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CHARACTERIZATION OF THE CHANGES IN THE LEVELS OF GABAERGIC AND CALCIUM CHANNELS MARKERS IN MICE PSYCHOSTIMULANT ADMINISTRATION MODELS

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Characterization of the changes in the levels of GABAergic and calcium channels markers in mice psychostimulant administration models

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ABSTRACT

The thalamocortical somatosensory system is the responsible for processing information coming from the sensory nerves so as to generate a somatosensory cognitive experience, which is the same, to produce a concrete sensation from the environment. This system consists of a highly coherent and differenced neuronal circuit, which allows perception to be carried on in our daily life. The alteration of the different components of the system by psychostimulants, such as cocaine or methylphenidate, induces distinct anomalies and/or pathologies in sensory perception, as it is possible to observe in the thalamocortical dysrhythmia syndrome suffered by chronic cocaine abusers.

The visualization of such alterations in the protein and electrophysiological components of the systems due to the administration of these drugs is essential to understand their effect on the thalamocortical somatosensory system. Concretely, those agents implicated in the excitation and inhibition of the components of the circuit, being the case of the T-type calcium channels and the GAD enzyme (glutamic acid decarboxylase), two proteins that, for their role in the system, are key modulators for the correct excitability and rhythm of each part of the system and, thus, to generate cognitive experiences. On the one hand, T-type calcium channels, also called low voltage activated or LVA, are responsible for the generation of the I_T current, needed to trigger action potentials in the thalamic ventrobasal nucleus. On the other hand, the GAD enzyme (in its two isoforms GAD 65 and GAD 67) is responsible for the synthesis of the inhibiting neurotransmitter GABA, being important in the thalamic reticular nucleus, which modulates the excitability of the ventrobasal nucleus and the transmission of information from this part of the thalamus to the somatosensory cortex. In addition, GABA is the main inhibiting neurotransmitter in the adult nervous system, being also important in the somatosensory cortex.

The alterations of these markers can be measured following two different approaches: the molecular biology approach, evaluating their protein levels by means of the Western Blot technique; or the electrophysiological approach, by the whole-cell patch-clamp technique, which allows the comparison of the densities of low (LVA) and high (high voltage activated, HVA) activation threshold calcium currents, the so called LVA/HVA ratios, and also the visualization of the GABAergic miniature currents (“minis”) caused by spontaneous GABA release.

Key words

GABAergic Transmission; Thalamic Reticular Nucleus; Ventrobasal Thalamic Nucleus; Somatosensory Cortex; Methylphenidate; Cocaine; T-type Calcium Channels; GAD 65/67; Thalamocortical Dysrhythmia

Caracterización de los cambios en los niveles de marcadores GABAérgicos y canales de calcio en modelos de administración de psicoestimulantes en ratón

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RESUMEN

El sistema talamocortical somatosensorial es el encargado de procesar información procedente de los nervios sensoriales para generar una experiencia cognitiva somatosensorial, es decir, para producir una sensación definida del medio que nos rodea. Dicho sistema consta de un circuito neuronal sumamente coherente y diferenciado, que permite que la percepción sea llevada a cabo de manera continua en nuestro día a día. La afectación de las distintas partes del mismo por psicoestimulantes, como la cocaína o el metilfenidato, induce distintas anomalías y/o patologías en la percepción sensorial, como se puede apreciar en el síndrome de disritmia tálamocortical en pacientes con consumo crónico de cocaína.

La visualización de las alteraciones de los distintos componentes proteicos y fisiológicos del sistema debidas a la administración de dichas drogas es primordial para el entendimiento de su afectación al sistema tálamocortical somatosensorial. En especial, aquellos agentes implicados en la excitación e inhibición de los distintos integrantes del circuito, como es el caso de los canales de calcio tipo T y la enzima GAD (*glutamic acid decarboxylase*), dos proteínas que, por su rol en el sistema, son esenciales para la correcta excitabilidad y ritmo de los componentes por separado y, por tanto, para producir experiencias cognitivas. Por una parte, los canales de calcio tipo T o de bajo umbral de activación (*low voltage activated*, LVA) son los responsables de la corriente I_T, necesaria para la generación de potenciales de acción en las neuronas del núcleo de relevo ventrobasal del tálamo somatosensorial. Por otra parte, la enzima GAD (en sus dos isoformas GAD 65 y GAD 67) es la responsable de la síntesis del neurotransmisor inhibitorio GABA, el cual cobra especial importancia en el núcleo reticular, que modula la acción del núcleo ventrobasal y la transmisión de información de éste a la corteza somatosensorial. Además, GABA es el principal neurotransmisor inhibitorio del sistema nervioso, siendo también importante en la corteza somatosensorial.

Las alteraciones de dichos marcadores se pueden medir siguiendo dos aproximaciones: o bien a nivel molecular, mediante la observación de sus niveles proteicos por la técnica de Western Blot, o bien mediante técnicas de electrofisiología, como el *patch-clamp* en configuración de célula entera, que permite comparar las densidades de las corrientes de calcio de bajo (LVA) y alto umbral de activación (*high voltage activated*, HVA), los llamados ratios o cocientes LVA/HVA, así como la visualización de los eventos de miniaturas GABAergicas (“minis”) debidas a la liberación espontánea de GABA.

Palabras clave

Transmisión GABAérgica; Núcleo Talámico Reticular; Núcleo Talámico Ventrobasal; Córtex Somatosensorial; Metilfenidato; Cocaina; Canales de Calcio tipo T; GAD 65/67; Disritmia Talamocortical

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ABBREVIATIONS

5-HT: 5-hydroxytryptamine (serotonin)

ADHD: attention deficit/hyperactive disorder

CL: centrolateral nucleus

GABA: γ-aminobutyric acid

GAD: glutamic acid decarboxylase

HVA: high voltage activated

MPH: methylphenidate

LVA: low voltage activated

OLAW/ARENA: Office of Laboratory Animal Welfare/Applied Research Ethics National Assoc.

PLP: pyridoxal phosphate

SI: primary somatosensory cortex

SII: secondary somatosensory cortex

TRN: thalamic reticular nucleus

VB: ventrobasal nucleus

VPM: ventral posteromedial nucleus

VPL: ventral posterolateral nucleus

1. INTRODUCTION

1.1. The thalamocortical somatosensory system

The thalamocortical system is a group of neuronal nuclei, including thalamic nuclei and diverse layers of the cortex, which carries out the function of integrating the sensory inputs that the brain receives and generates the subjective sensations as a whole (Llinás *et al*., 2002; Jones, 2007). The current opinion in this field of study points to the presence of two different thalamocortical circuits: the ones based on the specific or somatosensory (ventrobasal, VB) and non-specific or "contextual" (centrolateral, CL) thalamic nuclei. Somatosensory information travels from the peripheral somatosensory receptors via parallel pathways for mechanical sensation and for the sensation of pain and temperature, being transmitted, passing through the spinal cord and the brainstem, to the thalamus, from which it is relayed to the sensory cortex (figure 1.1) (Llinás *et al*., 2002; Jones, 2007; Purves, 2012). Therefore, the thalamus integrates much of the sensory information that the brain receives and acts as a "communications hub" between different brain regions. What these two different thalamocortical systems have in common is that they rely in almost the same scheme of feed-back inhibition and feed-forward stimulation. It has also been reported that none of the two thalamocortical circuits alone can support cognition (Llinás *et al*., 2002).

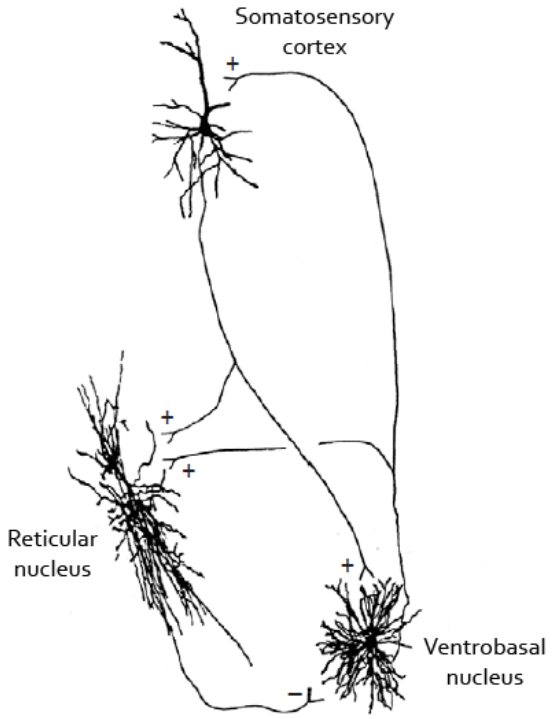

Figure 1.1. *Simplified scheme of the thalamocortical somatosensory system circuit* (modified from Steriade, 2005).

Regarding the thalamocortical somatosensory system, the stimulation of the VB nucleus simultaneously generates an excitatory input to the somatosensory cortex and to the thalamic reticular nucleus (TRN), which does not project to the cortex. On the other hand, the somatosensory cortex (SS) sends back a feed-forward excitatory input to the VB nucleus and, collaterally and simultaneously, to the TRN. The circuit is closed by the inhibitory (GABAergic-based) input exerted by the TRN over the thalamocortical thalamus, thus acting as a negative

feed-back switch off GABAergic component (Arcelli *et al*., 1996; De Biasi *et al*., 1997; Jones, 2002; Llinás *et al*., 2002; Steriade, 2005). This circuitry represents a mechanism for the global temporal binding required to generate single, cognitive events from the large number of sensory inputs arriving at the brain at any particular time (Llinás *et al*., 2002). The main neurotransmitters released by the axons (or afferents) of the neurons composing this system are glutamate and γ-aminobutyric acid (GABA), which exert excitatory and inhibitory effects in adult organisms, respectively (Erlander & Tobin, 1991; De Biasi *et al*., 1997; Jones, 2007).

The administration of cocaine on this system has been reported to dramatically alter the cognitive perceptions of the individuals causing even pathologies, like the thalamocortical dysrhythmia syndrome, thus remarking its essential role in the chronic abuse of cocaine (Urbano *et al*., 2009; Goitia *et al*., 2013). Previous studies have confirmed that the levels of T-type calcium channels increase in the VB nucleus after cocaine treatment and so do the levels of GAD (the enzyme responsible for GABA synthesis; reviewed in Erlander & Tobin, 1991) in the TRN (Goitia *et al*., 2013). Methylphenidate (MPH) and cocaine administration have been shown to induce higher locomotion in mice, as well as higher spontaneous GABA release (GABAergic miniature currents or "minis") from the TRN (Urbano *et al*., 2009; Goitia *et al*., 2013). Therefore, T-type calcium channels and TRN modulation of VB nucleus via GABA release are key components of the action of several psychostimulants, as cocaine and, probably, MPH. There is also evidence of the implication of this system in distinct pathologies, such as absence epilepsy or Alzheimer's disease (De Jong *et al*., 2008; Gigout *et al*., 2013).

1.2. Anatomical characterization of the thalamocortical somatosensory system

1.2.1 Anatomy of the somatosensory thalamus

The human thalamus is a two-lobule structure composed of a group of neuronal structures that originate in the diencephalon (a division of the embryo's prosencephalon) and it is the most voluminous structure of this zone. It is found in the middle of the brain, above the hypothalamus and separated from it by the hypothalamic sulcus of Monro. Traditionally, it is divided in epithalamus, ventral thalamus and dorsal thalamus (Hirai & Jones, 1989; Jones, 2007). The main thalamic nuclei composing the thalamocortical somatosensory system are the VB and the TRN, located both in the dorsal and ventral thalamus, respectively (Jones, 2007).

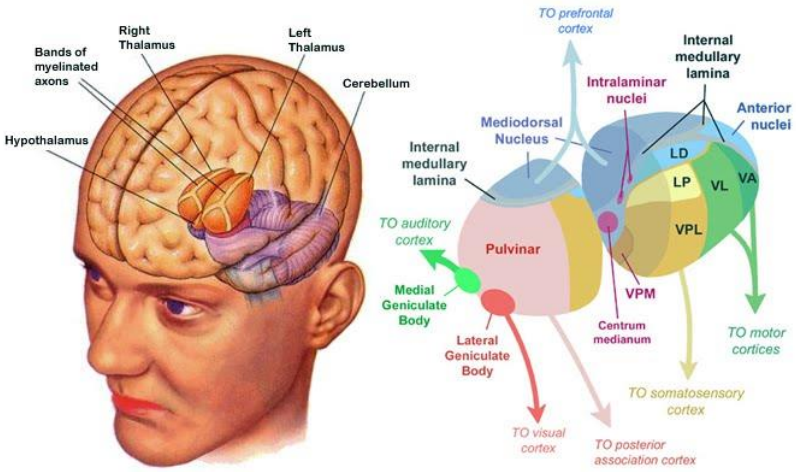

Figure 1.2. *Detail of a lobule of the thalamus and many of its main nuclei.* It is worth mentioning the ventral posteromedial (VPM) and ventral posterolateral (VPL) nuclei, which together build the ventrobasal (VB) nucleus (adapted from Rudyard, 2012).

1.2.1.1 The thalamic reticular nucleus

The thalamic reticular nucleus is a ventral-derived neuronal sheet that covers most of the rostral, lateral and ventral parts of the thalamus (Spreafico *et al*., 1991). As described in Jones (2002), GABAergic TRN neurons are innervated by collateral branches of both thalamocortical (VB) and corticothalamic (SS) fibers. TRN neurons are interconnected by a network of GABAergic chemical and electrical synapses which allows their coordination in the modulation of the VB nucleus (Landisman *et al*., 2002; Deleuze & Huguenard, 2006).

1.2.1.2 The thalamic ventrobasal nucleus

The ventrobasal nucleus, located in the dorsal thalamus, is determined by the confluency of the ventral posteromedial (VPM), which relays somatic sensory information carried by the trigeminal system from the face, and the ventral posterolateral (VPL), which relays somatic sensory information from the rest of the body nuclei in humans (Koyama *et al*., 1998; Purves, 2012). The neurons integrating the VB complex are multipolar cells of big diameter, whose glutamatergic (excitatory) axons mainly end in the primary (SI) and secondary (SII) somatosensory cortex. On the other hand, almost every region of the cortex reciprocally connects to the thalamic nuclei from which they originally received the synaptic stimuli (Llinás *et al*. 2002; Jones 2007).

It is worth mentioning that there is a lack of GABAergic interneurons in the VB nucleus of rodents, and the inhibition necessary for proper sensory perception in these animals is provided by GABAergic TRN afferents. In rodents, VB relay nucleus receives information from whiskers, projecting afterwards to somatosensory cortex (De Biasi *et al*., 1997).

1.2.2 Anatomy of the somatosensory cortex

Somatosensory cortex, which is widely accepted to be composed by six layers of neurons, is often divided into primary (SI; receives direct inputs from the thalamus) and secondary (SII; higher-order somatosensory cortex) somatosensory cortices (reviewed in Douglas & Martin, 2004). The SI cortex is located in the postcentral gyrus of the parietal lobe of the brain. The SII cortex is adjacent to the SI, from which receives inputs and sends them in turn to limbic structures such as the amygdala and hippocampus (Purves, 2012). The VB projects to the layer 4 on the primary somatosensory cortex, in which neurons acting co-ordinately are organized into barrels or clusters, and collaterally to inhibitory interneurons present in this region of the brain, which act as a negative feed-back modulators. This layer projects to the superficial layers, in which superficial pyramidal neurons in turn project to layer 5, which projects to layer 6. The neurons of this layer 6 project to the thalamic relay neurons via corticothalamic fibres of the SI and SII somatosensory cortex, simultaneously exciting the TRN via collateral fibres, which in turn generates a feed-forward inhibition over the VB. (Llinás *et al*., 2002; Steriade, 2005; Lambert *et al*., 2014; Douglas & Martin, 2004).

1.3. Physiological characterization of the thalamocortical somatosensory system

It has been observed that both VB and TRN neurons can exhibit rhythmic active and inactive states in isolated conditions in vitro, being this states dependent on the rhythmic inputs coming from the cortex and other brain regions. These activate or inactive states depend on the type of ion channels present in both VB and TRN neurons and are maintained by an activation of the metabotropic glutamate receptors, determining the information that the SS cortex receives (reviewed in Amarillo *et al*. 2015).

1.3.1. Activity of the thalamic reticular nucleus

Located strategically in the circuit between the the cortex and the relay thalamic nuclei (Jones, 2002; Llinás *et al*., 2002), the TRN plays an essential role in the thalamocortical somatosensory system modulating VB excitability, thus acting as its "switch off" component. As commented before, there is a lack of GABAergic interneurons in the VB nucleus of rodents (present in humans and called local circuit neurons) and the inhibition necessary for proper sensory perception in these animals is provided by GABAergic TRN afferents (Arcelli *et al*., 1996; De Biasi *et al*., 1997).

Depending on the state of vigilance, GABAergic TRN neurons are characterized by two firing modes. These modes of firing are opposite to that of VB neurons, which will be explained in detail later, thus remarking the duality of the system: while TRN neurons are active, VB neurons are inhibited, and viceversa (Steriade *et al*., 1986). Activation of TRN is mediated by glutamatergic (excitatory) afferents coming from the somatosensory cortex and the VB nucleus, which induce depolarizations on TRN neurons via T-type calcium channels ($Ca_V3.2$ and 3.3; Talley *et al*., 1999), coordinating the neurons of this nucleus to exert delayed and long-lasting feed-forward inhibition in thalamic relay cells (Steriade 2005; Sun *et al*., 2012).

1.3.2. Activity of the ventrobasal nucleus

Thalamic relay neurones also present intrinsic rhythmicity between two firing modes, due to the presence of a depolarizing pacemaker potential, the I_h current (Huguenard & McCormick, 1992; McCormick & Pape, 1990a). Depending on whether the membrane potential of the thalamocortical neurons is depolarized or hyperpolarized respecting to the resting potential (approximately -60 mV; Coulter *et al*., 1989), thalamocortical neurons have two regimes of excitability (figure 1.3): (1) tonic firing mode, which occurs at depolarized membrane potentials (positive to about -60 mV) and characterized by the continuous firing of action potentials of Na^+/K^+ (McCormick & Feeser, 1990; McCormick & Pape, 1990); and (2) burst firing mode (or oscillatory mode), with the generation of bursts (small groups) of action potentials of Na^+/K^+ at high frequency at hyperpolarized membrane potentials (negative to about -65 mV) (Jahnsen & Llinás, 1984). Regarding the burst firing mode, if thalamocortical neurons at hyperpolarized membrane potentials receive a depolarizing stimulus, a slow depolarizing response is elicited in these cells, mainly caused by a Ca^{2+} current (also called the I_T current), which in turn is mediated by the opening of the T-type voltage-dependent calcium channels (also called low voltage activated channels or LVA). This current is activated at hyperpolarized membrane potentials ranging from -65 mV to -90 mV, but is inactivated at membrane potentials above the resting potential (Coulter *et al*., 1989; Huguenard & McCormick, 1992). The presence of the I_h current, which activates on hyperpolarization and which contributes to generate a "pacemaker" potential, might produce such firing mode or stabilize the membrane potential in order to facilitate the action of I_T (McCormick & Pape, 1990a; reviewed in Amarillo *et al*., 2014).

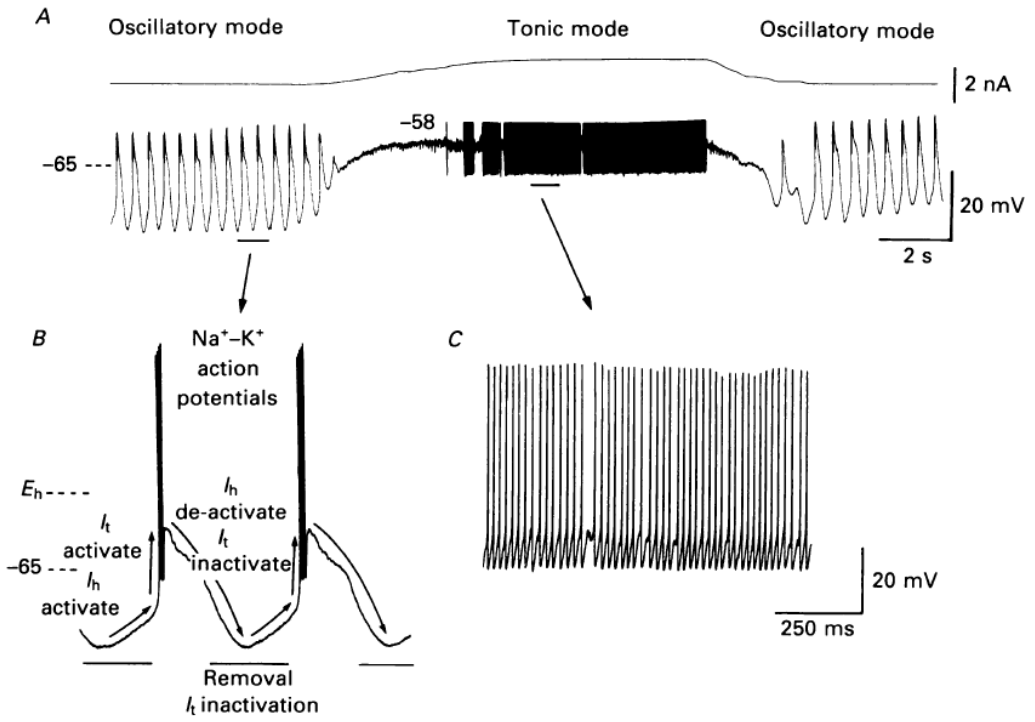

Figure 1.3. *Action potential firing modes of thalamic relay neurons.* **(A)** Electrofisiological register showing both tonic and oscillatory modes. At a hyperpolarized membrane potential of -65 mV with respect to the resting potential of -60 mV, the neuron generates spontaneous bursts of action potentials (burst or oscillatory mode) with a frequency of 2 Hz intraburst. However, if this neuron is depolarized to -58 mV applying electrical current, the firing mode becomes tonic. Removal of the depolarization reinstates the oscillatory activity. **(B)** Detail of the burst (or oscillatory) firing mode in the region indicated in (A), showing the involvement of I_T and I_h in the rhythmic action potential firing. Activation of the low-threshold calcium current, I_T, depolarizes the membrane towards threshold for a burst of Na^+- and K^+-dependent fast action potentials. Repolarization of the membrane due to I_T inactivation is followed by a hyperpolarizing depression, due to the reduced depolarizing effect of I_h. The hyperpolarization in turn de-inactivates I_T and activates I_h, which depolarizes the membrane towards threshold for another Ca^{2+} spike. **(C)** Detail of the tonic firing mode in the region indicated in (A) (modified from McCormick & Pape, 1990).

Because the return to an activated state of the I_T is kinetically slow, the generation of another action potential requires that the membrane of the neuron remains at hyperpolarized potentials (<-65 mV) a period of time of the order of milliseconds (Huguenard & McCormick, 1992). Together with the long-lasting inhibitory potentials that TRN neurons elicit over the thalamocortical neurons, these conditions allow the blockade of the relay neurons in the oscillatory mode, which is the same, in an “inactive” state (reviewed in Amarillo *et al*., 2015).

Tonic firing is said to be a relay mode that allows information to be transmitted from the relay nuclei (VB nucleus) to the targeted cortical areas (SS cortex), being VB neurons acting as sensory signal integrators or “hubs”, a state which mainly occurs during active cognitive states. On the other hand, the burst firing mode of thalamocortical neurons is related with brain states characterized by cognitive arrest (deep sleep, absence seizures), being VB neurons under repetitive burst firing mode acting as “resonators” (reviewed in Amarillo *et al*., 2015).

1.3.3. Activity of the somatosensory cortex

Almost all cortical areas receive thalamic inputs and relay such stimuli to lower motor centres. These cortical areas communicate between them directly (between cortical areas) or indirectly (via the thalamus). These connections can also be classified as "driver", if it transmits information, or as "modulator", if it controls this transmission. Although the pathways involving the thalamus belong to the first class, communications between cortical areas can be performed in either of the two classes of transmission. Thalamic pathways provide a regulatory checkpoint that can be more subtly modulated, being these inputs generally branches of axons with motor functions (reviewed in Sherman & Guillery, 2001).

The pyramidal cells of layer 5 are thought to drive subcortical structures involved in action (e.g., basal ganglia, ventral spinal cord) and decide the output of the cortical circuits. The same layer 5 pyramidal cells influence this output by their connection to layer 6 pyramidal cells that connect to the thalamic input layers (reviewed in Douglas & Martin, 2004). The primary somatosensory cortex has also been linked to the primary motor area, thus providing the required cross-talk for normal motor activity (McGeogh *et al*., 2014; Ueta *et al*., 2013), and to be involved in the modulation of the sensory aspects of pain and discrimination of pain intensity (Kim *et al*., 2014). However, further insights in this area are needed in order to clarify how the somatosensory cortex works and how it interacts with the rest of the circuitry.

1.4. Biomarkers of the activity of the thalamocortical somatosensory system

1.4.1. T-type calcium channels

Voltage-gated calcium channels play a central role in mediating calcium-dependent synaptic transmission. Many subtypes have been described (T, N, L, P, Q and R) and classified by their electrophysiological and pharmacological properties. One of the classifications is based on their activation threshold, being called as HVA (High Voltage Activated) or LVA (Low Voltage Activated). All subtypes except from T-type belong to the first one, being the T-type channels the only subtype belonging to the LVA group (Catterall, 2000).

Regarding T-type calcium channels, they are homomultimeric proteins formed by the α_1 subunit, containing four homologous domains (I-IV) connected by cytoplasmic regions. Each of these four domains consists of six transmembrane helixes (S1-S6), plus a loop region that creates the pore. The α_1 subunit is the responsible for the most important functional property: voltage sensing (Catterall, 2000). Moreover, there are three types of α_1 subunits: α_1G or $Ca_V3.1$ (262 KDa), α_1H or $Ca_V3.2$ (259 KDa) and α_1I or $Ca_V3.3$ (245 KDa) (UNIPROT).

The expression of the three members of the T-type calcium channel family has been described in multiple brain regions in rodents. The $Ca_V3.1$ subunit is the main subunit carrying I_T current in VB neurons in mice (Talley *et al*., 1999; Huguenard & McCormick, 1992), being the $Ca_V3.2$ and $Ca_V3.3$ subunits present in TRN GABAergic neurons, allowing their electrophysiological coordination (Talley *et al*., 1999; Landisman *et al*., 2002; Deleuze & Huguenard, 2006).

1.4.2 Glutamic acid decarboxylase

GABA is the most widely distributed known inhibitory neurotransmitter in the vertebrate brain, where its actions are mediated by $GABA_A$ and $GABA_B$ receptors (reviewed in Erlander & Tobin, 1991; De Biasi *et al*., 1997; Jones, 2007). $GABA_A$ receptors are ionotropic receptors, made up of hetero-oligomeric combinations of polypeptide subunits that form a Cl^- channel, which favours a rapid entrance of this ion inside the cell causing its hyperpolarization, thus inhibiting the target neuron. Their density is high in all thalamic nuclei except the TRN nucleus. $GABA_B$ receptors are metabotropic receptors, being present in high levels in the dorsal thalamus. These receptors mediate a relatively slow but long-lasting K^+-dependent inhibitory effect, favouring the exit of this ion out the neuron and, by that, hyperpolarizing the target neuron (Jones, 2007).

The brain contains two isoforms (derived from two different genes; Erlander *et al*., 1991) of the GABA synthetic enzyme, glutamate decarboxylase (GAD), which differ in molecular size, amino acid sequence, antigenicity, cellular and subcellular location, and interaction with the GAD co-factor pyridoxal phosphate (PLP). GABA synthesis by GAD is primarily regulated by its interaction with the cofactor PLP. Their distribution also differs, being GAD67 the predominant isoform found in the terminals and GAD65 the one found in the soma of the neurons (reviewed in Erlander *et al*., 1991). GAD has been reported to be present in the VB nucleus, the TRN and the SS cortex (Doetsch *et al*., 1993; Goitia *et al*., 2013)

1.5. Psicoestimulants affecting the thalamocortical somatosensory system

1.5.1 Addictions

Addictions have been considered a disease since 1960s (Dole *et al*., 1966). According to the National Institute on Drug Abuse (NIDA, USA), addiction is defined as a chronic, relapsing brain disease that is characterized by compulsive drug seeking and use, despite harmful consequences. It is considered a disease because of the physiological and structural changes that causes in the brain. Such changes can be long lasting and lead to the harmful behaviours seen in chronic abusers (NIDA, 2010a). Drug consumption usually begins at adolescent stage, which is considered the riskier age for drug intake regarding the high numbers of addicts belonging to this group (Schramm-Sapyta *et al*., 2004).

1.5.2 Cocaine

Cocaine is a powerfully addictive stimulant that directly affects the brain, being capable of passing through the blood-brain barrier (Sharma *et al*., 2009). Although it has been labelled as the drugs of the 1980s and 1990s, it is one of the oldest drugs known, as it comes from the coca leaves (*Erythroxylon coca*) that have been consumed for thousands of years. It is a highly addictive drug because of its short lifespan and of its reinforcement effects, which lead to its compulsive seeking by abusers (NIDA, 2010b; Zimmerman, 2012). Cocaine can make a person feel euphoric, but when the effect is lost it makes the consumer feel restless, scared, angry and even depressed in chronic abusers (NIDA, 2010b), having local anaesthetic properties, too (Zimmerman, 2012).

Regarding statistics, cocaine has prevalence on Spanish male population between 25 and 34 years old of 5.9% of the total population (PNSD, 2013). In Argentina, such prevalence is approximately the same, 5.8% of the total population (Plan nacional contra las drogas, 2007).

Regarding USA, the number of cocaine users reached 2.4 million in 2006, decreasing to 1.5 in 2010 (Zimmerman, 2012). The prevalence of cocaine worldwide is estimated to be 0.3-0-5% between 15 and 64 years old. In addition, North and South America register the highest amount of people in treatment due to cocaine abuse, taking into account that opioids are the most consumed drug worldwide (UNODC, 2015).

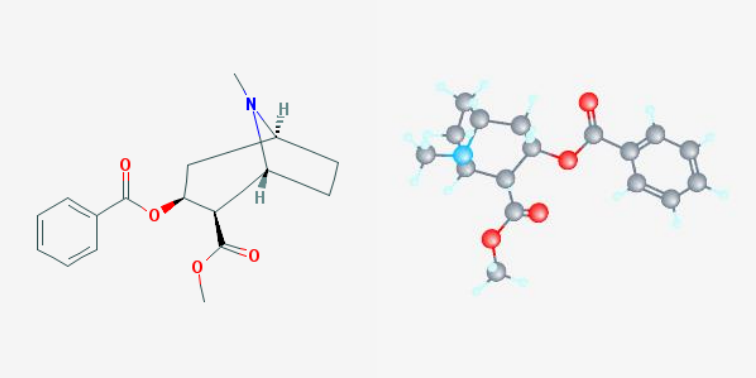

Figure 1.4. *Cocaine 2D and 3D structures* (PUBCHEM).

Cocaine, also called benzoylmethylecgonine (M_f: $C_{17}H_{21}NO_4$; M_w: 303.35 g/mol), is a white powder, which can be snorted up the nose or mixed with water and injected with a needle. Cocaine can also be produced in small white rocks, called crack. Cocaine has a short half-life of 0.7 to 1.5 hours, being rapidly metabolized by plasma and liver cholinesterases (NIDA, 2010b; Zimmerman, 2012; PUBCHEM).

Chronic abuse of cocaine is associated with important neuro-psychiatric conditions. The most important toxicities of cocaine use results from the inhibition of presynaptic monoamine transporters of dopamine (Ritz *et al*., 1987), serotonin and norepinephrine (Howes *et al*., 2000; reviewed in Zimmerman, 2012), elevating the synaptic levels of these neurotransmitters. Repetitive cocaine exposure has been shown to induce GABAergic thalamic alterations (Bisagno *et al*., 2010), cerebral hypoxia, seizures, and delirium sometimes associated with hallucinations (Devlin & Henry, 2008).

Higher LVA/HVA calcium current ratios, changes in I_h current density and voltage dependence of T-type currents were observed in thalamic VB neurons from cocaine-injected mice. GABA-mediated miniature ("minis") currents, which reflect the spontaneous exocytosis of GABA loaded vesicles (Fatt & Katz, 1952; Trigo *et al*., 2010), were also affected by cocaine treatment, being the amplitude and frequency of their currents on VB nucleus increased (Urbano *et al*., 2009). Western blot analysis after sub-chronic administration of cocaine in mice also revealed an increase in VB $Ca_V3.1$ (24 hours after three-day binge protocol) and TRN GAD 67 levels (after four-day binge protocol) (Goitia *et al*., 2013). Therefore, higher LVA/HVA calcium current ratios could suggest the existence of a compensatory expression of T-type channels by VB to overcome the higher TRN inhibition. In addition, higher GAD 67 levels in TRN could explain the increase in both frequency and amplitude of GABA miniature currents. These protein expression alterations are clear examples of the long-lasting cocaine effects on the thalamocortical system, since plasma cocaine levels are supposed to be completely washed out after 24 hours (Goitia *et al*., 2013; Goitia, 2015).

Taking all this together, it has been proposed that cocaine-mediated effects on thalamocortical neurons could result in the hyperpolarization of thalamic cortical projecting neurons, leading to a switch from the tonic-firing to burst-firing mode in the thalamocortical system. Such switch, previously suggested to be mediated by serotonin receptors (Urbano *et al*., 2009; Bisagno *et al*., 2010; Goitia *et al*., 2013), is thought to be caused by two different mechanisms: (1) by increasing the bursting activity of TRN neurons, which would trigger more frequent depolarizations on this nucleus and, thus, an increase in the GABAergic inhibition onto the VB nucleus, and (2) by the hyperpolarization of postsynaptic specific VB neurons, increasing their burst-mode activity (Urbano *et al*., 2009; Bisagno *et al*., 2010). The prolonged burst firing mode of thalamocortical neurons during awaken states has been suggested to induce ineffective sensory processing (McCormick & Feeser, 1990).

1.5.2.1 Thalamocortical dysrhythmia syndrome

The unbalance in LVA/HVA calcium currents was previously observed in mice lacking P/Q-type calcium channels and is involved in multiple psychiatric and neurological diseases, grouped as "thalmocortical dysrhythmia syndrome" (Llinás *et al*., 2007; Bisagno *et al*., 2010). Regarding this knockout model, VB neurons presented abnormally large LVA/HVA ratios (Llinás *et al*., 2007). As commented before, cocaine "binge" administration induced rapid and simultaneous increments of both LVA/HVA ratios, which were reversible after 24 hours (Urbano *et al*., 2009), thus resembling a thalmocortical dysrhythmia syndrome state.

1.5.2.2 The role of ketanserin

As serotonin is thought to play an important role in cocaine-mediated deleterious effects in the thalamus after sub-chronic protocols and act as one key reinfoncer of cocaine effects (Munzar *et al*., 2001; Bisagno *et al*., 2010; Goitia *et al*., 2013), the blockade of its receptors would theoretically minimize or eliminate some of the adverse effects of cocaine sub-chronic treatments. Ketanserin, an antihypertensive medication, is a serotonin receptor 5-HT_{2A} antagonist (Broderick *et al*., 2004; excitatory receptors in TRN as hypothesized in Goitia, 2015), binding to histamine and adrenergic receptors, too, and weakly to 5-HT_{1A} receptors (inhibitory as hypothesized Goitia, 2015); ketanserin does not bind to DA receptors at all. It has already been used to discriminate the cocaine effects caused by the activation of these receptors in the nucleus accumbens, blocking the increase in locomotion suffered by cocaine-administered mice (Broderick *et al*., 2004; Goitia *et al*., 2013) and shifting the cocaine dose-response curve (Munzar *et al*., 2001).

1.5.3 Methylphenidate

Methylphenidate (MPH; commercially sold as Concerta, Methylin, Medikinet, Ritalin, Equasym XL, Quillivant XR, Daytrana or Metadate, among others) is another psychostimulant widely used to treat children and adolescents diagnosed with attention deficit/hyperactive disorder or ADHD (Biederman *et al*., 1999). It helps to focus attention and learning, but when consumed frequently it can increase the period of wakefulness and produce euphoria in the consumer (NIDA, 2010c). An annual increment of 8% in MPH consume has been reported in Spain between 1992 and 2001, data that is far behind the first consumer worldwide, USA (Criado-Álvarez & Romo-Barrientos, 2003).

Administration of MPH to younger children raises concerns regarding its effects on the developing brain and rise concerns about the facilitation of drug abuse in these children. Many studies have documented an increased risk for drug use disorders in youth with untreated ADHD (Biederman *et al*., 1999; Clarke et al. 2003; Volkow & Swanson, 2003; Guerriero *et al*., 2006). However, the methodological limitations of such studies, including small sample sizes and nonrandomized study designs, show the need of further research in this area (NIDA, 2010d; Goitia *et al*., 2013). Although sharing mechanistic similarities to cocaine and amphetamine, MPH effects on the sensory thalamic nuclei and the somatosensory cortex still remain unclear (Akenasy *et al*., 2007; Goitia *et al*., 2013).

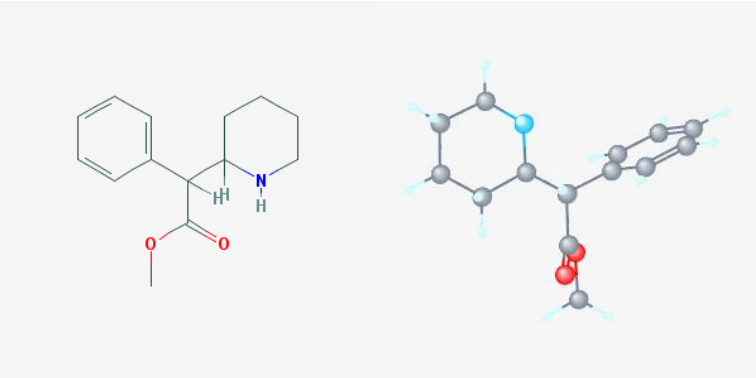

Figure 1.5. *MPH 2D and 3D structures* (PUBCHEM).

MPH (M_f: $C_{14}H_{19}NO_2$; M_w: 233.30 g/mol), like cocaine, can block dopamine and norepinephrine transporters, but not serotonin ones, inducing rapid increases in extracellular dopamine levels, thus becoming a great target for the study of the serotonin-mediated effects elicited by chronic abuse of cocaine in the thalamus. MPH affinity for dopamine transporters in vivo is comparable to that of cocaine (Pan *et al*., 1994; Kuczenski & Segal, 1997). Contrarily to cocaine, its wash out time from the body ranges from 48 to 96 hours and its half-life is longer, too, ranging from 1 to 4 hours, which could explain its lower risk of addiction (DRUGBANK).

Acute and sub-chronic effects of MPH over the thalamocortical system have been reported. For example, MPH increases locomotion in mice after sub-chronic protocols, in a similar way to cocaine, suggesting that this effect is dopamine- and/or norepinephrine-mediated. In addition, cocaine administration treatment induces lower GABAergic minis frequencies than MPH administration to mice, being the LVA/HVA not altered in the presence of MPH, but of cocaine. However, its effects over the thalamocortical somatosensory system seem to be milder than those of cocaine, suggesting a key role of serotonin in the deleterious cocaine-mediated effects in chronic abusers (Goitia et al., 2013). Further research is needed to obtain insights in this particularly drug which use is currently rising.

2. OBJECTIVES

The main objective of this study is the evaluation of the effects of several drugs in the thalamocortical somatosensory system, mainly cocaine and methylphenidate, and, collaterally, ketanserin. For such purpose, two complementary approaches for each drug treatment, based on the system's biomarkers, are established:

- Molecular Biology approach: the analysis of the protein levels of two essential proteins in the system, T-type calcium channels (LVA channels) and GAD.

- Electrophysiological approach: the analysis of the electrical currents related to these proteins, GABAergic miniature currents and LVA currents, the latter usually measured as LVA/HVA ratios.

3. MATERIALS AND METHODS

3.1. Animals

SV129/SvEv adolescent mice (between 25 and 45 days old) were employed for the studies. They were obtained from the Bioterio Central de la Facultad de Ciencias Exactas y Naturales of the Universidad Autónoma de Buenos Aires (UBA). The animals were treated according to the currently existent regulations of laboratory animal care of the CONICET (2003), which were determined with respect to the OLAW/ARENA normative (NIVE, USA). The access for food and water was *ad libitum*, and the day/night cycle was of 12h/12h, with lights being on at 7:00h.

3.2. *In vivo* drug administration

Five groups of study were designed in order to observe the different effects of all drugs involved in the current work:

veh (vehicle): administration of saline solution alone (NaCl 0.9%). This group builds up the negative control for all the rest of the groups.

MPH: administration of MPH. This group allows the observation of MPH effects.

CC: administration of CC. This group allows the observation of CC effects.

veh+K: injection of ketanserin followed by a saline solution binge protocol. This group allows the observation of ketanserin effects alone with a binge of saline solution. Such protocol serves as a control condition for the following group of ketanserin together with cocaine and is also useful for determining the effects of ketanserin alone.

CC+K: injection of ketanserin followed by a cocaine protocol. This group allows the observation of the effects of ketanserin injection in the cocaine protocol, in order to determine any effect that alters the normal effects of cocaine alone. Indeed, ketanserin allows observing the effects of cocaine with the 2A subtype serotonin transporters theoretically blocked by ketanserin.

The administration of all drugs of the study was performed via previous dilution in physiologic solution (NaCl 0.9%). Cocaine chlorhydrate (CC) as well as methylphenidate chlorhydate (MPH) were administered at a dose of 15mg/kg, while the ketanserin tartrate was administered at a dose of 3mg/kg. The schedule follow for such administration was a binge one followed during three days (Spangler *et al*., 1993; Urbano *et al*., 2009; Bisagno *et al*., 2010), which consists of three intraperitoneal (i.p.) injections of 15mg/kg of CC or MPH per day, delayed one hour between them; in the case of the ketanserin, just one injection of 3mg/kg per day was administered one hour before such treatments of CC or MPH. The control and veh+K group were administered equivalent volumes of physiologic solution (10µl per gram approximately). The animals were sacrificed 24h after the last injection. This mode of administration, considered as a sub-chronic binge protocol, tries to mimic the human behaviour of binge and crash, which is usually present in the chronic abusers of psychostimulants, trying to keep the euphoric feeling lasting longer, given the fact that the pleasure effects disappear before any significant reduction of drug levels in blood (NIDA, 2010b).

Table 3.1. *Administration protocol of a common binge used in this work.*

Time	Day 1	Day 2	Day 3	Day 4
0h	veh, CC or MPH	veh, CC or MPH	veh, CC or MPH	
1h	veh, CC or MPH	veh, CC or MPH	veh, CC or MPH	
2h	veh, CC or MPH	veh, CC or MPH	veh, CC or MPH	
24h				**Sacrifice**

Table 3.2. *Administration protocol of veh+K and CC+K groups.*

Time	Day 1	Day 2	Day 3	Day 4
0h	Ketanserin	Ketanserin	Ketanserin	
1h	veh or CC	veh or CC	veh or CC	
2h	veh or CC	veh or CC	veh or CC	
3h	veh or CC	veh or CC	veh or CC	
24h				**Sacrifice**

3.3. Slicing protocol

The mice were anesthetized with tribromoethanol (250mg/kg, i.p.) 24h after the last injection of the respective protocol, followed by an intracardiac perfusion with 15-20 ml of a cold solution, containing rich amounts of antioxidants and low sodium content (being in mM: 200 sucrose, 2,5 KCl, 26 $NaHCO_3$, 1,25 NaH_2PO_4, 20 glucose, 0,4 ascorbic acid, 2 sodium pyruvate, 1 kynurenic acid, 1 $CaCl_2$ y 3 $MgSO_4$) and under constant bubbling of carbogen gas (5% CO_2 – 95% O_2) at pH 7.3.

The different components of the cardiac perfusion solution have distinct functions which contribute to neuronal survival:

Sucrose: osmolarity control, specially, equiosmolar substitution of Na, preventing the generation of action potentials that can facilitate calcium entrance into the neuron and, thus, induce neuronal death (Thomas, 1968).

KCl: membrane potential control to hyperpolarizing resting potentials due to the action of both K^+ and Cl^- (Hodgkin & Horowicz, 1959).

$NaHCO_3$: pH buffer in equilibrium with carbogen gas and small contribution to the amount of Na in the solution (OECD SIDS, 2002).

NaH_2PO_4: pH buffer that, together with bicarbonate ($NaHCO_3$), keep pH at physiological values. Bicarbonate alone is slightly more acid than when combined with phosphate (Schrödter *et al*., 2012).

Glucose: nutrient source for neurons (Shimizu et al., 2012).

Ascorbic acid: antioxidant (Davies *et al*., 1991).

Sodium pyruvate: neuron nutrient and neuroprotective agent (Gonzalez *et al*., 2005).

Kynurenic acid: it blocks glutamate receptors unspecifically by increasing their desensibilization to glutamate. This effect prevents neuronal death by glutamatergic transmission over activation or excitotoxicity (Csapó *et al*., 2014; Lemieux *et al*., 2015).

$CaCl_2$: calcium supply needed for correct calcium channel functioning. It also serves a source for Cl^- ions.

$MgSO_4$: magnesium supply and partial blockade of L-type calcium channels. It also stabilizes the plasma membrane (Park *et al*., 2014).

The combination of low sodium and calcium concentrations, together with low temperatura and glutamate receptor blockade by kynurenic acid helps to prevent neuronal death by hypoxia or by excitotoxicity, pathological process by which neurons are damaged by over activation of the glutamate receptors (Thomas, 1968; Csapó *et al*., 2014; Lemieux *et al*., 2015)

After perfusion, the mice were decapitated and the brains dissected in cooled slicing solution (in mM: 2,5 KCl, 26 $NaHCO_3$, 1,25 NaH_2PO_4, 10 glucose, 0,5 ascorbic acid, 3 myo-inositol (neuroprotector and antioxidant; Mashhoon *et al*,. 2013; Chiappelli *et al*., 2015), 2 sodium

pyruvate, 250 sucrose, 10 $MgCl_2$, 1 $CaCl_2$). The cerebellum was separated from the brain, which was locked in a platform to obtain the coronal slices of 280-300 µm width by a vibratome (Campden Instruments, Reino Unido). The obtained slices were then incubated at 35ºC in artificial cerebrospinal liquid (aCSF; in mM: 2,5 KCl, 26 $NaHCO_3$, 1,25 NaH_2PO_4, 10 glucose, 0,5 ascorbic acid, 3 myo-inositol, 2 sodium pyruvate, 125 NaCl, 3 $MgCl_2$, 0,5 $CaCl_2$) for its immediate manual dissection.

3.4. Drugs

Ph. D. Bisagno of the ININFA (Instituto de Investigaciones Farmacológicas) has been authorized by the ANMAT (Administración Nacional de Medicamentos, Alimentos y Tecnología Médica, Ministerio de Salud de la Nación, Certificado 7561) to work with animal models of drug addiction. In addition, the ANMAT has also authorized the use of abuse drugs for basic research protocols in the IFIBYNE (INSTITUTO DE FISIOLOGÍA, BIOLOGÍA MOLECULAR Y NEUROCIENCIAS) in collaboration with the ININFA. Cocaine chlorhydrate was obtained from Sigma-Aldrich and methylphenidate chlorhydrate (Mallinckrodt Inc., USA) was generously donated by Osmotica Pharmaceutical Argentina S.A. (Buenos Aires, Argentina).

3.5. Molecular biology experiments

3.5.1. Tissue processing and quantification

Brain dissection was performed under a magnifying glass (15x) on a metallic plate surrounded by ice in order to keep the temperature low. The VB and TRN nuclei, together with the somatosensory cortex (figure 3.1), were separated from such slices and kept in eppendorf tubes at -80ºC until its processing (similar protocols in literature: Seo & Leitch, 2014).

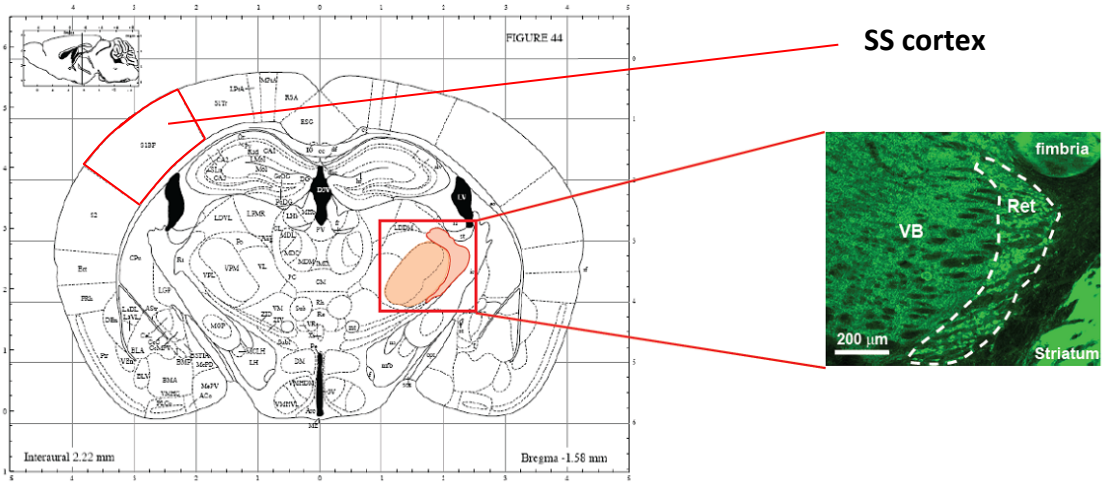

Figure 3.1. *Location of the ventrobasal nucleus* (VB in the figure), *the thalamic reticular nucleus* (TRN, Ret in the figure) *and the somatosensory cortex* (SS cortex in the figure) *in mice brain* (Paxinos & Franklin, 2001).

Sample homogenization was performed in radioimmunoprecipitation buffer (RIPA) which composition is: 50mM Tris-HCl pH 8 (buffer), 150mM NaCl (osmolarity control), 1% v/v NP-40

(non-ionic surfactant; ThermoFisher, USA), 0,1% p/v sodium dodecyl sulphate (SDS; anonic surfactant that induces protein denaturalization to avoid protein degradation, due to the denaturalization of the active sites of proteases, and to facilitate gel migration by negatively charging the proteins; PUBCHEM), 0,5% p/v sodium deoxycholate (anionic detergent useful for extraction of membrane receptors and other plasma membrane proteins; Sigma Aldrich, USA). This RIPA buffer was supplemented with a mixture of protease inhibitors in order to minimize protein degradation by these enzymes, mainly present in lysosomes (Luzio *et al*., 2014): 10µM ethylenediaminetetraacetic acid (EDTA; bivalent cation chelating agent; Sigma Aldrich), 2mM phenylmethylsulfonyl fluoride (PMSF; serin protease inhibitor; Jones, 1978; Estévez *et al*., 2012), 10µg/ml aprotinin (serine protease inhibitor; Royston, 2015), 100µM leupeptin (serine/cysteine peptidase inhibitor; Kim *et al*., 1998), 1µg/ml pepstatin A (aspartyl protease inhibitor; Matúz *et al*., 2012). Tissue immersed in this mixture was then mechanically disrupted with a plastic embolus. Homogenized tissues were incubated at 4ºC using a rotor during 2 hours and afterwards centrifuged for 20 min at 4ºC and 21500g. Supernatant were recovered and protein quantification was performed using a bicinchoninic acid-based kit (BCA kit; Pierce, USA), taking as reference its own BSA concentration pattern. Quantified protein samples were stored at -80ºC in aliquots of 25µg.

3.5.2. SDS-PAGE and Western blotting

In order to run the samples in a polyacrylamide gel, samples were incubated for 3 min at 100ºC in Laemmli buffer, which composition is: 62,5mM Tris-HCl pH 6,8 (buffer), 2% p/v SDS (facilitates protein migration by negatively charging them; PUBCHEM), 0,01% p/v bromophenol blue (protein dye; Sigma Aldrich) 2% v/v β-mercaptoethanol (reduces disulphide bonds between protein and inside protein complexes, allowing the visualization of each subunit separately; Sigma Aldrich), 10% v/v glycerol (avoids sample diffusion by increasing its density) (Laemmli, 1970). Afterwards, samples were quickly cooled down in ice and charged in denaturing (SDS) polyacrylamide gels (10% concentrated) and the SDS-PAGE (polyacrylamide gel electrophoresis in SDS denaturing conditions) was run in order to separate proteins by their size, given the fact that their electronic density is almost the same due to the presence of SDS.

SDS-PAGE was performed at a constant voltage of 120V during approximately one hour and a half and proteins were transferred to nitrocellulose membranes (SIGMA-ALDRICH, USA) at constant amperage of 250mA for 2 hours keeping temperature low with ice, thus forcing negatively charged proteins to migrate from the negative to the positive pole, as during the SDS-PAGE (figure 3.2). Ponceau S dying was performed later on to visualize the correct transfer of proteins and unspecific binding sites of the membrane were blocked either by commercial blocking buffer (Thermo Scientific, USA) or milk (SANCOR, Argentina), depending on the primary antibody (see antibody solutions later on), for 1 hour at room temperature with constant agitation. Membranes were then cut accordingly to the desired molecular weights regarding the molecular weight marker (Fermentas Page Ruler, ThermoScientific Pierce, USA; All blue, BioRad) which was run together with protein samples. Finally, membranes were incubated at 4ºC with constant agitation either overnight and mixed with the following primary antibodies, all made in rabbit: anti-GAD65/67 (dilution 1:1000 in blocking buffer-Tween20 (0.05%); Sigma Aldrich, USA), anti-actin (dilution 1:100 in blocking buffer-Tween20 (0.05%); Sigma Aldrich, USA), anti-$Ca_V3.1$ (dilution 1:200 in PBS-milk-Tween20 (0,05%);

Alomone, Israel). Previous checking of the primary antibodies was performed in order to visualize the correct binding to the proteins using commercial antigens provided together with the antibodies.

The next step was the washing up of the primary antibody at room temperature with two incubations of 5min with TBST (10mM Tris (buffer), 150mM NaCl (osmolarity control), 0,05% Tween-20 (detergent), pH 8) and two incubations of 5min with TBS (50mM Tris, 150mM NaCl, pH 8), in order to eliminate any remaining Tween-20 from the membranes, which can negatively affect antibody binding (Hoffman & Jump, 1986).

Following the protocol, secondary antibody incubation (anti-rabbit IgG coupled to HRP (horseradish peroxidase), dilution 1:1000, Dako, Denmark) was realized at room temperature for one hour and a half with constant agitation. A second washing step, as the previous one with the primary antibodies, was performed. The developing of the samples was then realized using commercial ECL by means of chemiluminescence (Immobilon Western, EMD Millipore Co., USA). For visualizing the developing, a fixed fast f0.95 camera (16 bit resolution) with auto focus and stage zoom was used (G:BOX Chemi XX6, Syngene, USA) and signal intensity was quantified using the program ImageJ 1.43m (http://imagej.nih.gov/ij/index.html, NIH). Bands corresponding to GAD 65, GAD 67 and CaV3.1 were normalized with respect to their own actin (loading control), and all data was then relativized to the average of the control group.

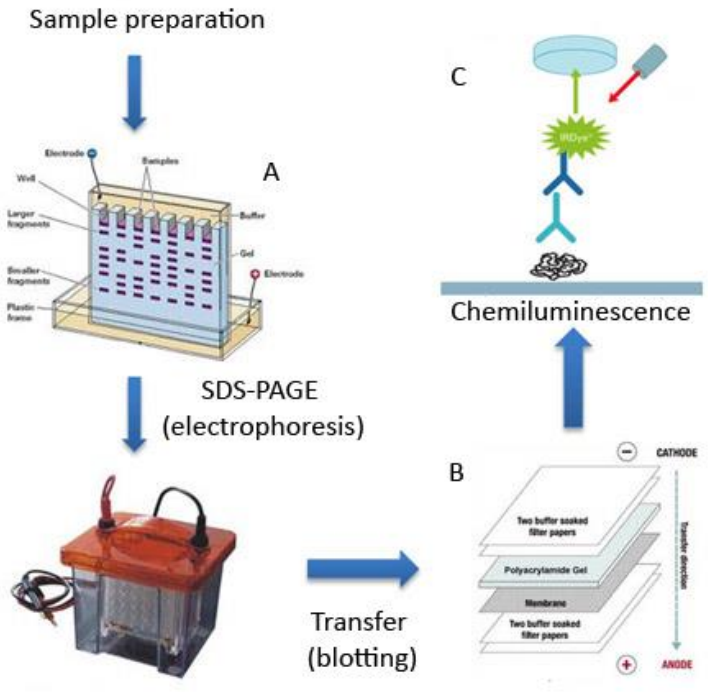

Figure 3.2. *Workflow of the Western Blot protocol followed in this study* (modified from CASE WESTERN RESERVE UNIVESTITY). **(A)** After sample preparation, an SDS-PAGE protocol is performed in order to separate the proteins present in the sample by their size. **(B)** Transference of the proteins to a nitrocellulose membrane (blotting) is followed to facilitate protein visualization in the next step. **(C)** Chemiluminescence visualization of the proteins via primary (specific to the protein) and secondary (labelled) antibody binding to the sample.

3.5.3. Statistical analysis

The programs used for statistical analysis were the InfoStat (Universidad Nacional de Córdoba, Argentina) and the SigmaPlot (Systat Software, USA). Depending of the amount of groups to be compared, t-test or one-way ANOVA test was used with two or more groups, respectively. Following comparisons between groups were performed either by the LSD Fisher method or by the Tukey method (a more restrictive method). If the assumptions of normality or homoscedasticity (homogeneity of variances) were not passed, non-parametric tests were used for comparisons. The main ones employed in the study were the Wilcoxon-Mann-Whitney U test (instead of the t-test) and the Kruskal-Wallis test (instead of the one-way ANOVA). Differences between groups were considered as significant with a p-value under 0.05. All data is shown as an average together with the standard deviation of the mean.

3.6. Electrophysiological experiments

3.6.1. Patch-clamp register devices

The patch-clamp technique allows visualizing electrical currents affecting cells, which can be really useful for determining different pathologies, drug effects or physiological processes. These experiments were realized in a set up composed by a Olympus BX50WI vertical transilluminated microscope, two heads (pre-amplifiers) controlled by micromanipulators for the positioning of pipettes and stimulation electrodes, a perfusion system by peristaltic pump for the recirculation of the carbogenous register solution (aCSF supplemented with blocking compounds described later) and an digitalizing system (Axon Instruments, Molecular Devices). The microscope as well as the heads, micromanipulators and the peristaltic pump are surrounded by a Faraday cage (which minimizes environment electrical background) and placed on an anti-vibrational table. The register electrode is filled with intracellular solution and connected to a pre-amplifier that amplifies the signal, acts as a first background filter and is also connected to a neutral electrode in the bath where the slice in under study. This head is connected to a MultiClamp 700B amplifier, which allows amplifying even more the signal and its filtering in order to eliminate the high frequency background. Moreover, the amplifier controls the current injection and holds the feed-back circuitry required for voltage control. In addition, this amplifier is connected to a Digidata 1440A digitalizer that allows digitalizing the register and connects to a computer where data is acquired and processed.

Registers of total intracellular currents were performed in the soma of VB neurons by voltage holding by means of patch-clamp in whole-cell configuration (whole-cell patch-clamp).

Patch pipettes were obtained by borosilicate glass capillary stretching with internal filament (Sutter Instrument, USA) and were filled with a Cs^{+}-rich solution, in order to block voltage dependent potassium channels (in mM: Hepes 40 (buffer), TEA-Cl 20 (blocks nicotinic acetylcholine neurotransmission by blocking the receptor-mediated K+ currents; Sigma Aldrich), phosphocreatine 12 (energetic substrate), EGTA 0,5 (chelant agent), Mg-ATP 1 and Li-GTP 1 (energetic substrates), MgCl2 1 (supply of Mg^{2+}), CsCl 110) supplemented with 1 mM of QX-314 (*N*-(2,6-Dimethylphenylcarbamoylmethyl)triethylamonium bromide, a modified quaternary lidocaine that intracellularly blocks the voltage dependent sodium channels. Inside

the pipettes, a previously clorured (with commercial bleach, NaClO 5.5%) silver wire is placed, thus acting as a Ag/AgCl electrode that can make the redox pair together with the CsCl internal solution, which allows electrical continuity between the pre-amplifier and the pipette. This circuitry is closed by another electrode Ag/AgCl submerged in the aCSF (in mM: 2,5 KCl, 26 $NaHCO_3$, 1,25 NaH_2PO_4, 10 glucose, 0,5 ascorbic acid, 3 myo-inositol, 2 sodium pyruvate, 125 NaCl, 1 $MgCl_2$, 2 $CaCl_2$) with soaks the slices and is connected to the same pre-amplifier which carries the patch pipette. The pipette resistance, measured in aCSF, was between 2 and 5 MΩ. Previous seal formation, the pipette tip potential was electronically neutralized.

3.6.2. Whole-cell patch-clamp

After localising the VB nucleus using a 4x objective, a search of suitable neurons is performed using the immersion 40x objective. Patch pipette is then lowered with a slightly positive pressure to keep clean the tip of the pipette and to visualize the moment at which the pipette is near by the plasma membrane of the target neuron. Afterwards, positive pressure is released and a soft negative pressure is then applied in order to create a seal between the tip of the pipette and the plasma membrane of the neuron (figure 3.3). The electrical resistance of this seal must reach at least 1 GΩ in order to guarantee the quality of the voltage holding. By means of short negative pressure pulses and by capacitive currents superiors to those that the plasma membrane can compensate, a rupture is produced in the region that entered the pipette, thus creating a continuity between the inside of the pipette and the cytoplasm, for which is called whole-cell patch-clamp. The cytoplasm is replaced in few minutes by the internal solution of the pipette.

The held potential membrane (holding) through the patch pipette was of -70 mV. Capacitance was compensated (25-40 pF) and also was the serial resistance between the pipette and the cell (6-12 MΩ, previous electronic compensation of about 20-30%).

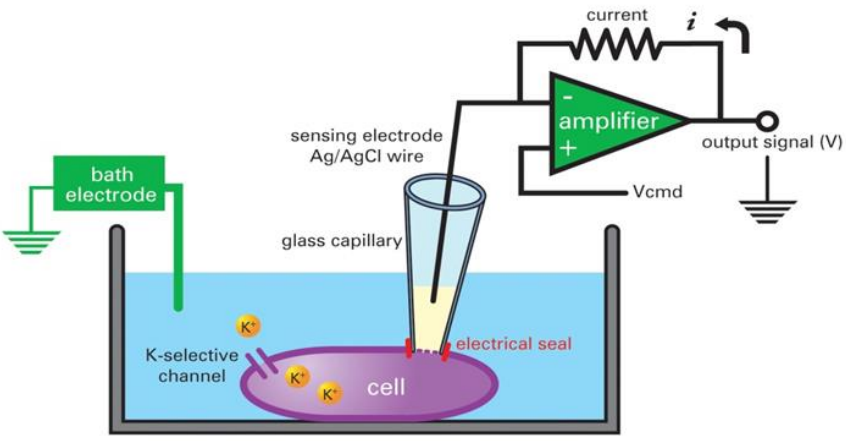

Figure 3.3. *Schematic representation of the whole-cell patch-clamp technique.* On the right, scheme of the electrical circuit integrating the amplifier and the pipette. The seal between the pipette and the plasma membrane must be high enough in order to avoid any leakage of current, which would alter the measurements. Adapted from Clare (2010).

3.6.3 Spontaneous GABA miniatures current register

By adding 3 µM of tetrodotoxin (TTX) to the bath, a voltage-dependent sodium channel blocker (Alomone, Israel), the firing of spontaneous action potentials is prevented in the neurons present in the slice. However, this agent does not avoid the GABA spontaneous release from the TRN neurons, which is registered in the post-synapsis (VB neurons) as miniature currents (referred as minis when spontaneous events are registered in the presence of TTX). The GABAergic nature of the minis was isolated from glutamatergic stimuli over the neurons by the addition of blockers in the register solution: 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, AMPA/Kainate receptor antagonist, 20 µM) y ácido (2R)-amino-5-phosphonovaleric acid (DL-AP5, NMDA receptor antagonist, 50 µM). Moreover, minis are $GABA_A$ receptor-dependent due to its inhibition by the addition of bicuculline or picrotoxin (50 µM) (reviewed in Erlander *et al*., 1991). Mini registers were realized under voltage holding (-070 mV) for 2:30 minutes, only in those cells with a leak current below to 500 pA and with an access resistance below to 10 MΩ. Otherwise, GABA identification becomes difficult due to the background.

The analysis of the registers obtained was realized with MiniAnalysis (Synaptosoft, USA). Accumulative probability graphs were created for every register for either the amplitude or the event interval (the inverse to frequency of events), which were later fitted to the following equation: $y = y_0 + a*\exp(-b*x)$, where y is the accumulated probability, x is the amplitude o event interval, and y_0, a and b are parameters calculated by the program used for the fitting (SigmaPlot; Systat Softwares, USA). From this fittings, median values (x values for y = 0.5) were obtained, which later were used as variables for statistical comparisons.

3.6.4. Calcium currents register

In order to measure calcium currents mediated by T-type and P/Q calcium channels, a protocol consisting in a voltage ramp was used. This ramp lasted for 500 ms, going from -90 mV to +50 mV. By doing this, serial activation of T-type channels (low-voltage-activated, LVA) and P/Q channels (high-voltage-activated, HVA) is allowed, thus facilitating the calculation of their densities without suffering any rundown (Urbano *et al*., 2009; Bisagno *et al*., 2010). Currents were quantified with Clampfit-pClamp 10.2 (Molecular Devices, USA).

4. RESULTS AND DISCUSSION

4.1. Effects of the sub-chronic administration of MPH in the thalamocortical somatosensory system

As it has been previously reported, cocaine-deleterious effects over the thalamocortical somatosensory system might be induced by serotonin-mediated mechanisms. To clarify such effects, MPH was the perfect candidate because, although sharing with cocaine the blockade of both dopamine and norepinephrine transporters, it does not block serotonin ones. Therefore, in order to visualize the effects of MPH in the thalamus, the same protocols that induced significant differences in mice treated with cocaine were followed, as described in Goitia *et al*., 2013. In addition, the same proteins were also studied (T-type calcium channels and GAD).

The sub-chronic administration of MPH (three-day binge, three injections per day) and the sacrifice of the treated mice at 24 hours post-binge (after the last injection of the binge) produced different effects in the levels of these proteins in the somatosensory thalamocortical system.

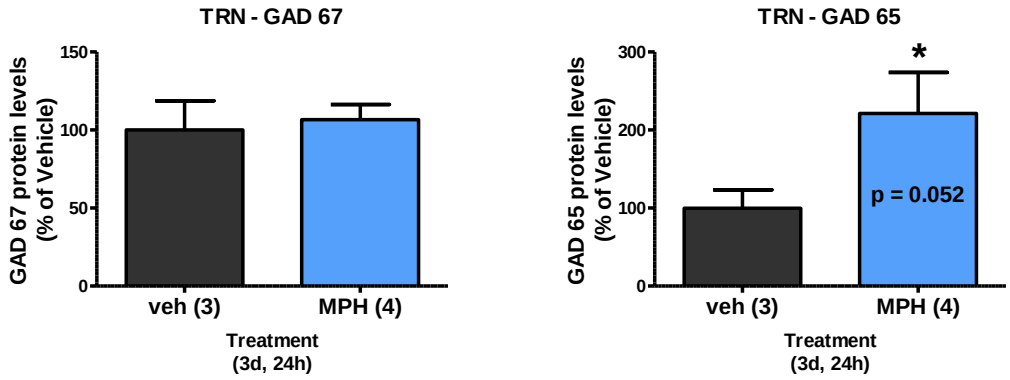

Figure 4.1. *GAD protein levels in the TRN after MPH binge.* For all the following figures, numbers in brackets represent the number of samples analysed; data is shown as percentage of the mean of the vehicles, as it is relativized to this value.

In the TRN, the administration of MPH induced a significant difference with respect to the control group in GAD 65 levels, the isoform located in the soma of the TRN neurons and actually the one thought to be implied in TRN coordination and inhibition (reviewed in Erlander & Tobin, 1991). An increase in this isoform might imply that the TRN nucleus is being over-inhibited by MPH treatment (hypothetically, increasing GABA levels in the synaptic clefts of the TRN neurons), thus blunting the inhibitory component of the system, which results in a higher amount of relayed information from the VB to the SS cortex. This effect could explain the proven clinical effects of such drug in patients, helping to focus attention and improving their performance (NIDA, 2010d).

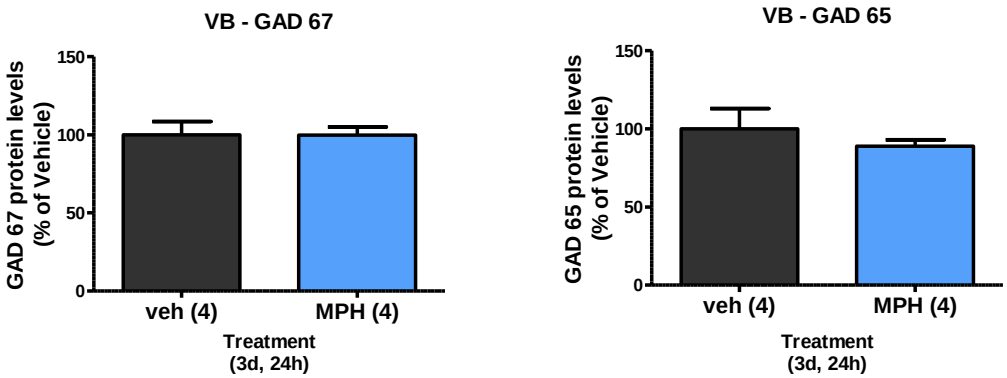

Figure 4.2. *GAD protein levels in the VB nucleus after MPH binge.*

In the VB nucleus, the administration of MPH did not induce any significant difference compared to the control animals, as previously described in Goitia *et al*. (2013). This could be explained by the lower physiological importance that the GAD enzyme has in this glutamatergic nucleus, being focused on the stimulation of other areas via its excitatory (and so, glutamate-based) afferents.

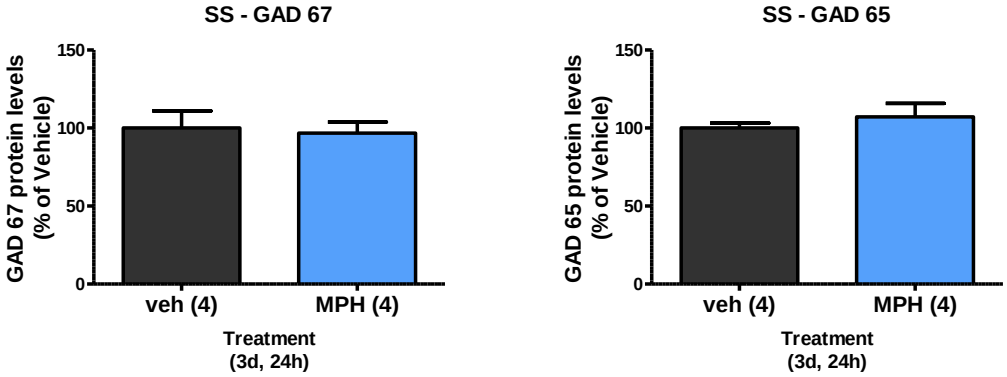

Figure 4.3. *GAD protein levels in the SS cortex after MPH binge.*

In the somatosensory cortex, the administration of MPH did not induce an increase of GAD protein levels with respect to the control group. This result would suggest that no strong effects are being exerted on the SS cortex, since there is an important population of GABAergic interneurons in this tissue.

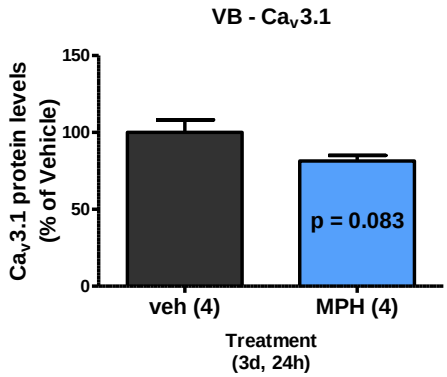

Figure 4.4. *$Ca_v3.1$ calcium channel levels in the VB nucleus after MPH binge.*

The protein levels of $Ca_v3.1$ calcium channels in the VB nucleus did not suffer significant differences with respect to the control animals, but the p-value was close to 0.05, suggesting that the commented effect of MPH of inhibiting the TRN is allowing the VB to be free of its inhibition. Such lack of inhibition would produce an excess over the normal levels of calcium channels required for proper information relay, forcing the homeostatic degradation of the excess. However, a higher amount of samples analysed is required to clarify this effect.

4.2. Role of ketanserin in the modulation of cocaine-mediated effects in the thalamocortical somatosensory system

The administration of ketanserin to mice, an antagonist of serotonin receptors 5-HT_{2A} (Broderick *et al*., 2004), would theoretically produce an inhibition or a softening of the serotonin-mediated effect of cocaine in the thalamocortical system. In fact, it is thought that serotonin binds to two main receptors in the TRN: 1A and 2A, supposed to be inhibitory and excitatory for the TRN, respectively (Goitia, 2015). Therefore, TRN would not be over activated by cocaine treatment if 2A receptors are blocked by ketanserin. Three different groups were analysed here in order to study this effect: veh+K (saline with ketanserin), CC+K (cocaine with ketanserin) and CC (cocaine alone).

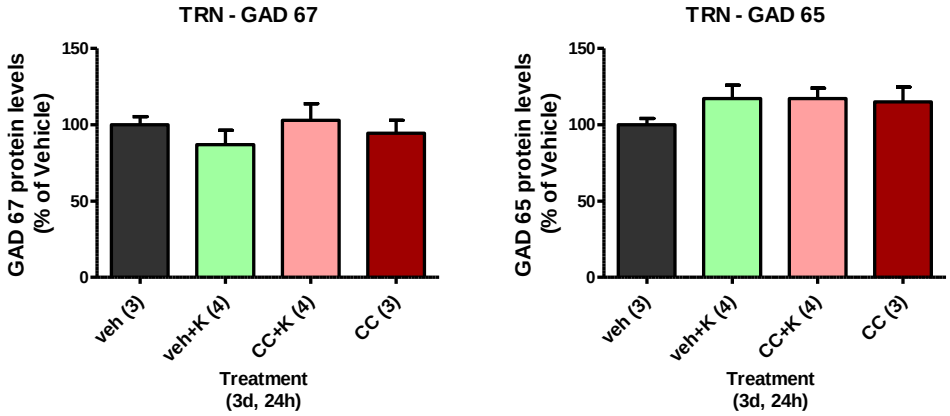

Figure 4.5. *GAD protein levels in the TRN after veh+K, CC+K and CC treatments.*

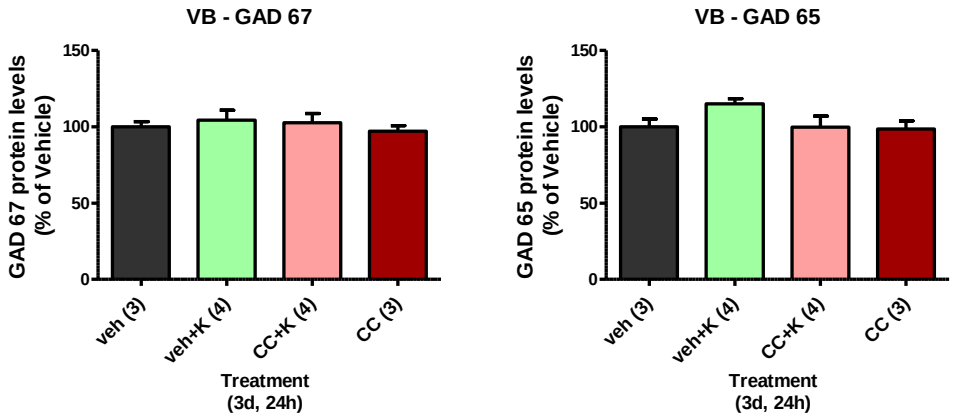

Figure 4.6. *GAD protein levels in the VB nucleus after veh+K, CC+K and CC treatments.*

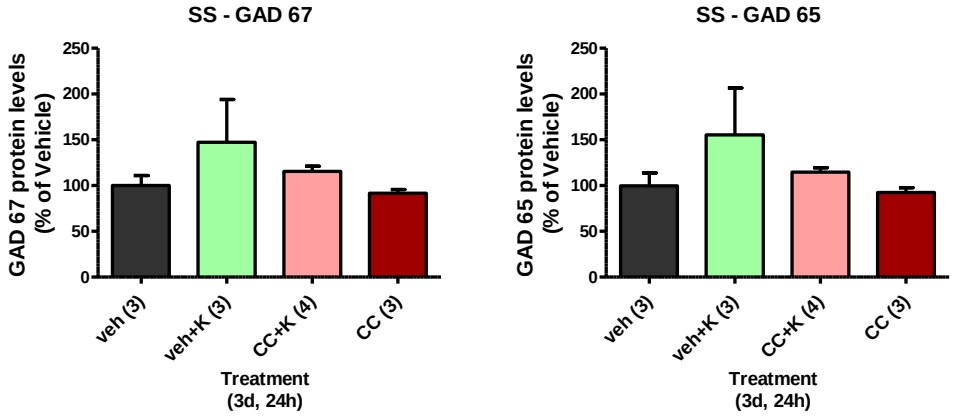

Figure 4.7. *GAD protein levels in the SS cortex after veh+K, CC+K and CC treatments.*

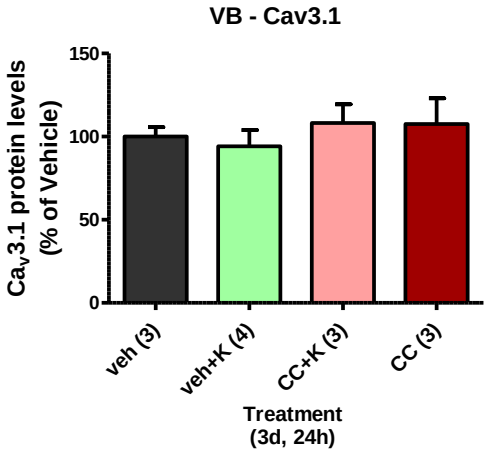

Figure 4.8. *$Ca_v3.1$ calcium channel levels in the VB nucleus after veh+K, CC+K and CC treatments.*

No significant differences in the proteins analysed were found between those animals treated with ketanserin and saline (veh+K) and those treated with ketanserin and cocaine (CC+K). There were not significant differences either with respect to control (veh) or cocaine-treated animals neither. Nevertheless, these results would imply that the blockade of 2A serotonin receptors is essential to prevent cocaine-mediated effects in the thalamocortical somatosensory system. The absence of increasing levels of GAD or $Ca_v3.1$ proteins supports the hypothesis that the effects of cocaine in this tissue are mediated by serotonin, given the fact that MPH does block norepinephrine and dopamine transporters as cocaine does, but not serotonin ones. However, since no significant differences in $Ca_v3.1$ levels were found between cocaine and control groups, as described in literature (Goitia *et al*., 2013), a higher sample size is required in order to confirm this hypothesis. Probably, ketanserin does not exerts a soft effect in this system or a stronger treatment with ketanserin (i.e. co-administration with saline solution or cocaine during the binge; longer treatments or higher doses) is required so as to observe its long-lasting effects (changes in protein expression) over the somatosensory thalamocortical system. Serotonin receptor 1A must also be taken into account in order to elucidate the full extent of these drugs in the system.

4.3. Differential effects of sub-chronic administration of MPH, cocaine and ketanserin treatments in the thalamocortical somatosensory system

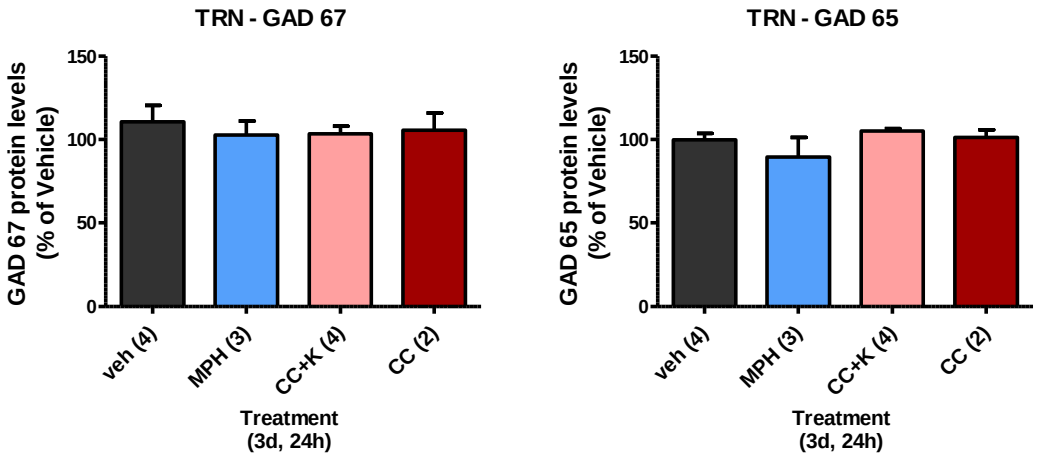

Figure 4.9. *GAD protein levels in the TRN after MPH, CC+K and CC treatments.*

No significant increases in GAD 65 were observed in the MPH group, as described before, suggesting either that the treatment is too weak to obtain homogeneous data or that a higher amount of samples is required to elucidate the effects of these drugs.

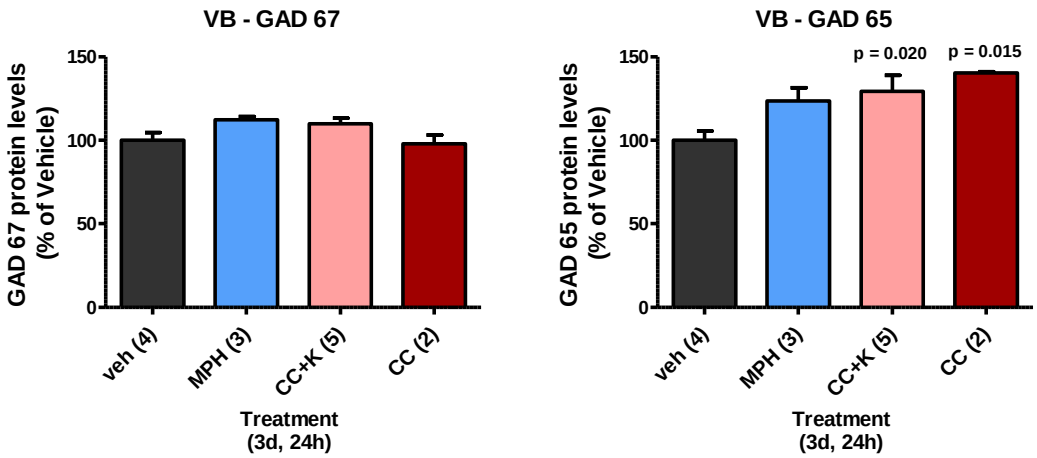

Figure 4.10. *GAD protein levels in the VB nucleus after MPH, CC+K and CC treatments.*

As shown in the graph, GAD 65 levels did change in the VB nucleus after cocaine and cocaine with ketanserin treatments. Due to the low number of samples composing the cocaine group (just 2 samples), significant levels should be treated carefully. However, the increase in GAD 65 levels after cocaine with ketanserin treatment should be taken into account. Further research is needed to clarify this effect.

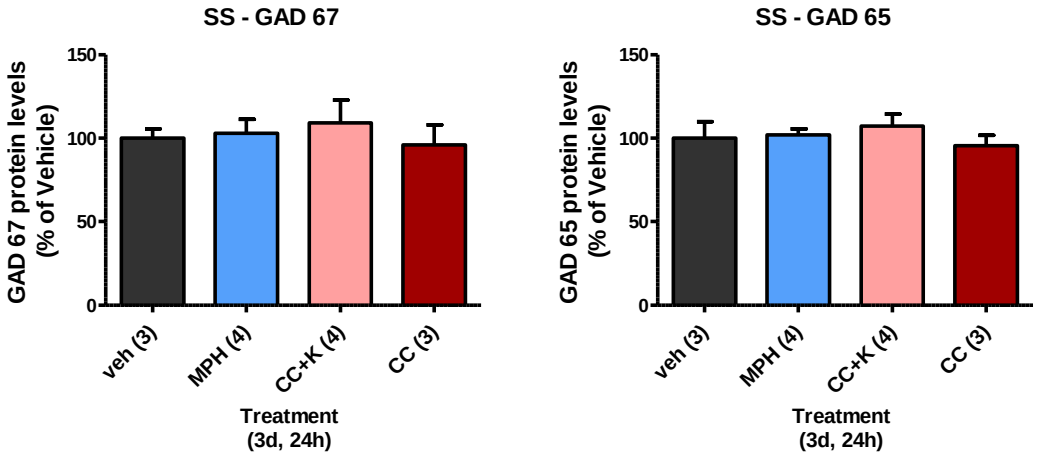

Figure 4.11. *GAD protein levels in the SS cortex after MPH, CC+K and CC treatments.*

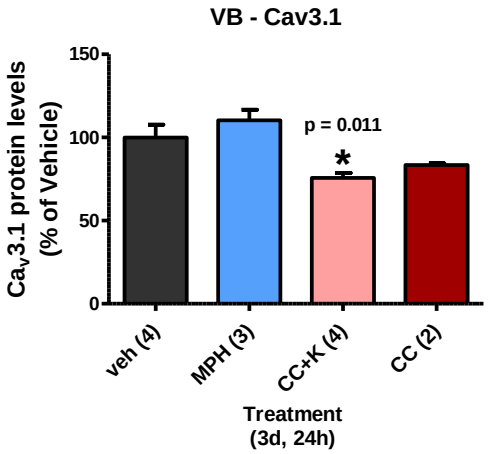

Figure 4.12. *$Ca_v3.1$ calcium channel levels in the VB nucleus after MPH, CC+K and CC treatments.*

As it is possible to observe in the graphs, GAD protein levels did not differ much in the majority of the treatments compared to the rest. However, it is interesting to mention that the levels of the $Ca_v3.1$ calcium channels in the VB nucleus did decrease significantly in the group of cocaine with ketanserin (CC+K) when compared to the control group, similarly to when mice were treated with MPH (figure 4.4), This tendency in MPH is not observed here, probably because of the experimental variability and low number of samples. The significant reduction that was not observed in previous graphs in the CC+K group with respect to the control group (figure 4.8) may be caused by a stronger effect of ketanserin in these animals or by the low number of samples in the previous graphs.

Taking into account that further research is required in order to clarify the effects of these drugs on the somatosensory thalamocortical system, these data suggest that ketanserin could probably be either inhibiting the TRN or causing a lower activation of the TRN, resulting in a softer inhibition action over the VB and, thus, creating an useless pool of $Ca_v3.1$ receptors which are not essentially needed in order to elicit action potentials and to successfully make VB act as the relay nucleus it is. As explained before in figure 4.4, a lower amount of these channels would be required in order to cause the depolarization needed to begin the generation of an action potential, due to the lower hyperpolarizing (inhibiting) effect of TRN, which would be inhibited (or lower activated) by the blockade of serotonin receptors $5\text{-}HT_{2A}$ exerted by ketanserin. This excess of T-type channels in the VB nucleus would be recycled or eliminated in order to achieve the homeostatic levels of this protein. Although there is an accumulation of serotonin in the synaptic cleft of the TRN, the blockade of its receptors would be preventing the generation of the harmful effects that cocaine exhibits alone.

4.4. Effects of the sub-chronic administration of MPH and cocaine with ketanserin over the spontaneous GABAergic transmission in the ventrobasal nucleus

Another important aspect of the effects of psicostimulants over the thalamus is the measurement of the GABAergic miniature currents, hyperpolarizing currents produced by the spontaneous release of GABA from TRN terminals (Fatt & Katz, 1952; Trigo *et al*., 2010), which would reflect the TRN basal activity (and would be related to GAD 67 levels).

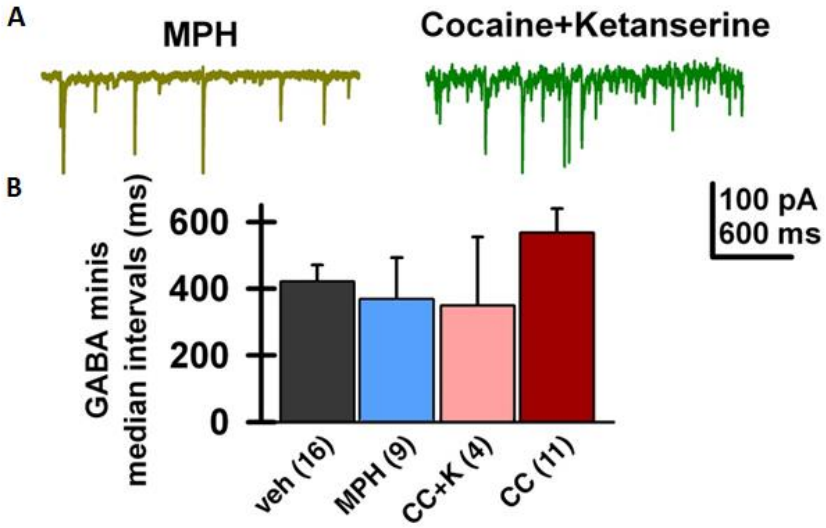

Figure 4.13. *Effects of the sub-chronic administration of MPH and cocaine with ketanserin on the spontaneous GABA release over the VB nucleus.* **(A)** Representative registers of the GABAergic miniature currents measured in the VB nucleus in animals treated with MPH and cocaine with ketanserin. It is worth mentioning the higher background measured in the latter group. **(B)** Medians of the intervals between GABA miniatures belonging to neurons from mice treated with MPH and cocaine with ketanserin. Mice were sacrificed 24 hours after a 3-days binge following the protocol detailed before. Numbers in brackets specify the number of neurons registered in each group. Data of saline (veh) and cocaine (CC) GABA minis is adapted from Goitia *et al*., 2013.

It has been reported that both cocaine and MPH induce a similar increase in the frequency of GABA "minis" (smaller median interval) after sacrifice 1 hour after a three-day binge, effect that is lost when sacrificed at 24 hour after a three-day binge (Urbano et al. 2009; Goitia *et al*., 2013). Interestingly, no differences between MPH and CC+K groups were observed, supporting the idea that the blockade of serotonin receptors by ketanserin in the cocaine treatment mimics the situation of the MPH treatment, without any binding to serotonin transporters. Another important fact is that none of both groups suffered significant differences when compared to the control group, supported by the non-increased levels of GAD 67 reported in this study. However, a higher amount of registers in both CC and CC+K groups would be required to clarify the effects of these treatments.

4.5. Effects of the sub-chronic administration of MPH and cocaine with ketanserin over the post-synaptic calcium current ratios

Taking into account previous results of the research group, which point to the T-type calcium channels as one of the agents implied in the thalamocortical dysrhythmia syndrome induced in vivo by cocaine (Urbano et al. 2009; Bisagno *et al*., 2010; Goitia *et al*., 2013), the effects of the different drug treatments were studied on the current mediated by these channels (I_T), specially its density when compared to the current mediated by the P/Q calcium channels, using voltage ramps that allow activating T-type channels first (low-voltage-activated, LVA) and, later on, P/Q channels (high-voltage-activated, HVA). It has been observed a reduction of the LVA/HVA ratio in mice which undergone a three-day binge cocaine protocol and were sacrificed 24 hours after the last injection. This reduction was caused by a strong increase of P/Q current rather than by a reduction of the T-type one (Goitia *et* al., 2013). This over expression would be a way of how these neurons cope with cocaine's mediated over-activation of T-type channels. Indeed, such increment in P/Q channels can be seen as "compensatory homeostatic" mechanism in response to cocaine's effects (Driscoll *et al*., 2013).

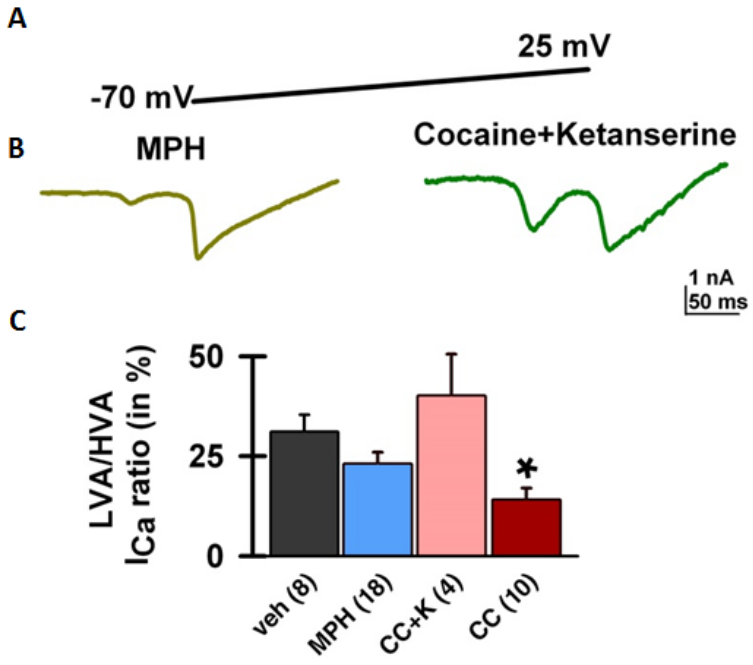

Figure 4.14. *Calcium current ratios on post-synaptic VB neurons measured after sub-chronic protocols of MPH and cocaine with ketanserin.* **(A)** Detail of the ramp applied to the patched VB neurons. **(B)** Detail of the low-voltage-activated (LVA, corresponding to T-type calcium channels; left peak in each graph) and of the high-voltage-activated (HVA, corresponding to P/Q calcium channels; right peak in each graph) calcium currents measured in patched VB neurons after MPH and cocaine with ketanserin administration. **(C)** LVA/HVA ratios corresponding to each treatment group. Mice were sacrificed 24 hours after a 3-days binge following the protocol detailed before. Numbers in brackets specify the number of neurons registered in each group. Data of saline (veh) and cocaine (CC) GABA minis is adapted from Goitia *et al*., 2013.

5. CONCLUSIONS

1. The sub-chronic administration of MPH induced both a decreasing tendency in T-type calcium channels in the VB nucleus and a significant increase in GAD 65 protein levels in the TRN. These results could imply an inhibition or lower activated state of TRN mediated by MPH, lowering its inhibitory effect on the VB nucleus.

2. Ketanserin induced a significant increase in GAD 65 levels in the VB nucleus, a controversial result that would be indicating VB inhibition and that needs further research in order to be clarified.

3. Ketanserin also induced a significant decrease of T-type calcium channels in the VB nucleus when co-administered with cocaine, resembling the tendency that triggered MPH in the same nucleus. Although no differences in both of the electrophysiological experiments between MPH and cocaine with ketanserin groups were found, thus supporting such hypothesis of similarity, this situation requires further research to elucidate the similarity between both treatments.

4. No remarkable effects were observed in the SS cortex, probably because of its role in generating the cognitive experience itself rather than relaying or modulating the transmission of the sensory information, as VB nucleus and TRN do, respectively. However, further electrophysiological experiments, like whole-cell patch-clamp, are required to understand the role of the SS cortex in the thalamocortical somatosensory system.

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