for 1 hour at room temperature prior to recording. A slice was placed into a recording chamber of about 0.5 ml where it was submerged and continually perfused with carbogenated ACSF at a rate of 2-3 ml /min. Usually, one or two slice(s) were generated from one animal and one cell was recorded from each slice.

2.1.3. Perforated patch clamp recording

The recording was made within the confines of the VTA under a dissecting microscope (Leica MZ6). Because it is common that important cellular messengers are lost during conventional whole-cell recording as a result of diffusion of these messenger molecules into the recording pipette, whole cell recording utilizing nystatin to form current conducting channels was used to avoid dilution of diffusible messenger molecules. Patch electrodes were made by pulling KG-33 glass micropipette (OD 1.5 mm, Garner Glass Co., Claremont, CA, USA) on a P-97 Brown-Flaming micropipette puller (Sutter Instruments, Novato, CA, USA). The tip of the electrode was filled with the intracellular solution (see section 2.1.4) and then back filled with the same solution that also contains nystatin (see section 2.1.4). Typical electrodes had a range of resistance between 4 and 6 MΩ. The electrode was placed at an angle of approximately 45° relative to the slice and placed in the VTA. In a fresh slice, the VTA is a bilateral anatomical area between the substantial nigra on each side of the midline. The substantial nigra could be recognized by its semitransparent oval shape. Gigaohm seals were made using a MultiClamp 700B (Axon Instrument, Foster City, CA, USA) amplifier. In spontaneously active cells, the effectiveness of nystatin in providing access was based on size of action

potentials in either spontaneous or evoked by injecting a depolarizing current. It usually took 10-15 min for complete partitioning of nystatin into the cell membrane to give appropriate access which in current clamp mode was reflected by action potential of at least 50 mV that overshot 0 mV. I_h was recorded as the difference in voltage (in current clamp) between instantaneous and steady state readings evoked by a series of current pulses of 2 s in duration at intervals of 5 s for complete recovery of the channels. Data were acquired using pClamp 9 software, sampled at 5 kHz and digitized by DigiData 1320A. Analyses were made with Clamp fit software and Mini-Analysis (Synaptosoft Inc., Decatur, GA, USA).

Spontaneous and evoked firing patterns and action potential characteristics were examined in current clamp mode. Drugs were made as a stock solution and frozen in aliquots until bath application. DA cells were identified using a set of electrophysiological characteristics including: 1) a spontaneous, pacemaker-like firing at low frequencies; 2) a broad spike followed by a long-lasting AHP; 3) a large I_h current (which is identified by a prominent sag in voltage during a long hyperpolarizing current injection); and 4) a hyperpolarizing response to exogenous DA (Johnson and North, 1992). These criteria are practical with fairly good separation of the cell groups in the region, but in a small sample such as ours, it may compound interpretation of the results. After gaining appropriate access, baseline firing was recorded for 5 min before applying any drug. The inter-spike intervals (ISI) were measured with the mini-analysis program and were further used to calculate firing frequency (Hz). Changes in firing rate were evaluated as a percentage of baselines by comparing averaged firing rates over 2 min.

AHP components were defined based on following criteria (Koyama and Appel, 2006; see also Fig.2.2):

1) fAHP: peak amplitude of AHP in <5ms
2) mAHP: peak amplitude of AHP in 5-100ms
3) sAHP: peak amplitude of AHP in 100-300 ms

2.1.4. Chemicals

A) Artificial cerebrospinal fluid composition for slicing and recording (mM): 126 NaCl (Fisher Chemicals), 2.5 KCl (FisherChemicals), 1.2 NaH_2PO_4 (FisherChemicals), 1.2 $MgCl_2$ (FisherChemicals), 18 $NaHCO_3$ (Sigma), 2.4 $CaCl_2$ (Sigma) and 11 glucose (Sigma). pH was 7.4 when bubbled with carbogen.

B) Intracellular solution composition for the tip of electrode (mM): 120 potassium acetate (Fisher Chemicals), 40 Hepes (Sigma), 5 $MgCl_2$ (Fisher Chemicals), and 10 EGTA (Fisher Chemicals), with pH adjusted to 7.35 using 0.1 N KOH.

C) Nystatin solution for back filling of the electrode: Intracellular solution containing 450 μg/ml nystatin (Sigma) and Pluronic F127 (Sigma)

D) Drugs:

M channel blockers: linopirdine (Tocris Cookson Inc), 10, 10-bis (4-pyridinylmethyl)-9-(10H)-anthracenone (XE-991) (Tocris Cookson Inc).

Tabel of content:

Sodium metabisulfite (Sigma), dopamine (Sigma)

2.2. Distribution of KCNQ3 channels in the midbrain of nVH model

2.2.1.1. Animals

Male Sprague-Dawley rats at postnatal day 7 (where PND 0 is the day of birth) were obtained from the Memorial University of Newfoundland Vivarium with their mother and housed on a 12/12 hr light/dark cycle with food and water available *ad libitum*. Litters were culled to 10 pups/litter to eliminate the risk of unequal access to breast milk, which in turn would affect the growth and development of the pups. After surgery on PND 7, described in the following section, pups were returned to their mother. After weaning (approximately PND 21) the rats were housed in groups of 2-3 (same sex) in each cage.

2.2.1.2. Neonatal ventral hippocampal lesion

I used bilateral neonatal ventral hippocampal lesion as a model of schizophrenia in the rats. The Lipska's (1993) and Flores's (1996) method surgery was followed. On PND 7, the pups were randomly divided into two groups: sham-operated and lesion. Hypothermia anesthesia was induced by burying the pups in wet ice for 8-10 min. A pup was secured to a stereotaxic apparatus and a longitudinal incision of 2- 2.5 cm was made in the midline on the dorsal surface of the head. One hole on each side was drilled up to

the dura mater (relative to Bregma: anterior-posterior -3.0 mm, medio-lateral ±3.5mm). A Hamilton syringe needle (26 gauge) was lowered to the ventral hippocampal area (ventro-dorsal -5.0 mm from the dura), 0.3 µl (10 µg/µl) ibotenic acid in bacteriostatic sodium chloride 0.9% or 0.3 µl of bacteriostatic sodium chloride 0.9% was bilaterally injected at a rate of 0.15 µl/min respectively. In order to prevent back flow of the neurotoxin through the needle track, the syringe was left in place for 3 additional minutes before withdrawal. The incision was then sutured and pups were warmed up on a heating pad until they started to breath and their skin color turned pink. Then pups were returned to their mother.

2.2.1.3. Prepulse inhibition test

Prepulse inhibition test (PPI) measures the magnitude of startle reflex and its modulation by a prepulse. At PND 56, rats were transferred to the 20.32 cm plastic cylinder to test their startle response to an acoustic stimulus. This cylinder then was put on the standard sample startle chamber (San Diego Instrument), which was fitted with a speaker for producing acoustic sound bursts. The chamber was equipped with a fan ventilator. The motion of rats in response to each sound burst was detected by a piezoelectric transducer, which was placed directly below the cylinder. Detected motion through transducer was transferred to a computer for recording.

Before each PPI testing, rats were acclimatized to the chamber for 5 min with a background noise level of 60 dB. Each PPI test session included 3 trials: Startle trial (Pulse-alone), Prepulse trial (Prepulse+Pulse) and no-stimulus trial (no-stim). A total 60 trials were performed. The test sessions began and ended with five presentations of the

pulse-alone trial. The same course of pulse alone trails was randomly presented in the middle of PPI trials. Each of pulse alone presentation consisted of 50 msec burst of 120 dB. After this course of pulse alone trails, PPI was measured by prepulse+pulse trials, which consisted of 69, 73 or 81 dB prepulse, following 50 msec 120dB burst (PPI was measured by prepulse+pulse trials which consist of 9, 13 and 21dB above background 60 dB noise).

The no-stim trial consisted of background noise only which in between sessions was presented 10 times in a pseudorandom order. Interval between each presentation was 5. All trils were conducted in the dark.

Average transducer output just prior to the noise burst and peak startle amplitude within each sample was saved as baseline (V_{start}) and V_{max}, respectively. Peak startle amplitude was expressed as V_{max} -V_{start}, which was used in further analysis. After each PPI test session, the cylinder was washed with detergent and water to avoid the effect of released odor in response to stress on the next rat.

2.2.1.4. KCNQ3 expression in the midbrain

The distribution of KCNQ3 was studied by 3, 3'-diaminobenzidine (DAB) immunohistochemistry and immuno-flourecence double labeling on paraformaldehyde-fixed coronal sections. Some of the sections were also Nissl stained for histological estimation of ventral hippocampal damage.

2.2.4.1. Transcardial Perfusion

After completion of PPI test, rats (PND 56, 290-400 g) were deeply anesthetized with thiopental sodium. A midline incision of approximately 4 cm was made from the sternal angle to the xyphoid process. The ribs were bilaterally cut and retracted to expose the heart. A small incision was made in the left ventricle close to the apex. A 26-gauge needle was used for perfusion. This needle was guided to the ascending aorta and was clamped in place for effective perfusion. The right atrium was cut to facilitate perfusion. Approximately 150 ml phosphate buffer (0.1 M) was perfused through the heart using a peristaltic perfusion pump (Master flex, Cole-Parmer Instrument Co.) at a rate of 30 ml/min until the liver turned pale and outflow from the right atrium was clear. At this point, 300 ml paraformaldehyde 4% in 0.1 M phosphate buffer was perfused. The brain was then removed from skull and left in paraformaldehyde 4% at 4° C for 2 hours before transferring to 20% sucrose in 0.1 M phosphate buffer. The brain were left overnight at 4° C until they sunk.

2.2.1.5. Sectioning

The fixed brains were frozen quickly by dry ice and 40 μm coronal sections of the brain were cut frozen using a cryostat at the level of the VTA -5.20 mm to -6.72 mm from Bregma (Paxinos-Watson 1998). Two sections at the level of rostral, middle and caudal VTA (a total of 6 sections per animal) were collected. Sections were mounted

onto cooled gelatin-coated slides where they were left to dry at room temperature for 10 min and then used for immunohistochemical study or Nissl staining.

2.2.1.6. Histological lesion scoring by Nissl (Cresyl violet) staining

Mounted sections were stained using a standard Nissl stain. The identity of the animal was marked on the slide and this was covered when scoring of the lesion was assessed under light microscope (Leica DM LS2) with 4x objective lens. Sections at appropriate levels were chosen to score the size of the lesion on each side according to the Weinberger rating system (Sams-Dodd, 1997) as follows: In each hemisphere, no lesion i.e. cell loss or cavitations in the ventral hippocampus scores 0, small lesion 1, medium lesion around the middle portion scores 2, and lesion in middle side plus lesion in the center scores 3 (Fig.3.3). Bilaterally, each brain could have a score between 0-6. The animals with a total score of less than 4 were excluded from analysis.

2.2.2. Immunohistochemistry

The primary KNQC3 antibody raised in rabbit was diluted 1/400 in phosphate buffered saline (PBS) containing 0.2 % Triton X-100, 2% normal goat serum and 0.02% NaN_3. Sections through the midbrain at the VTA level were incubated on slides for 18-24 hours in primary antibody at 4°C. The slides were then rinsed 3x10 min with PBS at room temperature, followed by incubation in biotinylated goat anti-rabbit secondary antibody for 1 hour. This was followed by another 3x10 min washes in PBS. The sections

were further incubated with Adivin-biotin complex (Vectastain Elite ABC kit) for 1 hour. After 3x10 min rinses in PBS, sections were visualized by a brief exposure (3-4 min) to 0.05% 2, 4 diaminobenzidine (DAB) with 0.01% H_2O_2. Paired groups of nVH lesioned and sham-operated rats were processed in the same batch in order to guarantee identical staining conditions for both groups. Finally, sections were immersed in a series of graded alcohol concentrations, cleared in xylene, and coverslipped.

2.2.3. Expression of M channels in DA cells in the VTA:

2.2.3.1. Immunoflorecence double labeling

To confirm the expression of KCNQ3 channels in DA cells in the VTA and evaluate the proportion of this expression in DA cells in this area, three sections from the rostral, middle and caudal part of the VTA were double labeled with anti-TH and anti-KCNQ3. In this part of the study, free-floating sections were incubated in a cocktail of rabbit anti-rat KCNQ3 (1:100) and mouse anti-rat TH (1:500) primary antibodies for 16-20 hours. The primary antibodies were diluted in PBS containing 4% normal goat serum (NGS), 0.2 % triton X –100 and 0.02% NaN_3. After 3x15 min rinsing in PBS, sections were then incubated in a secondary antibody cocktail of Cy3-conjugated goat anti-rabbit (1:800) and Cy2-conjugated goat anti-mouse (1:200) antibody for 90 min. They were rinsed again in PBS for 3x15 min and were mounted on gelatin-coated slides and coverslipped using an anti-fade mounting gel (Aqua poly/Mount). The slides were stored in dark at 4 °C until analysis.

2.2.3.2. Image analysis

The following analyses were done blindly, i.e. the identity of experimental groups (lesion or sham) was hidden while observing the labeled sections.

A) Camera Lucida

The distribution of KCNQ3 channels in the midbrain was compared in lesioned and sham groups using camera lucida. DAB-stained sections for KCNQ3 were examined under light microscope with an x4 objective. The labeled cells in coronal sections at the level of the VTA (-6.30 from Bregma) were marked.

B) Neuro Lucida

Also using DAB-stained sections, the number of KCNQ3-positive cells in three areas of the midbrain (the VTA, oculomotor nucleus and red nucleus) were quantified using NeuroLucida® (MicroBrightfieldeild). The identity of the slide was obscured during quantification. The area was first defined by the NeuroLucida software; labeled cells were marked individually by hand under objective magnification of x20 and counted by the software. Upon revealing slide identities, the number of labeled cells for each nucleus was compared between the lesioned and sham groups.

C) Confocal microscopy

Confocal microscopy (FV300 scan head, BX50WI upright microscope; Olympus) was used to investigate the colocalization of TH-positive (as a marker for DA cells) and KCNQ3-positive cells and to evaluate the proportion of DA cells containing KCNQ3 in the VTA. The confocal microscope was equipped with blue argon (488 nm) and green helium neon (543 nm) to visualize Cy2 and Cy3 labeled cells, respectively. High magnification images were captured using a 20x objective lens and a zoom setting of 3x. Captured images were imported into Photoshop version 8 where brightness and contrast were adjusted. Labeled DA cells were counted separately in two main anatomical subdivisions of the VTA, the dorsal and ventral tier. These two tiers were defined based on their morphological feature: ventral tier consisted of sheet of highly dense packed angular cells located in ventral zone of VTA. In contrast dorsal tier cells were located in dorsal part of VTA with scattered pattern of distribution (Prensa and Parent, 2001; Thompson *et al.,* 2005).

Total labeled cells were counted from two blocks (250 μm x 25 μm) in the ventral tier and three blocks (250 μm x 250μm) from the dorsal tier. The percentage of colocalized cells with anti-TH was estimated in each group: [(Total co-labeled cells with anti TH and KCNQ3 in defined blocks of each tier in the same blocks) x 100] / (Total labeled cells with anti-TH). The non-colocalized percentage was obtained as follow: [(Total labeled cells with anti-KCNQ3 – total co-labeled cells with anti-TH and KCNQ3) x 100] / Total labeled cell with anti-KCNQ3.

2.2.5. Chemicals and antibodies:

A) **Antibodies:** Mouse anti-TH monoclonal (Sigma), Rabbit anti-KCNQ3 (Alomone Labs), Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), Cy2-conjugated goat anti-mouse IgG (Jackson ImmunoResearch)

B) **Chemical and drugs:** 2, 4-diaminobenzidine-tetrahydrochloride dihydrate (Amresco), Thiopental sodium (Vetoquinol), Normal goat serum (Vector), ABC kit (Vector), bacteriostatic sodium chloride 0.9% (Abbott Laboratories), Aqua poly/Mount (Polysciences, Inc) .

2.2.6. Statistics

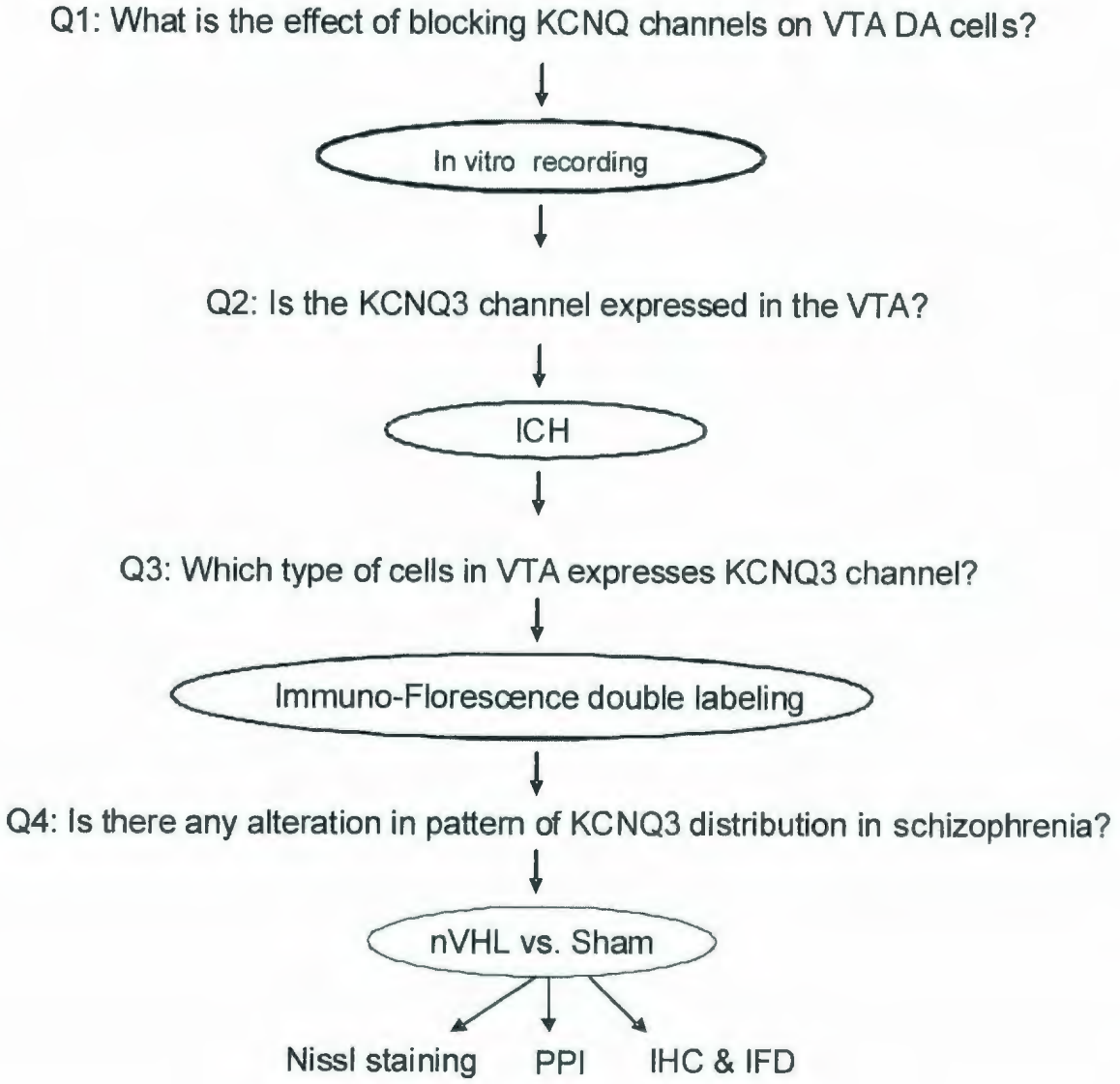
For all experiments except the PPI test, data were compared statistically with two-tailed Student *t*-Test (paired or unpaired as appropriate). In the electrophysiological part statistical comparisons were made using paired *t* tests, with a minimal number of 6 cells in each group.

For IHC, results from a minimum of 8 rats in each group were required for statistical comparisons. Rats in the lesioned groups would needed to have a lesion score of at least 4 (see section 2.2.1.6 and also fig 2.1) to be included in the analysis. In PPI test using two-tailed Z test, median V_{max} -V_{start} of sham and lesion group were compared. In addition, with Fisher test average of V_{max} -V_{start} of 5 first, within and last pulse alone

trials were compared to study the possibility of habituation. $p < 0.05$ was considered significant. Results are expressed as mean ± SEM.

2.7.7. Summary of study plan

The plan of the present study is summarized in figure 2.1.


Q1: What is the effect of blocking KCNQ channels on VTA DA cells?
In vitro recording
Q2: Is the KCNQ3 channel expressed in the VTA?
ICH
Q3: Which type of cells in VTA expresses KCNQ3 channel?
Immuno-Florescence double labeling
Q4: Is there any alteration in pattern of KCNQ3 distribution in schizophrenia?
nVHL vs. Sham
Nissl staining
PPI
IHC & IFD

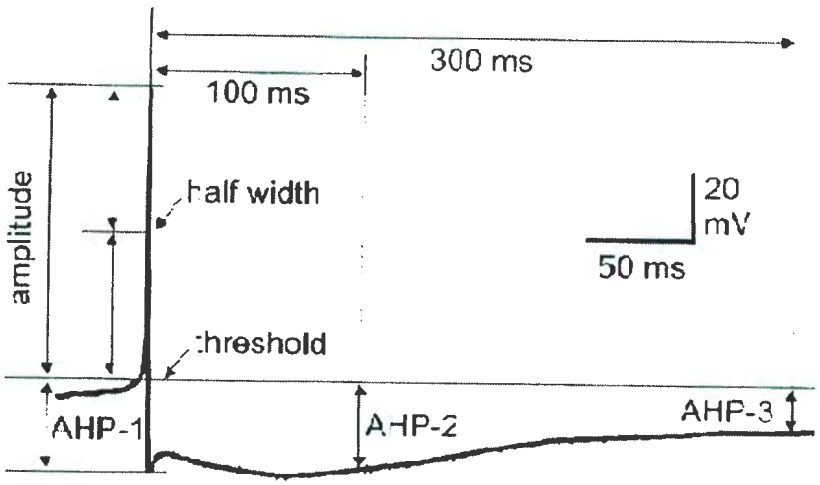


Fig.2.2) Diagram illustrating measurement of the AHP components in VTA DA neurons.

Adapted from: J Neurophysiol 96: 535-543, 2006

Chapter 3

Results

Part A- Electrophysiology

In the electrophysiological part of the present thesis, I evaluated the effect of blocking M channels on the excitability of VTA cells. Based on their response to exogenous DA and their intrinsic membrane properties, I classified VTA cells into two groups: putative DA projection neurons and GABAergic interneurons as illustrated in Fig.3.1. DA cells displayed relatively slow firing frequency and wide action potentials (Fig 3.1a), a prominent afterhyperpolarization-activated current (I_h) (Fig 3.1c) and a hyperpolarization in response to exogenous DA (Fig 3.1b). Putative GABAergic cells reveal fast firing without a prominent AHP or hyperpolarization-activated I_h and are hyperpolarized by the $\bar{\mu}$-opioid receptor, DAMGO (Fig 3.1d,e,f).

According to these criteria, using nystatin–perforated current clamp recordings we examined 14 DA cells and 4 GABA cells in the VTA. A majority of recorded cells were spontaneously active (17 of 18, 94.4%). After recordings became stable, a period of 5 min baseline was recorded for measurement of resting membrane potential, basal firing rate and action potential characteristics. Membrane voltage and firing responses to DA

(50 μM) and/or DAMGO (1 μM) was measured by applying the drugs to the bath for a brief period. After washing out the effects of DA or DAMGO, linopirdine (n= 10, all DA cell) or XE-991 (n=8: 4 DA and 4 GABA cells) was tested.

DA cells displayed a low basal firing rate (0.16 ± 0.02 Hz, n=14) while GABA cells had a significantly higher firing rate (0.8 ± 0.07 Hz, n=4). Resting membrane potential was higher in DA cells (-47.7 ± 0.7 mV) than in GABA cells (-56.0 ± 0.4 mV). DA cells showed relatively wider action potentials (half width: 2.8 ± 0.3 ms for DA cells vs. 2 ± 0.1 ms for GABA cells).

Fourteen of 18 cells (75%) responded to a brief (average 40 s) application of 50 μM DA with a significant hyperpolarization (-6.4 ± 03 mV) accompanied by a complete inhibition of firing. The remaining 4 cells that did not respond to DA responded to DAMGO with a hyperpolarization (-2.2 ± 0.3 mV). A much larger I_h voltage sag (34.6±3.9 mV) was recorded in DA cells than in non-DA cells (1.9 ± 0.7 mV, $p< 0.05$, Fig .3.1).

3.1. Blocking M channel excites DA VTA cells

M channel blockers linoperdine (10 μM) and XE-991 (5 μM) were tested on 10 and 4 putative DA cells, respectively. The effect of XE-991 was also tested on 4 GABA cells. The effect of these blockers on firing rates was assessed by comparing using paired t-test average firing rates at the peak of the response with basal firing rates of at least 5 min stable recording.

3.1.1. Linopredine and XE-991 excite VTA DA cells

Bath application of 10 μM linopirdine excited 90% DA cells tested (9 out of 10) by 37.5 ± 4.2% at the peak of the response. The onset of the increase in firing rate was slow; ranging 5-15 min following continuous drug application. The response was reversible following 7-15 min washout. In 3 of 4 DA cells, XE-991 (5 μM), another M channel blocker, induced a similar increase in firing rate (28.6 ± 2.7%) that was irreversible upon washout for 50 min. This increase in firing appeared to be related to a depolarization following drug application (2.1 and 2.9 mV depolarization after linopirdine and XE-991 application, respectively) accompanied by a reduction in the medium components of the AHP following each action potential (25.2 ± 3.6 mV for control vs. 21.3 ± 2.8 mV following linopirdine, $p< 0.05$). In contrast application of XE-991 for 25 min did not excite GABA cells (Fig .3.2; n=4).

3.1.2. M-channel blockers induce burst firing.

Besides increasing the firing rate, the two M channel blockers also induced a change in firing patterns. Linopirdine induced a change in firing pattern from regular spiking to burst firing in 7 out of 10 cells tested (Fig. 3.2). Burst firing induced by linopirdine, occurred after 30.7 ± 4.5 min following the start of drug application and was reversible following washout of 12 ± 2.4 min. Burst firing was preceded by an increase in firing rate. XE-911 induced similar switching in the firing pattern of 2 of 4 DA cells.

However, it caused no burst firing in the 4 GABA cells tested. The onset of burst firing following XE-991 application was 19.5 ± 4.5 min, this effect of XE-991 was not reversible after 50 min washing in both of these cells.

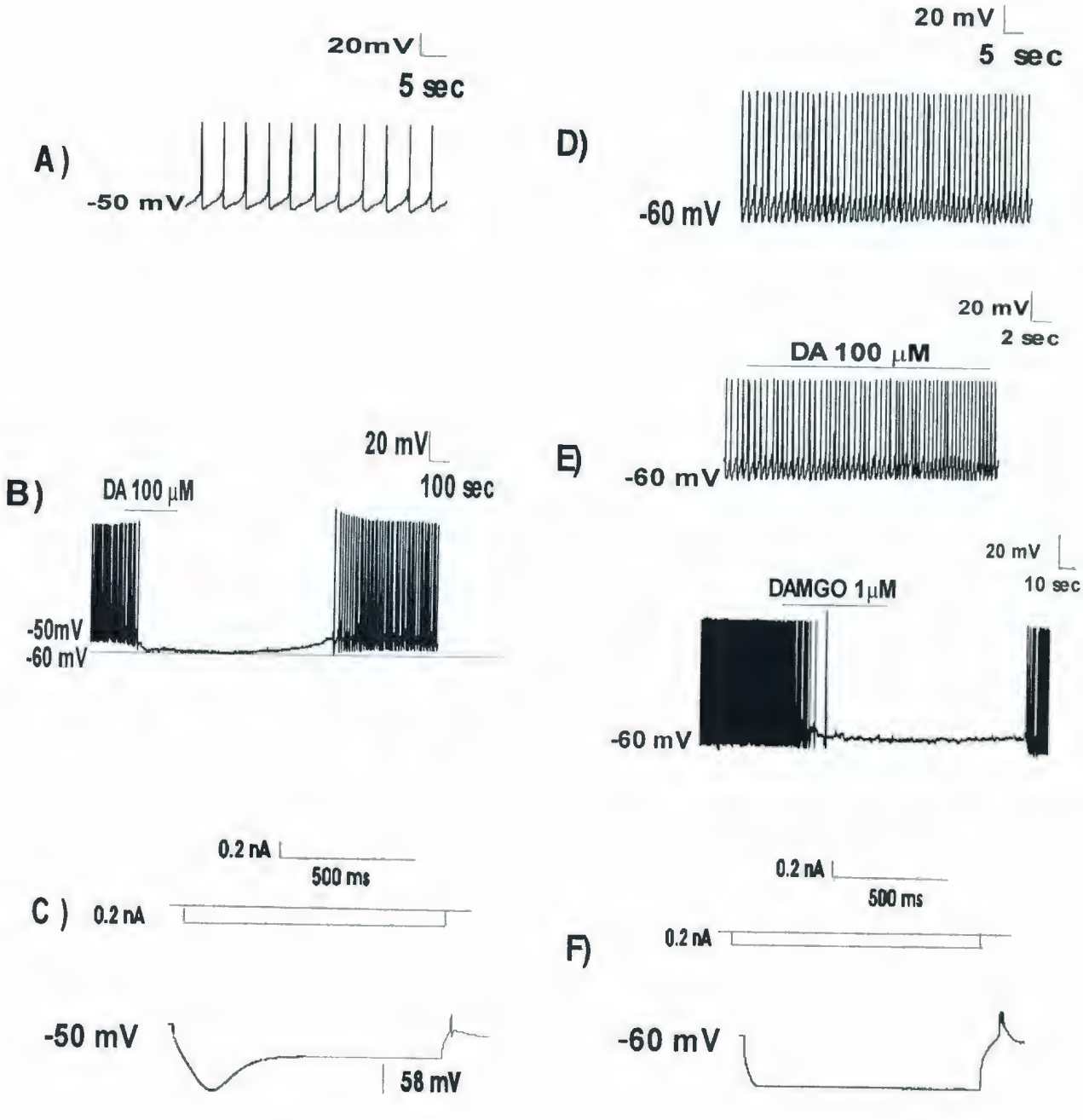

20mV
5 sec
A)
-50 mV
20 mV
5 sec
D)
-60 mV
20 mV
2 sec
DA 100 μM
E)
-60 mV
20 mV
100 sec
B)
DA 100 μM
-50mV
-60 mV
20 mV
10 sec
DAMGO 1μM
-60 mV
0.2 nA
500 ms
C)
0.2 nA
-50 mV
58 mV
0.2 nA
500 ms
0.2 nA
F)
-60 mV

Fig.3.1. Characteristics of putative DA or GABA cells in current clamp

Classification of VTA cells was based on three main electrophysiological parameters in current clamp mode characterized as DA or GABA cells. Panels A-C belong to the same putative DA cell while panels D-E represent the same putative GABA cell.

A) At room temperature a putative DA cell showed slow regular firing (0.2-0.3 Hz) with RMP of approximately -50 mV. **B)** Hyperpolarization in response to 100 μM DA. **C)** Presence of a time-dependent inwardly rectifying I_h in response to hyperpolarization. **D)** GABA cell was spontaneously active with more negative RMP in comparison with DA cell (approximately -60) and faster firing (3-4 times faster). **E)** Application of 100 μM DA did not have any response, instead DAMGO (0.1 μM) induced hyperpolariztuon. **F)** The time-dependent inwardly rectifying current to hyperpolarization in GABA cell was not seen.

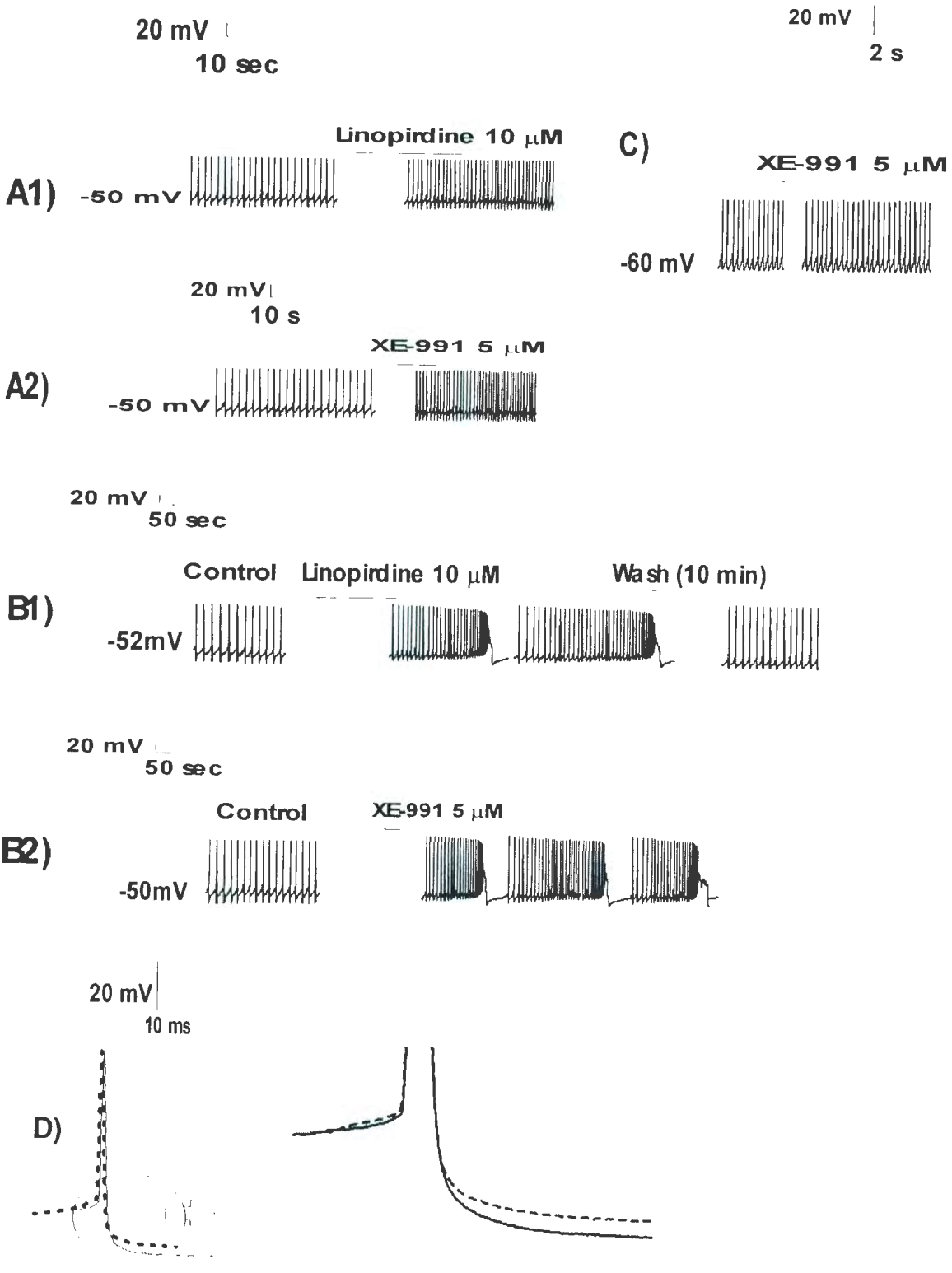

20 mV
10 sec
20 mV
2 s
Linopirdine 10 μM
A1)
-50 mV
C)
XE-991 5 μM
-60 mV
20 mV
10 s
XE-991 5 μM
A2)
-50 mV
20 mV
50 sec
Control
Linopirdine 10 μM
Wash (10 min)
B1)
-52mV
20 mV
50 sec
Control
XE-991 5 μM
B2)
-50mV
20 mV
10 ms
D)

Fig.3.2. Blocking M-current in VTA DA cells increases firing and induces burst firing

A1) A DA cell responded to the M channel blocker, linopiridine (10μM), with increased firing from 0.2 Hz to 0.5. **A2)** XE 991(5 μM) excited DA cell (from 0.2 to 0.9 Hz)

B1) Bath application of linopirdine (10μM) after 25 min resulted in burst firing which recovered after washing. **B2)** XE-991 (5 μM) induced burst firing which was persisted after 50 min washing.

C) Application of XE-991 (5 μM) could not change frequency or pattern of firing in a GABA cell.

D) Linopirdine or XE-991 reduced mAHP component of AHP. Solid and dashed are AP before and 15 min after application of linopirdine respectively.

Part B- Immunohistochemistry

This part describes the expression of KCNQ3 channels in the VTA of sham and nVH lesioned rats. The first section summarizes histological assessment of the hippocampal lesion, followed by behavioral validation of the model based on PPI tests, and finally, the immunohistochemical visualization of KCNQ3-positive cells in the VTA along with their neurochemical identification.

Ibotenic acid was injected into bilateral ventral hippocampus of 20 male and 6 female pups from four litters to induce lesion. In each of the 4 litters, 2-4 (total of 12) male pups received the same amount of normal saline using the same coordinates to constitute the sham group. Of 20 male nVH lesioned rats that had adequate lesion scores and a significant deficit in PPI of startle reflex in comparison with sham group, 8 animals were further used for KCNQ3 immunohistochemistry.

In the second batch of animals, 10 males were lesioned with ibotenic acid and 6 males were sham-operated controls. 8 of the 10 lesioned rats had a score of 4 or above and were used in double florescence labeling for KCNQ3 and tyrosine hydroxylase.

3.2.1. Ventral hippocampal lesion assessment

Ventral hippocampal lesions induced by bilateral injection of ibotenic acid were assessed in Nissl stained coronal sections at PND 56. Of 20 male animals in the lesion

group, six rats showed unilateral or no lesions and were therefore excluded from the study. 14 brains showed bilateral lesions that were further scored based on Lipska's scoring system as described in the Materials and Methods section. In 9 of 14 brains, lesions started at the level of -4.52 (relative to bregma) and extended to -5.20mm; however, in the remaining 5 brains, lesion was visible at -5.20 through -6.72. The lesion was scored at the level that showed most damage. This yielded 8 male brains receiving a score of 4 or more (4.5 ± 0.3) that were included in the study (Fig.3.3).

A) Lipska's scoring system **B) Nissl stained sections in sham nVH rats**

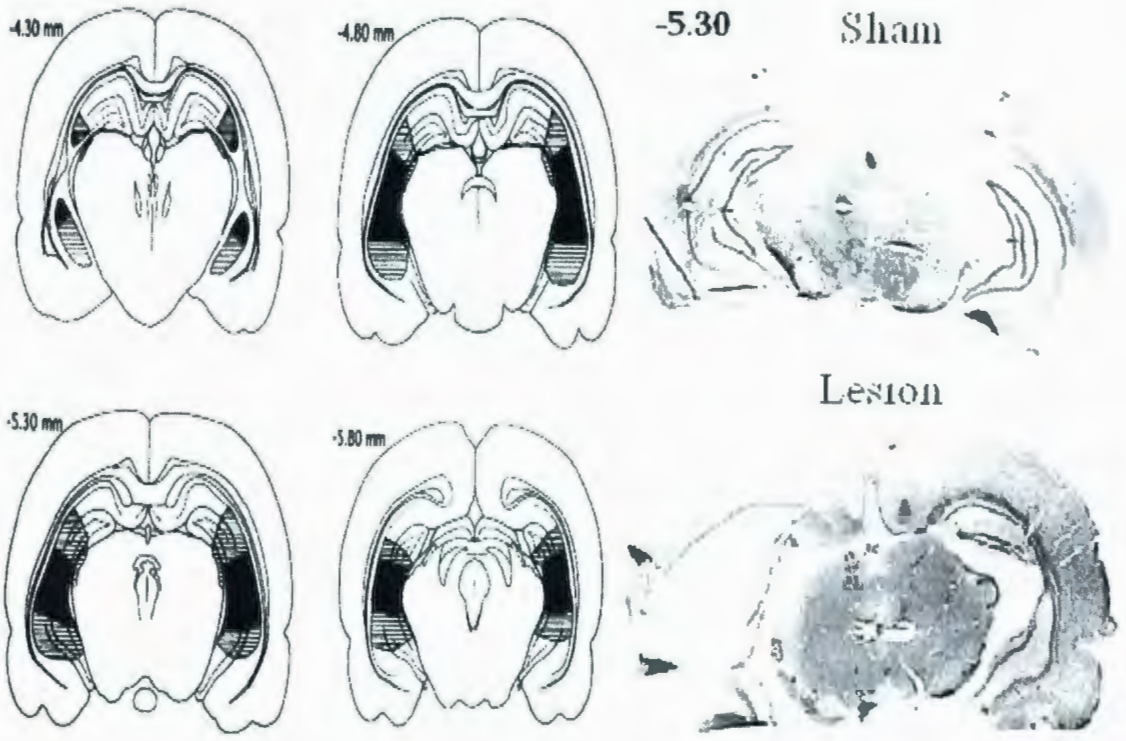


Fig.3.3. Scoring lesion in nVH rats based on Lipska's rating system:

A) Lesion boundaries defined as the area of neuronal absence and determined from thionin-stained coronal sections from rats with nVH lesions. Coordinates refer to distance (mm) posterior to bregma. Black and striped areas indicate the smallest and largest lesions, respectively.

Adapted from: Proc. Natl. Acad. Sci. USA .Vol. 92, pp. 8906-8910, September 1995,Neurobiology

B) Representative Nissl stained image of nVH lesion at the midbrain level

3.2.2. Neonatal lesion of the ventral hippocampus results in PPI deficit in adult rats

In the first series of PPI tests, developmental changes in PPI were evaluated in 12 male rats in lesion group and 6 male rats in sham group. The PPI test was repeated at PND 35, PND 49 and PND 56. In each experiment the peak of startle reflex in response to pulse alone (120 dB) and pulse (120 dB) following 69 dB, 73 dB and 81 dB prepulse between lesion and sham group were compared. An increase in the magnitude of the response over the weeks in both groups was detected for all trials. Nevertheless, only at PND 56 did the differences between sham and lesion animals become significant with a deficit in inhibition following all three prepulses tested in lesion animals ($p< 0.05$, Fig.3.4).

To study the possibility that alteration in body weight over the weeks affected the startle reflex response, mean body weight of the rats were compared. Although there was a significant increase in body weight from PND 35 to 56 in both groups (non-paired Student's *t* test $p< 0.05$, Fig.5), in each period of experiment (35, 49 and 56 PND) the difference was not significant between sham and lesion rats (2 tail z, Mann Whitney U test for differences in medians, $p<0.05$, Fig.3. 5).

It is also possible that animals habituated to the auditory stimulus. To test this possibility, additional two litters underwent sham or nVH lesions. The nVH animals fulfilled the same histochemical and behavioral criteria. Habituation was tested by giving a series of 120 dB pulse before, during, and after PPI tests. The result indicates that PPI has not induced significant habituation of the startle response to the 120 dB stimulus (Fig.3.6).

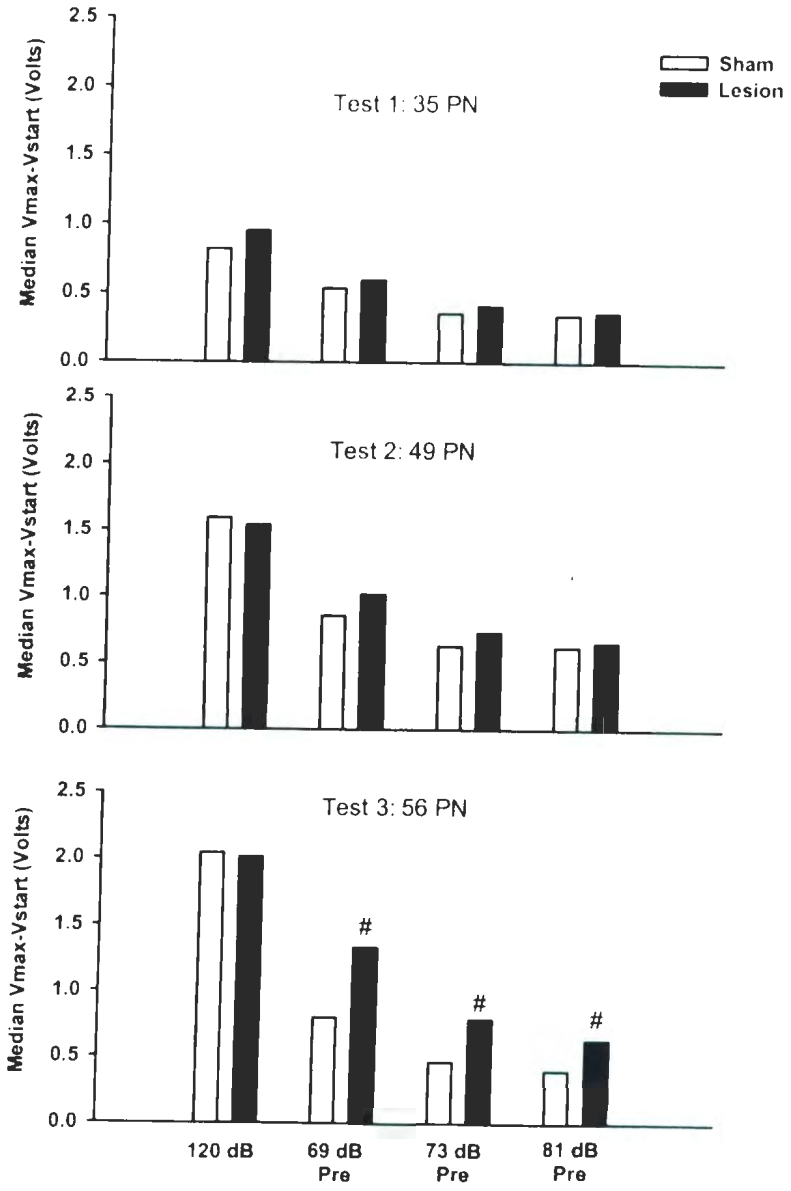


Fig.3.4. PPI at different PND

In nVH lesion rats PPI for all three prepulses (69, 56 and 81 dB) showed a gradual decrease of PPI magnitude over PN 35-56 days. This deficit of inhibition was significantat 56 PND in comparison with sham for all three prepulses.

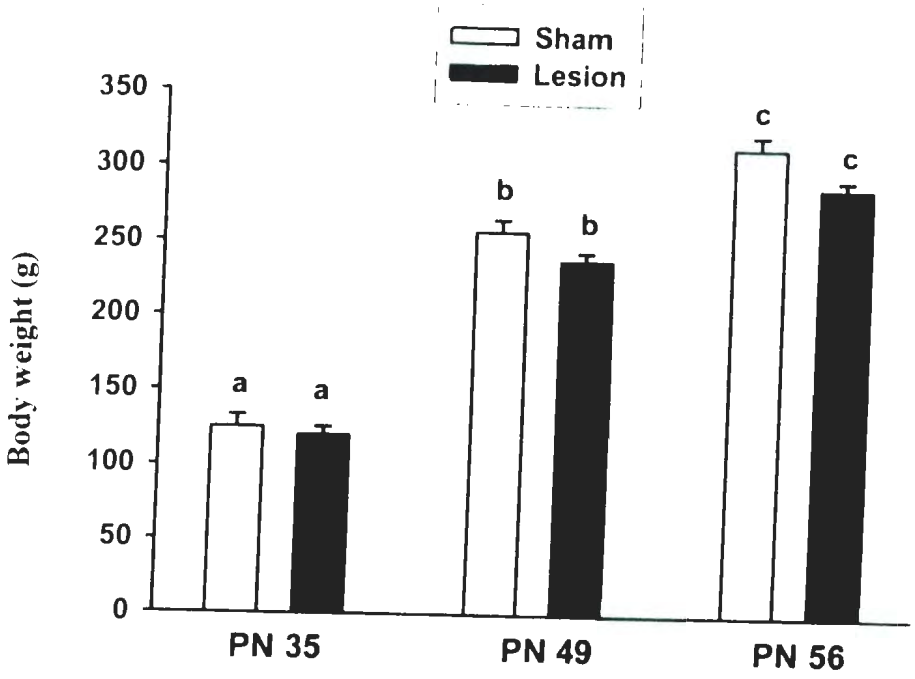


Fig.3.5. Bodyweight changes over PND 35-56

Both sham and nVH lesion rats gained weight body over the testing period, but no difference was noted at any of the testing points.

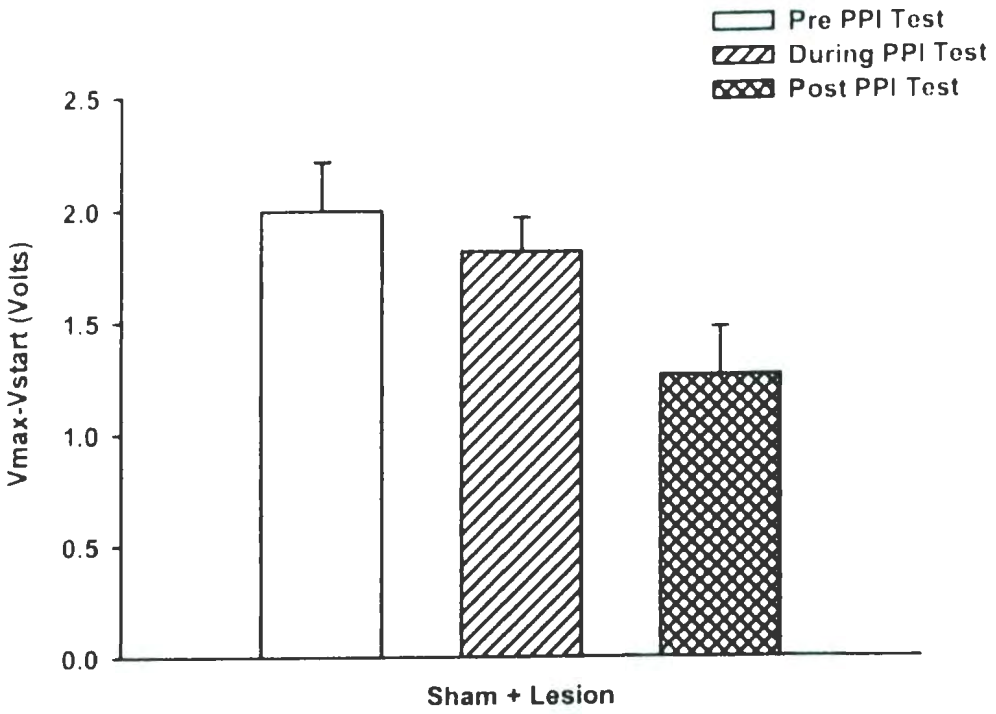


Fig.3.6. 120 dB trial analysis pre, during and post PPI trials

Comparing magnitude of response to five 120 dB trials during and post PPI trials with same series of pulses prior to PPI test showed a decrease in response which was not significant.

3.2.3. Changes in KCNQ3 channel expression in the VTA of nVH lesioned animals

The expression of KCNQ3 channels in the midbrain was first analyzed in DAB stained coronal sections. Using camera lucida, the number of VTA cells positive for KCNQ3 was quantified (Fig.3.7). The specificity of staining was confirmed by preincubation of anti-KCNQ3 with KCNQ3 peptide. Brain sections incubated with the cocktail of anti-KCNQ3 and the peptide did not show labeled cells in any part of the section.

For quantifying KCNQ3 expression, Neurolucida was used (Fig.3.7). Because the area of the defined VTA varies from section to section, the number of positive cells was as expressed number of cells per 10^4 μm^2. The average defined area for VTA was 40000 ± 7000 and 45000± 4000 μm^2 in shams and nVH lesion animals respectively which was not statistically different (n=8 in each group). The number of labeled cells in the VTA was significantly less in lesioned animals (2.0 ±0.2 cell/10^4 μm^2, n=8) than in the sham-operated (3.4± 0.4 cell/10^4 μm^2, n=8), ($P<0.02$, Fig.3.8).

3.2.4. Increase in KCNQ3 channels in the red nucleus and oculomotor nucleus of nVH lesioned animals

There was an intense staining for KCNQ3 channels in other midbrain areas, most notably the red nucleus and the oculomotor nucleus. KCNQ3 labeled cells were large and clustered in these areas.

Quantification of KCNQ3-positive cells with neurolucida revealed that in both the red nucleus and oculomotor nucleus alterations in KCNQ3 expression was significant. In the red nucleus, normalized number of positive cells was 2.4 ± 0.2 cell/$10^4\mu m^2$ in sham-operated group (n=8). This number increased to 3.7 ± 0.4 cell/$10^4\mu m^2$ in nVH lesioned animals (n=8) ($p<0.02$ Fig.3.8). Using the same method, nVH lesioned rats showed 7.8 ± 1.1 positive cells in the oculomotor nucleus compared to 3.8 ± 0.67 cell/$10^4\mu m^2$ in sham-operated rats ($p<0.001$ Student's *t* test, Fig.3.8).

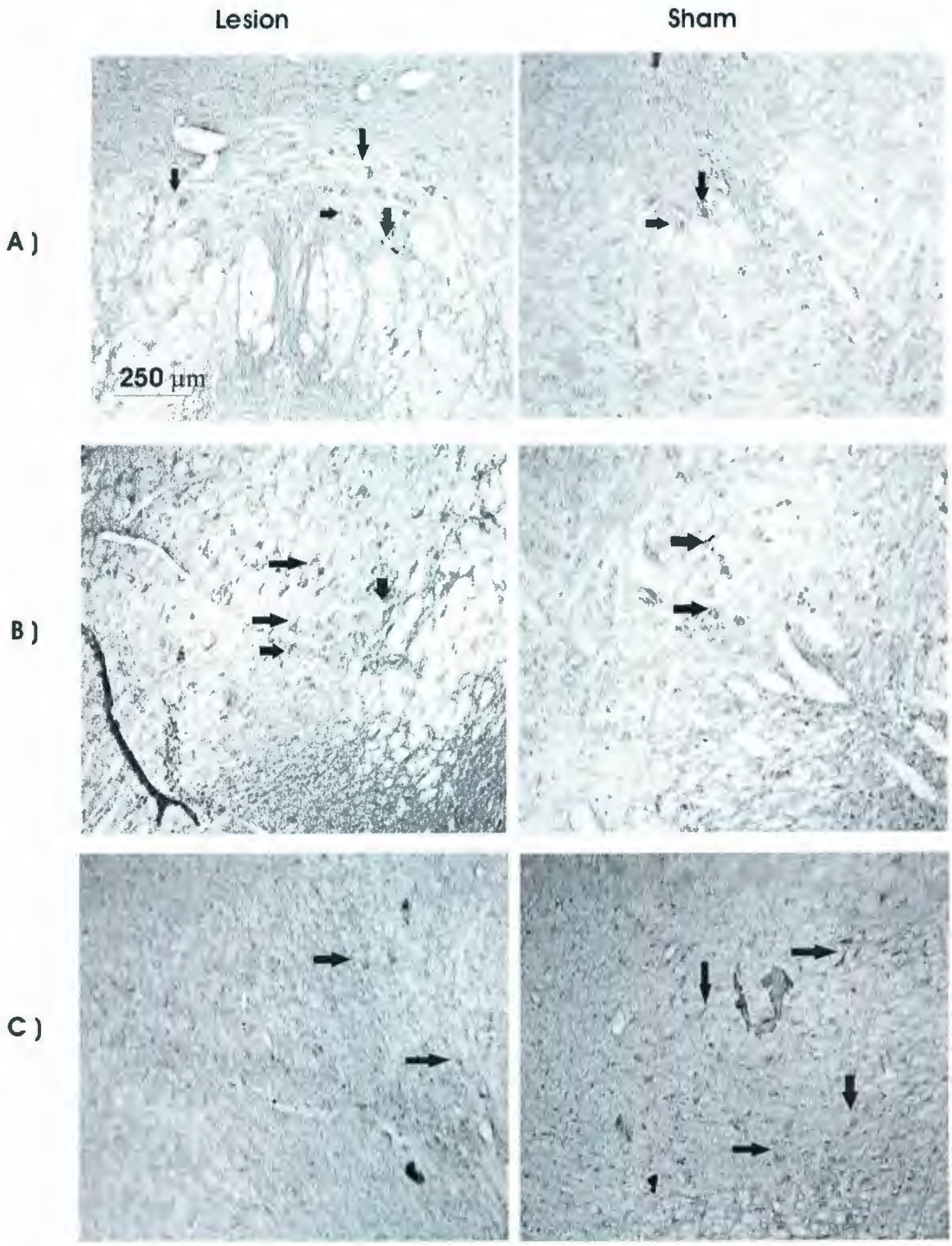
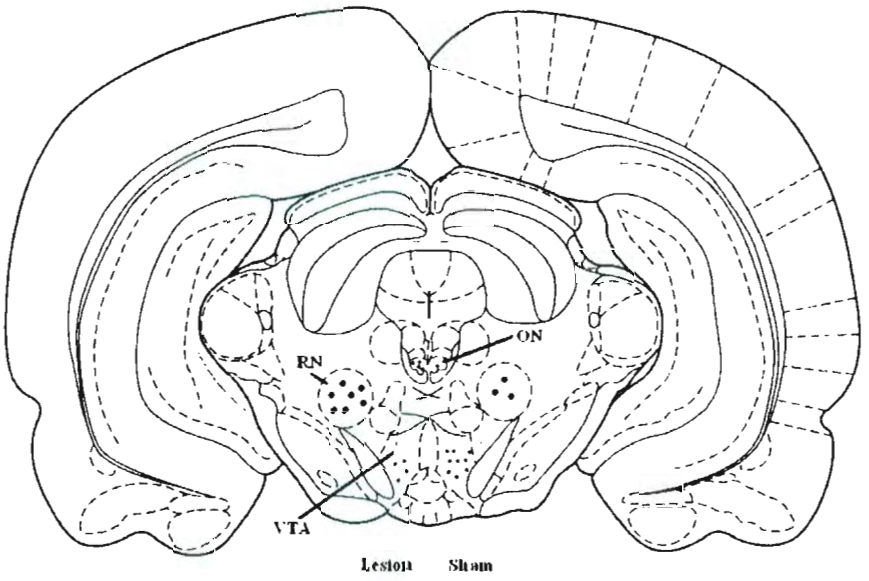

Lesion
Sham
A)
B)
C)
250 µm

Fig.3.7. Expression of KCNQ3 channel in red nucleus, VTA and oculomotor nucleolus in sham and nVH lesion rats

Expression of KCNQ3 labeled cells oculomotor nucleolus (A) and red nucleus (B) of nVH lesion (left) rats, using neurolucida, was higher than sham (right) group. This expression in VTA (C) of nVH lesion (left) rats, in contrast, was lower than sham (right) group.

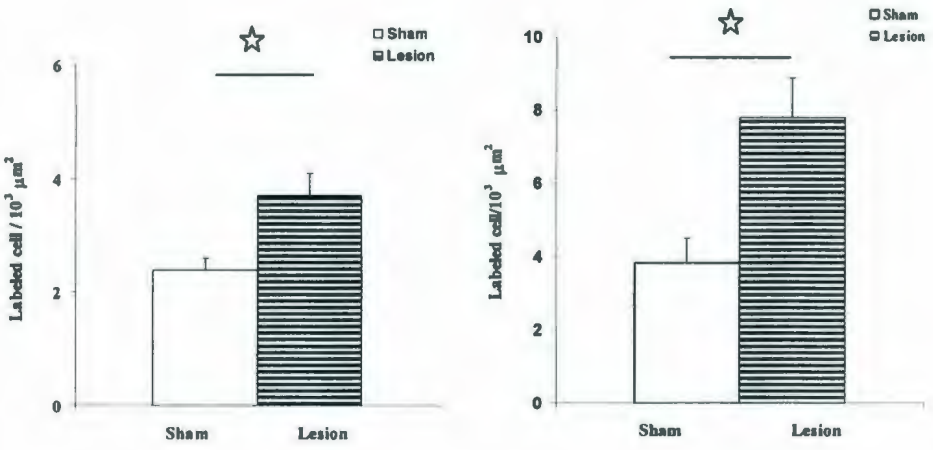
D) Camera lucida drawing at x4 magnification showing patterns of distribution of KCNQ3 labeled cells in the VTA, oculomotor nucleus and red nucleus (-6.04 from bregma). nVH lesion rats (presented at the left side of the schematic brain section) had fewer labeled cells in the VTA in comparison with sham group (presented at the right side of the schematic brain section). The expression in oculomotor nucleus and red nucleus was higher in nVH lesion rats than sham group.

D)



A)

B)



C)

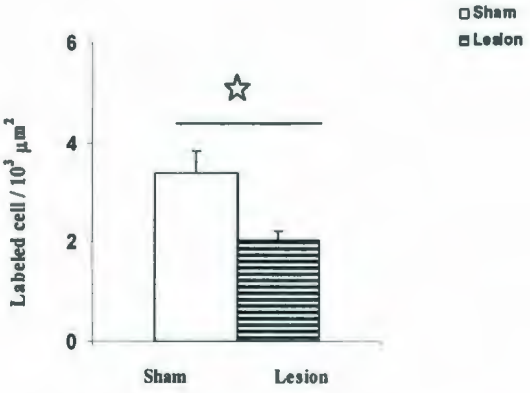


Fig.3.8. Distribution of KCNQ3 channel in oculomotor nucleus, red nucleus and VTA cells

Using neurolucida with x10 magnification the labeled cells with anti-KCNQ3 in the red nucleus (A), oculomotor nucleus (B) and VTA (C) were counted. Comparing the average number of labeled cells in two groups, there was a significant increase in red nucleus ($P<0.05$) and oculomotor nucleus ($P<0.001$) and decrease in the VTA ($P<0.05$) in nVH lesion group.

3.3. KCNQ3 expression in DA and non-DA cells in the VTA

Another set of experiments was performed on 16 rats to study KCNQ3 expression in DA and non-DA cells in the VTA using confocal microscopy. Of the 10 nVH lesioned rats, 8 rats received a minimum score of 4 (4.9 ± 0.4) and were included in the immunoflorescence study. TH and KCNQ3 labeled cells and the percentage of double-labeled cells were assessed by confocal microscopy (Fig 3.9). The VTA was divided into the ventral and the dorsal tier as described in the Methods section; positively labeled cells were counted in each of the two tiers. Counting was made in 2 blocks in the ventral tier and 3 blocks in the dorsal tier (each block was defined as the total field of a 20x objective lens with a zoom setting of 3x which was 25 x 250 μm). Average number of labeled cells with each antibody was derived for the ventral and the dorsal tier separately, the percentage of colocalization of the two antigens was also compared between the lesioned (n=8) and sham operated rats (n=6).

3.3.1. KCNQ3 expression in DA cells declines only in the dorsal tier of the VTA in nVH lesioned rats

Double labeling with anti-KCNQ3 and anti-TH in the VTA showed that both DA and non-DA cells expressed the KCNQ3 antigen. Percentage of colocalization of KCNQ3 and TH to total TH-positive cells declined significantly in the dorsal tier of the VTA in lesioned rats (56.30± 3.66, n=8) in comparison with sham animals (n=6, 73.01 ± 2.8,

$p<0.001$, Fig.3.10). No such difference was detected in the ventral tier (77.1% in sham vs. 81.0 in nVH lesion).

3.3.2. KCNQ3 expression in non-DA cells in the VTA does not change in nVH lesioned rats

In sham and lesion groups, 40.92 % ± 2.1 and 41.2 %± 1.6 of VTA dorsal tier cells which were TH negative, labeled with anti KCNQ3, respectively. On the other hand, in the VTA ventral tier 55.9 ± 2.2 % and 54.2 ± 2.7% of TH negative cells in sham and lesion rats were also KCNQ3 positive. In each tier, differences in two groups were not significant. However, when ventral and dorsal tiers were compared with each other, the percentage of non-colocalized KCNQ3 positive cells were significantly higher in the ventral tier for both sham and nVH lesion rats ($p<0.001$ for sham and $p<0.05$ for nVH lesion, Fig.3.11).

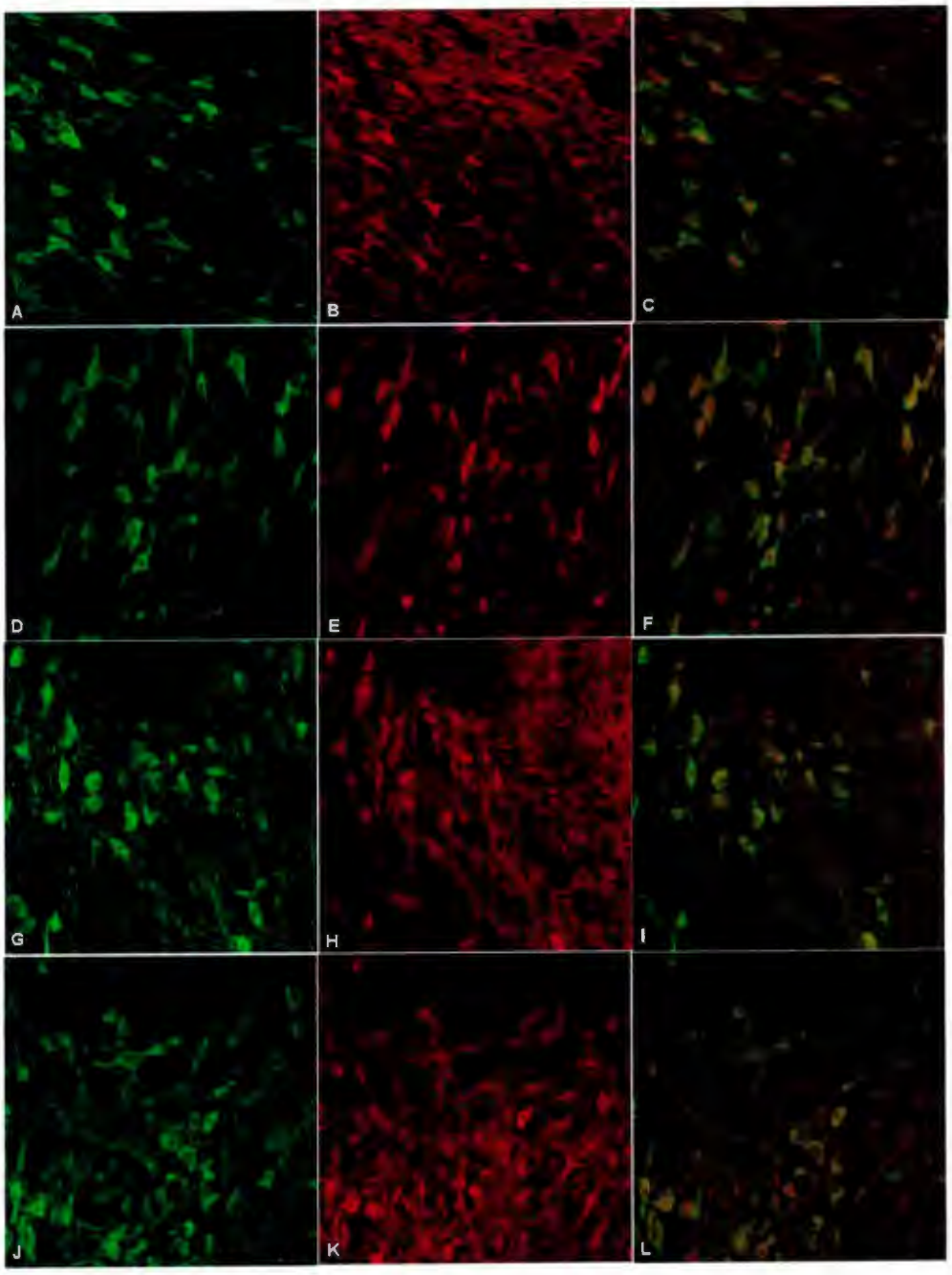

A
B
C
D
E
F
G
H
I
J
K
L

Fig.3.9. Distribution of KCNQ3-positive cells and their colocalization with TH in the ventral and dorsal tiers of the VTA in sham and nVH lesioned animals

Labeled cells with anti-TH in sham (A) and anti-KCNQ3 (B) showed less co-localization (C) in comparison with nVH lesioned rats (F) in the dorsal tier. Panels D and E show immunoreactive cells with anti-TH and KCNQ3 in nVH lesioned rats, respectively.

In the ventral tier, positive labeled cells with anti-TH (sham, G; nVH lesioned rats, J) and anti-KCNQ3 (sham, H; nVH lesioned rats, K) revealed that percentage of colocalization of TH and KCNQ3 was the same in sham (I) and lesioned rats (L).

A) B)

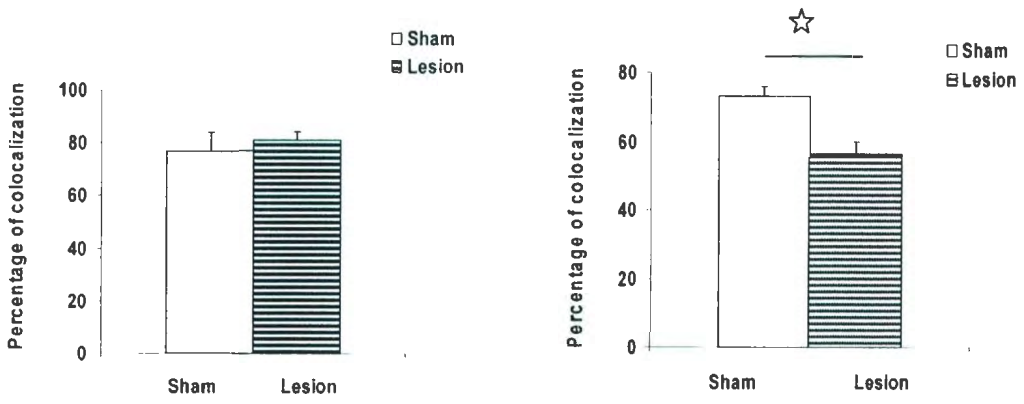


Fig.3.10. Expression of KCNQ3 channel in DA cells of the ventral and dorsal tiers of the VTA

A) Percentage of colocalization of KCNQ3 and TH in the ventral tier was similar between sham and nVH lesion groups. The percentage in each group was estimated by counting colocalized labeled cells with anti- KCNQ3 and TH in two blocks of ventral tier with magnification of x20 of confocal microscopy.

B) This percentage in the dorsal tier was significantly lower in nVH lesion group ($p<0.001$).

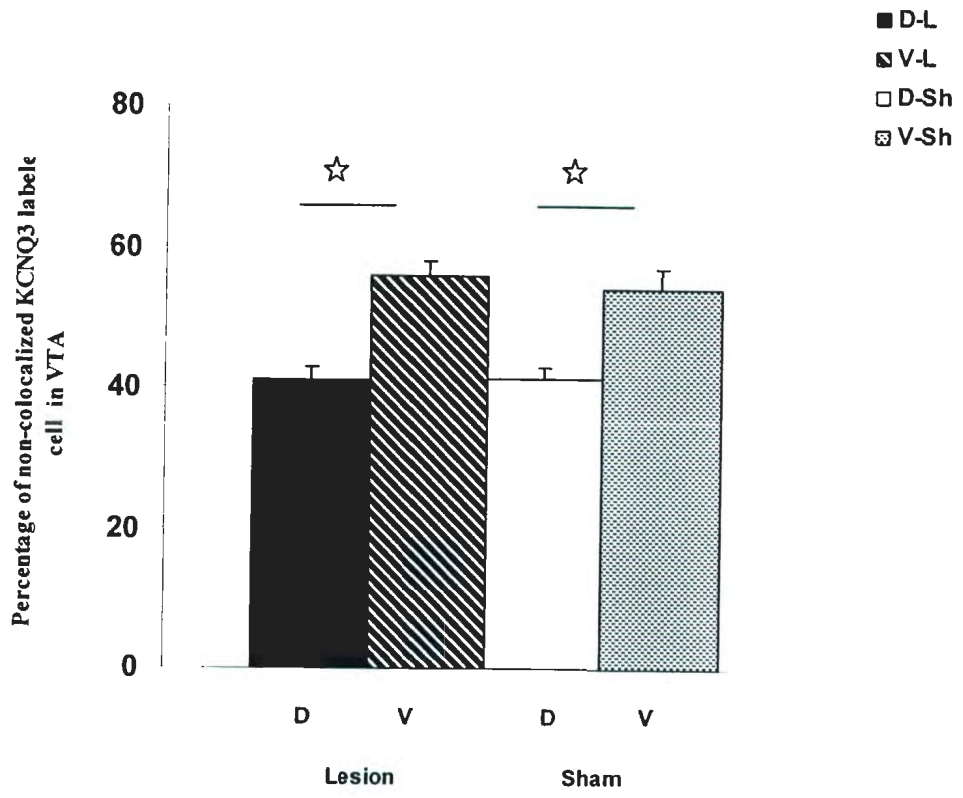


Fig.3.11. Percentage of non-colocalized KCNQ3 labeled cells in dorsal and ventral tiers of the VTA

Percentage of TH negative cells which labeled with anti-KCNQ3 was similar in the ventral or dorsal tier of sham and nVH lesion groups. This percentage was significantly was higher in the ventral tier ($p<0.001$) than the dorsal tier for both groups.

Chapter 4

Discussion

In this thesis, I found that blocking M channels increased the firing rate and changed the firing mode of DA cells in slices. This is correlated with a reduction in the number of M channel positive DA cells in the dorsal tier of the VTA in animals that had neonatal ventral hippocampal lesion and displayed the characteristic PPI deficit. Here we discuss our results in two main parts of electrophysiological and anatomical experiments.

4.1. Electrophysiological Experiments

4.1.1. The effect of recording conditions on the cell

I used nystatin-perforated patch clamp recording method because it provides maximal preservation of cytoplasmic molecules from being diluted by the micropipette solution (Mazzarella, 1999). This property results from partitioning of nystatin into the membrane to create small holes in the cell membrane rather than rupturing membrane as in conventional whole recording. This is particularly important in this thesis because the KCNQ/M channel is regulated by intracellular molecules such as cyclic nucleotides, Ca^{2+} and protein kinases that are diluted or lost during conventional whole cell recordings (Lo and Numann, 1999; Higashida *et al.,* 2005; Nakajo and Kubo, 2005). The electrophysiological properties of putative DA and GABA cells were consistent with

other reports using this method (Zhang *et al.,* 2005, Neuhoff et al., 2002). Another factor which affects the activity of the cell is temperature. Temperature has been shown to alter spontaneous action potential trains due to sodium and potassium channel fluctuations, i.e. temperature changes the mean open rates of these channels leading to different rate and duration of action potentials at different temperatures (Yang and Jia, 2005). A numbers of other channels are affected by temperatures, e.g. gating kinetics of I_h channels are sensitive to temperature (Yanagida et al., 2000). Since I was studying the firing behavior of DA cells which express a prominent I_h, and in particular burst firing of these cells that depends on many ion channels that are regulated by diffusible messenger molecules, choice of experimental approach is therefore important.

All the recordings were done at room temperature (22-24° C). A majority (94.4%) of VTA DA cells in this series identified by their electrophysiological properties were spontaneously active without bursting although they are capable of bursting *in vivo*. Given that in awake animals activity levels are correlated with natural burst firing, this lack of burst firing *in vitro* could arise from deprivation of sensory stimulation or interruption of synaptic input (Overton, 1999). Technically speaking, this could also be due to dilution of the cytoplasm during whole-cell recording and to temperatures (which are invariably lower than 37° C) at which the recordings are made. Although it has been contended that perforated patch is better for recording burst firing, it should be noted that burst firing can also be induced in conventional whole cell recordings (Korotkova *et al.,* 2003; Govindaiah and Cox , 2006; Zhu *et al.,* 2006) and there appears to be no difference in the characteristics of burst firing induced under these conditions. Temperature has been known to alter I_h current with it being much larger at higher temperatures. The

presence of a prominent I_h has been used as one of the electrophysiological finger prints for DA cells (Neuhoff *et al.*, 2002). I_h is one of the primary forces moving the membrane potential out of a hyperpolarizing afterpotential that follows each and every action potential in DA cells and is one of the currents at subthreshold voltages to contribute to generation of different firing patterns. Indeed, it has been reported that burst firing is much slower and more protracted at lower temperatures, but the overall features of a bursting cycle remain the same (Moore *et al.*, 2001).

4.2. Blocking M channels excites DA cells

The M channel generates a potassium current termed M current which contributes to the excitability of many neurons in the central nervous system (Geiger *et al.*, 2006; Norbert *et al.*, 1998). Existence of M current recently has been reported in DA VTA cells and blocking the current increase the firing rate of these cells (Koyama and Appel, 2006). Whether this channel blocks burst firing has not been documented although it is the case in other CNS regions such as the hippocampal pyramidal cells (Yue and Yaari, 2004). Muscarinic agonists have been shown to block the M current (Nakajo and Kubo, 2005) and induce burst firing of DA cells in the VTA (Zhang *et al.*, 2005). However, since muscarinic agonists activate multiple signaling pathways, it is not conclusive whether its effect on burst firing is attributed to its blocking action on the M channels.

The M channel is activated by membrane depolarization to give rise to a non-inactivating current that persists to form a component of the AHP after an action potential. The appearance of the AHP is a limiting factor that forces single spike firing.

Removing one or more of these AHP components should disrupt regular single spike firing.

All three components of the AHP (sAHP, mAHP and sAHP) are due to K^+ currents that play a role in interspike interval and bursting (Maylie et al., 2003). However contribution of different types of K^+ channels to each component differ in different neurons, e.g. sAHP in nigral DA neurons is due to small conductance Ca^{2+}-dependent K^+ channels (Ping and Shepard, 1999) which contribute to mAHP instead in hippocampal neurons (Pedarzani et al., 2005). Blocking M channels reduces fAHP and sAHP in VTA DA cells (Koyama and Appel, 2006) but it reduces only fAHP in hippocampal neurons. The bee toxin apamin which blocks the slow component of the AHP has been shown to induce burst firing in DA cells. However, since in different cells AHP components have different underlying ion channels, it remains a question whether blocking M channels induces burst firing of DA cells. The reduction in slow and fast components of AHP in our experiments was consistent with other studies. The main and significant change in the medium AHP component accompanied by a depolarization and increased firing frequency is similar to the effects of this blockade on hippocampal neurons (Yue and Yaari, 2004).

The finding that blocking M channels induced burst firing of VTA DA cells is in good agreement with the channel's involvement in AHPs. The only similar such manipulation was reported to increase firing rate without any changes in firing patterns (Koyama and Appel, 2006). This could arise from the dissociated cell preparation used in Koyama's experiment that is devoid of distal dendrites. In general, M channels are

located in different parts of CNS neurons and in particular these channels are expressed on dendrites (Weber *et al.,* 2006; Yue and Yaari, 2005). The loss of M channel and many other channels, for example, Ca^{2+} and NMDA channels, may have caused a different response to M channel blockers. Alternatively, it could be due to the fact that burst firing induction takes time to develop (Liu *et al.,* 2007), the duration of linopirdine and X-991 application in Koyama's experiment was much shorter than our experiments (20-340 msec vs. 20-50 min). XE-991 induced burst firing in relatively shorter time than linopirdine could be due to its higher affinity (Robbins, 2001) and selectivity (Wang *et al.,* 1998) for M channels.

The striking result is that M channel blockers induced burst firing in at least 7 of 10 of DA cells tested, but not in the 4 GABA cells tested. It is clearly demonstrated (see results of this thesis) that both cell types express M channels. It is possible that the two cell groups express different channel subtypes that are differentially blocked by these blockers at the concentration used in the thesis. For example, cells that express KCNQ2/3 heterodimeric channels will give much bigger M currents that can be more readily blocked by low doses of linopirdine and XE-911 (Wang et al., 1998). It is, however, more likely that dichotomy in the response to M channel blockers is caused by different resting membrane potentials. In this series, GABA cells had an average resting membrane potential of 56.0 ± 0.4 mV compared to -47.7 ± 0.7 mV recorded from DA cells. As M channels operate at voltages between –30 and –50 mV (Selyanko and Brown, 1999), some of the channels are already active at rest in DA, but not GABA cells. Blocking the channel would therefore affect the firing of DA, but not GABA cells.

4.2. Behavioural and anatomical experiments

In my experiments, the surgical procedures and lesion scoring were in accordance with Lipska and Weinberger (Lipska et al., 1992; Lipska and Weinberger 1993). 10 % of rats did not show any hippocampal lesion and another 30% showed unilateral lesion. These unsuccessful surgeries may have happened because the skull was not set flat during surgery resulting in inaccurate targeting of injection site. Because the unilateral lesion does not produce behavioral changes in this model (Chambers and Self, 2002; Wood et al., 2003), these rats were excluded from analysis. In bilateral lesioned brains the hippocampal damage ranged from -4.52 to - 6.72 from Bregma. This difference in locality of damage could have resulted from a couple of possibilities. First the hippocampus is a relatively large structure in the brain and local injected ibotenic acid may not disperse to the entire structure. Secondly, the speed of injection and withdrawal of the needle, which differ from one pup to another, can affect the dispersion of ibotenic acid.

4.2.1. PPI shows progressive changes in nVH lesioned rats

The VTA has been proposed in regulating PPI in the rat brain (Klejbor *et al.,* 2006; Feifel and Reza 1999; Hart *et al.,* 1998) and PPI has been shown repeatedly to be modulated by DA (Vinkers *et al.,* 2007; Byrnes *et al.,* 2007; Swerdlow *et al.,* 2005; Bitsios *et al.,* 2005). The PPI deficit in the nVH model reported here further validates this connection. In this thesis, it was found that nVH lesioned rats had PPI deficit at PND 56

which is in accordance with other studies (Lipsaka et al., 1995; Le Pen and Moreau, 2002). It has been suggested that PPI deficits are only evident at PND 56, mirroring the fact that schizophrenic episodes usually have a time of onset after puberty in humans, such changes are completely absent before that time (Martinez *et al.* 2002; Lipska *et al.*, 1995). However, I tested PPI at 35, 49, and 56 PND and found there was a worsening of PPI of in nVH lesioned rats, as age proceeded. Given that PPI deficit in this model improves with antipsychotic treatment (Rueter et al., 2004; Le pen and Moreau, 2002) and a gradual worsening of PPI during development, it may reflect the gradual changes that occurs in the central DA system including the VTA that powers the system.

4.2.2. Distribution of KCNQ3 channels altered in nVH lesion midbrain

KCNQ3 channels was widely expressed in different parts of the rat midbrain, in particular strong somatic staining in the red nucleus, which is comparable with the mouse brain (Geiger *et al.,* 2006), although the intensity of staining was weaker in our experiment. Difference in staining intensities could result from different KCNQ3 antibodies used (Geiger *et al.,* 2006; Weber *et al.,* 2006). Camera lucida used to quantify KCNQ3 positive cells has advantages as well as limitations, e.g. this method quantifies KCNQ3 distribution for the entire area of the midbrain. However, it is not easy to accurately define an anatomical region and distinguish weakly labeled cells with low magnification. So I also used other methods for more accurate anatomical evaluation of KCNQ3 expression, taking advantage of cell counting with higher microscopic magnification.

Increased quantity of KCNQ3 channels in the midbrain nuclei of the nVH lesioned rat can be an indicator of decreased excitability of the cells in these nuclei. One of the most notable changes in nVH rats is an increase in KCNQ3 immunoreactivity in the red nucleus. The most important efferent projections of the red nucleus are to the contralateral spinal cord that is mainly involved in the control of upper extremities and fingers. However, lesioning of this nucleus with ibotenic acid does not impair reaching or grasping (Wishaw and Gorny, 1996). The behavioral changes are therefore unlikely to be due to KCNQ3 changes in the red nucleus.

Besides the red nucleus, the oculomotor nucleus and the VTA were also clearly stained for KCNQ3 which was altered by neonatal hippocampal lesion. In spite of TH immunoreactivity in the oculomotor nucleus, this structure is not associated with the DA system (Pearson *et al.,* 1990). Therefore, it is not clear how an increase in KCNQ3 expression in terms of the intensity of staining and the number of stained cells in the nucleus is related with the DA system in the nVH model. However, it has been well documented that the pupillary reflex in schizophrenic patients at the level of oculomotor nucleus is affected (Steinhauer *et al.,* 2000). Since abnormalities in pupillary reflex is positively correlated with the severity of symptoms especially the impairment in working memory (Morris *et al.,* 1997; Steinhauer *et al.,* 2000; Minassian *et al.,* 2004), the dramatic increase in KCNQ3 expression in this nucleus is worth studying. On the other hand schizophrenic patients suffer from number of eye movement abnormalities related to the oculomotor system such as smooth-pursuit eye movement dysfunction (Hutton *et al.,* 1998; Thaker *et al.,* 1998) and higher frequency of incorrect saccade inhibition during an antisaccade task(McDowell *et al.,* 2002; Thaker *et al.,* 2000; Hutton *et al.,* 2002). It is

not known whether increased KCNQ3 expression in the oculomotor nucleus reduces the excitability of the neurons in this nucleus to affect eye movement in schizophrenia.

The total number of KCNQ3-positive cells in the VTA of the nVH lesioned rats was reduced compared to sham-operated rats. KCNQ3 mRNA has been detected in several parts of midbrain involving VTA, red nucleus and oculomotor nucleus (Saganich et al., 2001). Therefore expression of KCNQ3 in all these three parts of midbrain is predictable. However, to our knowledge in the only immunohistochemistry study on this channel, no expression in ventral tegmental area and oculomotor nucleus was detected (Hansen *et al.,* 2006). Hansen and his coworkers in their study have used a dilution of 1:1000 which is more diluted than our protocol (1:1000 vs.1:400). This might account for our positive results for staining in the VTA.

DAB immunohistochemical labeling does not discriminate whether the reduction occurs in DA or GABA cells. Usually the size of GABA interneurons is smaller than that of DA cells; however, because of overlap in size and shape, neither shape nor size are valid for distinguishing the two cell groups (Domesick, 1988).

KCNQ3 staining patterns change with development, i.e. at PND 3 it is detectable at lowest level mainly in the cell body (Gieger *et al.,* 2006). During neurodevelopement, the quantity of KCNQ3 increases to reach its maximum at PND 45 with high expressions in axons and dendrites (Hadley *et al.*, 2003). Such developmental change in KCNQ3 expression is interesting in light of the fact that PPI deficit in nVH rats showed a developmental change in our study, which was associated with changes in the expression of KCNQ3 channels in some midbrain nuclei.

4.2.3. KCNQ3 expression is lower in the dorsal tier of VTA DA cells in nVH lesioned rats.

To study whether alterations in KCNQ3 expression occurs in DA cells in the VTA, double immunofluorescence labeling of TH and KCNQ3 was used. Due to different projections of DA cells in the VTA with the ventral tier projecting to the sensorimotor part of the striatum and the dorsal tier projecting to the ventral and dorsal striatum, limbic and cortical areas, we analyzed the two areas of the VTA. The tiers were identified based on anatomical location and density of cells. Ideally, this should be combined with the expression of ion channel protein GIRK2, landmark of the ventral tier (Schein *et al.,* 1998), and calbindin, landmark of the dorsal tier (Haber *et al.,* 1995). The limbic and cortical projections are implicated in schizophrenia, and specifically, the nucleus accumbens regulates PPI in rats (Swardelow et al., 2007; Byrnes et al., 2007; Yamada, 2000) and humans (Kumari et al., 2005) due to DA's action in the nucleus. Change in the excitability of DA neurons in the VTA projecting to the nucleus accumbens changes PPI in nVH lesioned rats.

The percentage reduction in KCNQ3-positive cells in the VTA was similar but not identical between the DAB-stained group and the immunoflorescence double labeling group. With confocal microscopy, counting of immunoflorescent cells with KCNQ3 and TH was clearer, giving a more accurate estimation of colocalized cell percentages. However, DAB staining can be more sensitive than the immunoflorescence method (Burnett *et al.,* 1997).

Sensorimotor striatum refers to the basal ganglia, which regulate PPI in healthy humans (Kumari *et al.,* 2007) and mice (Takahashi et al., 2006; Ingram et al., 2005). However, in the present study no significant changes in KCNQ3 expression was found in DA cells in the VTA (i.e. the ventral tier) that project to the basal ganglia. Meanwhile because of heavy projections from the ventral striatum to the sensorimotor striatum (Bjorklund and Dennett, 2007), it is possible that changes in the dorsal tier that projects to the ventral striatum are reflected by the connections between the ventral and dorsal striatum.

KCNQ3 immunoreactivity was also found in TH-negative cells. Since I did not stain these cells for glutamate decarboxylase (marker for GABA cells), it is not known if these were all GABA cells. Besides DA and GABA cells, it has been recently demonstrated that there is a group of cells that use glutamate as a transmitter (Geisler *et al.,* 2007). As GABA cells comprise a predominant proportion of non-DA cells in the VTA, it is very likely no significant changes in KCNQ3 expression occurs in GABA cells of the nVH rat.

Blocking M channels increases firing rate and induces burst firing in DA cells but not in GABA cells *in vitro*. Similarly, a reduction of KCNQ3 expression is present in DA cells but not in GABA cells in rats with nVH lesions. It is then logical to conclude that DA cells of nVH rats may fire at higher frequencies or in more frequent bursts. The specific reduction of KCNQ3 expression in DA cells in the dorsal tier signifies the involvement of DA projections to the limbic and cortical regions that have been known to be involved in the development of psychosis.

Conclusion

In this thesis, it was found that blocking M channels increased the excitability of DA, but not GABA cells in the VTA in an *in vitro* slice preparation. Moreover, blocking M channels induced burst firing in a percentage of DA cells. Because burst firing is more effective in raising DA levels at the synapse, I examined whether an altered expression of M channels was present in an animal model of schizophrenia where increased DA transmission is evident. The KCNQ3 subunit which forms KCNQ3/4 and KCNQ2/3 heteromeric channels to conduct M currents 11-15 times more efficiently than homodimeric channels of either subunit was expressed in both DA and GABA cells in the VTA. The channel was also present in other midbrain regions such as the red nucleus and the oculomotor nucleus. In an animal model of schizophrenia where ventral hippocampus was lesioned early in life, there was a progressive deficit in prepulse inhibition that has been shown to result from excessive DA activity. In these animals, there was an overall reduction in the number of cells KCNQ3 positive cells in the VTA. Double fluorescence labeling with TH and KCNQ3 revealed that the reduction of KCNQ3 positive cells only occurred in DA cells in the dorsal tier of the VTA. DA cells in the ventral tier or non-DA cells throughout VTA displayed similar KCNQ3 immunoreactivity as compared with sham controls. KCNQ3 expression in ventral hippocampal lesion animals showed a significant increase in the red nucleus and oculomotor nucleus.

Results in this thesis suggest that neonatal hippocampal lesion leads to a selective reduction of KCNQ3 expression in DA cells in the dorsal tier of the VTA. This would make these DA cells to fire at a higher frequency or in more frequent bursts, both of

which will enhance DA output in their cortical and limbic terminal fields to mediate symptoms of psychosis.

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