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# Identification Of The Cell Type Expressing The Glycine Transporter 1 (glyt1) In The Thalamus

Patricia A. Lozano

University of Texas at El Paso, palozano3@miners.utep.edu

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IDENTIFICATION OF THE CELL TYPE EXPRESSING THE GLYCINE  
TRANSPORTER 1 (GLYT1) IN  
THE THALAMUS

PATRICIA ANDREA LOZANO PRADO

Master's Program in Biological Sciences

APPROVED:

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Manuel Miranda, Ph.D., Chair

---

Siddhartha Das, Ph.D.

---

Giulio Francia, Ph.D.

---

Xiao Chuan, Ph.D

---

Stephen Crites, Ph.D.  
Dean of the Graduate School



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by

Patricia Andrea Lozano Prado

2019

To my beloved parents and brothers who have supported me in every moment.

IDENTIFICATION OF THE CELL TYPE EXPRESSING THE GLYCINE  
TRANSPORTER 1 (GLYT1) IN  
THE THALAMUS

by

PATRICIA ANDREA LOZANO PRADO, B. S.

THESIS

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The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

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## **Abstract**

Glycine acts as an inhibitory neurotransmitter and a co-agonist of NMDAR at the glutamatergic synapses. Termination of glycinergic neurotransmission is achieved by glycine transporter 1 (GlyT1) and glycine transporter (GlyT2). Both transporters are expressed in the spinal cord, brainstem and cerebellum, but GlyT1 is also expressed in the retina and the forebrain. Previous studies have described the role and cyto-architecture of GlyT1 in the caudal areas of the brain and retina, but not in the structures of the forebrain. Based on our preliminary data, GlyT1 is highly expressed in the thalamus, an area involved in sensory and motor signal relay. The function, mechanism and the cell type expressing GlyT1 in this area are poorly studied and deserves to be studied. Preliminary results demonstrate that GlyT1 immunoreactivity is expressed in neurons rather than glial cells. However, the characterization of the neurons expressing GlyT1 remains unknown. Due to the dual phenotype of GABAergic and glycinergic neurons in other regions of the brain, it is hypothesized that the cells expressing GlyT1 in the thalamus are GABAergic. To address this question, we performed conventional immunohistochemistry, fluorescent and viral tracing, and transgenic mice models. Given the expression of GlyT1 in areas of the thalamus, these results suggest a better characterization of the location of GlyT1 and suggest that it might have a different role aside the inhibitory role of the caudal structures of the brain. These findings will help understand the glycinergic kinetics and the overall role and function of GlyT1.

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# **Chapter 1**

## **Introduction**

### **1.1 Cell Communication in the Central Nervous System**

During the past few centuries, the central nervous system (CNS) has been studied by different approaches in order to understand its interactions, mechanisms, and functions. Nevertheless, it has not been possible to fully elucidate how it regulates the body functions because of the complexity of its millions of connections. Hundreds of questions are yet to be answered and joint efforts are still required to learn more about the mysteries of the CNS.

Just as every living organism finds ways of maintaining homeostasis for various mechanisms and processes, the CNS has to maintain a chemical balance in order to function properly. In the same manner within the neurotransmission process, neurotransmitters levels have to be regulated by specific proteins known as neurotransmitter transporters.

Neural communication can be compared to a follow-up letter that contains a specific message, where the writer sends the message in an enclosed envelope. The sender has to deliver the message with the exact content in order to be comprehended by the recipient. The message has to be clear, organized and sent on time; consequently, the recipient will receive it and understand the message. The same concept is applied for neural communication. There are presynaptic and postsynaptic neurons, which act as the sender and the recipient of the message, respectively. The neurotransmitters, which serve as the message, are stored in synaptic vesicles. The regulation of the message is a critical step during cellular communication. Vesicles and

neurotransmitter transporters are in charge of controlling and regulating the aforementioned process. In nature, glycine is one of the chemical messengers that transmits signals in the CNS (Zafra and Giménez, 2008).

## **1. 2 Glycine**

### **1. 2. 1 Glycine: Biosynthesis and Degradation**

Glycine is the simplest amino acid. It has one amino group, one carboxyl group and two hydrogen atoms bound to one carbon atom. It plays several important functions in metabolic regulation, anti-oxidative reactions and neurological function (Wang et al., 2013). In all mammals and other vertebrates, glycine acts a precursor for macromolecules and furnishes all the nitrogen atoms of purines (Nelson and Cox, 2005).

Several biological pathways yield on the production of this amino acid. Glycine is synthesized from different amino acids such as serine (via serine hydroxymethyltransferase (SHMT)), threonine, choline and hydroxyproline involving the liver and kidneys (Wang et al., 2013). Glycine biosynthesis and degradation are crucial processes. The glycine cleavage system (GCS, also called glycine synthase) is the principal enzyme that degrades glycine in mammals. It is worth mentioning that a genetic defect in the GCS might lead to a congenital metabolic disease known as glycine encephalopathy or non-ketotic hyperglycinemia. The accumulation of the amino acid in the fluids and tissues of the body, especially in the cerebrospinal fluid, characterize this rare metabolic disorder. Patients with this disease present severe brain malformation and neurological symptoms such as seizures, hypotonia, intellectual

disability, and myoclonic jerking (Applegarth and Toone, 2006). An important point to consider is that these serious symptoms are not manifested in any other known amino acid metabolic disorders; demonstrating that glycine has an essential and indispensable role in the brain development and function (Sato, 2018).

### **1. 2. 2 Glycine as a Neurotransmitter**

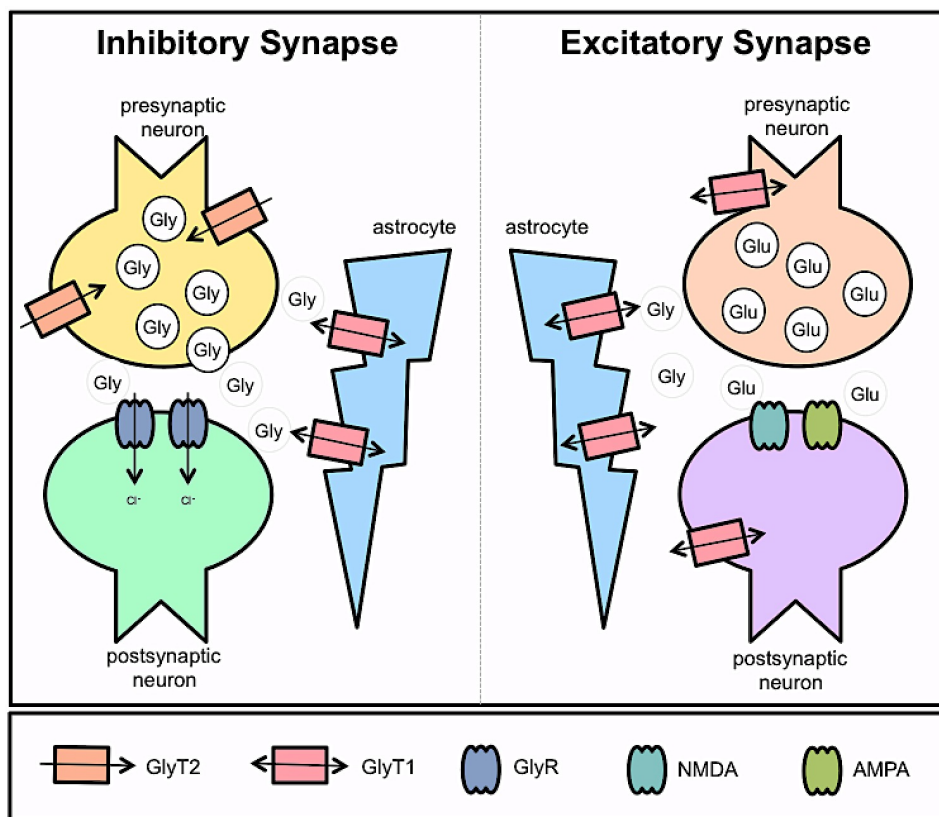
It was not until 1965, when Aprison and Werman proposed and described, for the first time, the presence of glycine as neurotransmitter in the spinal cord of cats (Aprison and Werman, 1965). Five years later, Shank and Aprison demonstrated that glycine could be synthesized by neurons. Simultaneously, Hopkin and Neal published their studies revealing that glycine was released from spinal cord slices after proper stimulation (Hopkin and Neal, 1970). Since then, glycine has been identified and recognized as a neurotransmitter (Bowerly and Smart, 2006). In recent years, glycine has been considered as the major inhibitory neurotransmitter in the posterior areas of the vertebrate central nervous system, especially in the brainstem, spinal cord (Aprison and Werman, 1965) and retina (Aragón and López-Corcuera, 2003, Vitanova et al., 2004). Glycine is involved in the coordination of reflex responses (muscle tone balance), processing of sensory signals, and sensation of pain (Zafra and Giménez, 2008). The presence of glycine in these caudal areas of the CNS has been studied and established. However, glycine has also been discovered in other regions of the brain such as the forebrain, where its function has not been fully addressed yet. In these areas, glycine may play a different function besides its well-known inhibitory role (Hernandes and Troncone, 2009).

## 1. 3 Glycinergic Synapse, Receptors and Transporters

### 1. 3. 1 Glycinergic Neurotransmission

The glycinergic neurotransmission needs to follow a series of steps. First, the storage of the molecule in the synaptic vesicles from the presynaptic neuron, neuron depolarization, glycine-release in the synaptic cleft and its binding to the receptors (Neal and Pickles, 1969)

In the CNS, glycine plays two physiological functions. It is involved in the inhibitory and excitatory synapse [illustration 1].



**Illustration 1. Neurotransmission at the glycinergic synapses.** Schematic representation of the glycinergic inhibitory (left) and excitatory (right) synapse. The legend shows the representation of each protein in the diagram. Adapted from Harvey and Yee, 2013.



### ***Glycinergic Inhibitory Neurotransmission***

Glycine is stored in the synaptic vesicles. Upon depolarization, it is released by the  $\text{Ca}^{2+}$ -mediated influx to the synaptic cleft. Glycine binds to specific glycine receptors (GlyRs). The GlyRs are located in patches in the postsynaptic neuron and they are ligand-gated chloride channels composed of  $\alpha$  and  $\beta$  subunits (Betz and Laube, 2006; Lynch, 2009). The binding of glycine within the ionotropic GlyRs allows the pore of the receptors to open and  $\text{Cl}^-$  ions to passively diffuse across the cell membrane of the postsynaptic neuron. Therefore, the intracellular  $\text{Cl}^-$  concentration increases and changes the postsynaptic membrane potential leading a neural hyperpolarization. This inhibition of action potentials prevents depolarization and neuronal firing induced by excitatory neurotransmitters (Stein and Nicoll, 2013). However, the glycinergic transmission does not stop at this point. Glycine has to return to the basal levels (Zafra and Giménez, 2008) and be prepared for the next required cycle. Glycine transporters (GlyTs) are plasma membrane transporters that terminate glycine signaling. These proteins regulate the extracellular concentration of glycine and facilitate the termination of glycinergic neurotransmission at the synaptic cleft.

In the mammalian-mature brain stem and spinal cord, inhibitory-glycinergic transmission has a well-established role in the regulation of motor control and reflex responses (Legendre, 2001). Glycine transmission focuses on vision (Vitanova et al., 2004), audition (Friauf et al., 1997), cardiovascular (Hildreth and Goodchild, 2010) and respiratory functions (Fortuna, Kugler and Hulsmann, 2018)

### ***Glycinergic Excitatory Neurotransmission***

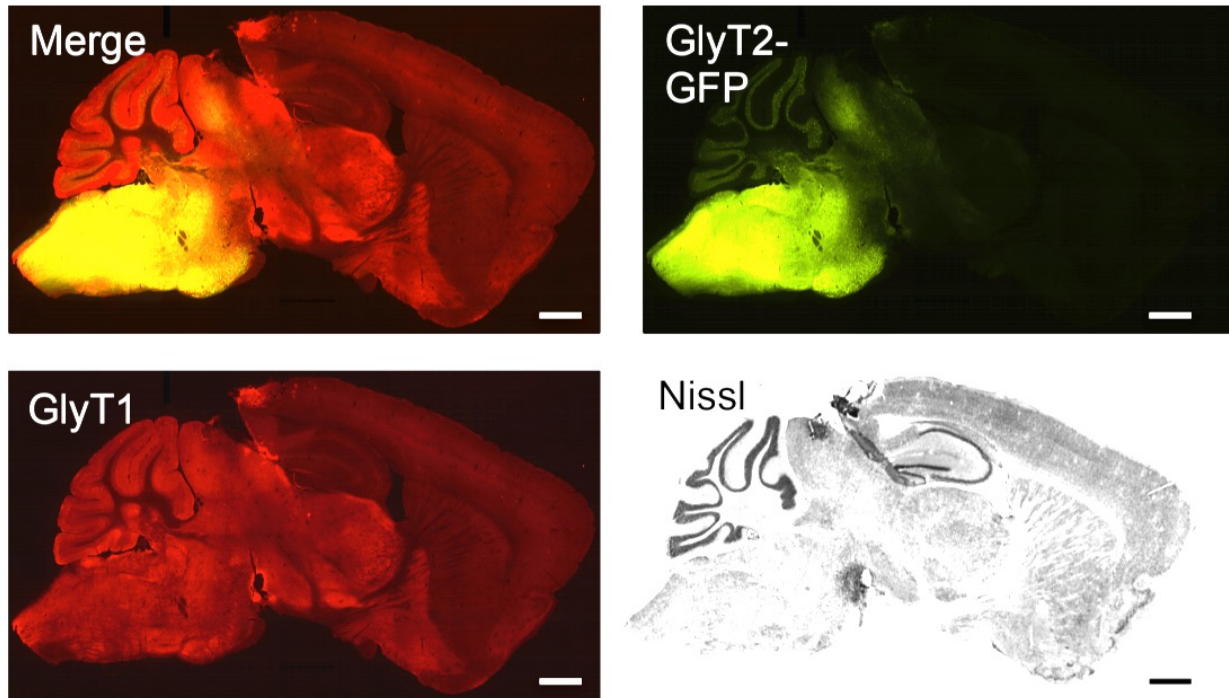
Glycine not only participates under inhibitory neurotransmission; it is also involved in glutamatergic neurotransmission. Glutamate is the primary excitatory neurotransmitter in the CNS, where it plays a critical role in synaptic plasticity. At the excitatory glutamatergic synapse, glycine and D-serine are obligatory co-agonists at NMDA (N-methyl-D-aspartate)-type glutamate receptors. The binding of glycine to the NMDA receptors is a pre-requisite for NMDA receptor activation by L-glutamate (Wolosker, 2007). In 1987, it was first demonstrated that glycine facilitated the excitatory transmission through the activation of the NMDA receptor. Johnson and Ascher performed experiments on primary cortical neuron cultures where these cells showed a stronger response to glutamate of NMDA when glycine was present (Johnson and Ascher, 1987). The ionotropic NMDA receptor is a ligand-gated  $\text{Ca}^{++}$  channel (Jewett and Thapa, 2018). This receptor is particularly important because it participates in a wide range of functions such as long-term potentiation, synaptic plasticity, learning and memory (Wilard and Koochekpour, 2013).

#### **1. 3. 2 Glycine Transporters**

Glycine transporters are endogenous regulators of glycine by controlling its extracellular levels at inhibitory and excitatory synapses (Harvey and Yee, 2013). They terminate the transmission.

Glycine transporter 1 (GlyT1) and Glycine transporter 2 (GlyT2), belong to the solute carrier 6 (SLC6) transport family. These are integral membrane-secondary active transporters characterized by the  $\text{Na}^{+}$ -dependent translocation of amino acids or amino

acid-like substrates. Other members of this family include gamma-amino butyric acid (GABA), dopamine (DAT), serotonin (SERT), and norepinephrine (NET) transporters. The SLC6 family has essential roles in eukaryotes where they modulate the intensity of signals in the central and peripheral nervous system. Members of this family are directly associated with several human disorders and diseases such as drug addiction, schizophrenia, Parkinson's disease, post-traumatic stress disorder, autism, attention deficit hyperactivity disorder, and mood disorders as depression, anxiety and obsessive compulsive disorder. For this reason, the SLC6 family are recognized as a critical target in the discovery and development of novel therapeutics (Promad et al., 2012). Throughout the past years, research has focused mainly on GABA, DAT, SERT and NET transporters. Experimental studies have demonstrated the importance of these proteins including cytoarchitecture, mechanism of function, phenotype, among others. By contrast, it has not been the same scenario for the full characterization of glycine transporters. Lack of highly specific antibodies and development of innovative methodologies might be the reason for the underrepresentation of these essential transporters. Research studies demonstrate that glycine transporters are also important and they have been recognized as novel therapeutic targets for schizophrenia and alcohol dependence (Harvey and Yee, 2013), regulation of pain (Dohi et al., 2009), and epilepsy (Shen et al., 2015). The coordinated and complementary activity of both glycine transporters is essential for the regulation of the glycinergic neurotransmission. Therefore, these transporters are potential targets for manipulating this neurotransmission (Harvey and Yee, 2013). Further research studies are critical for discovery and clinical development of novel drugs.



**Figure 1. Expression pattern of GlyT1 and GlyT2-GFP in transgenic mouse brain.**

A comparative distribution of the GlyT1 (red) and GlyT2 (green) in a sagittal section from a transgenic mouse that expresses GFP (green fluorescent protein) under the control of GlyT2 (Zeilhofer et al., 2005). Mouse brain sections were stained with the corresponding antibodies. The reactivity of GlyT1 and GlyT2-GFP exhibit similar distribution and overlaps with a high density in the caudal areas and a very low density in the forebrain brain, whereas GlyT1 has a widespread distribution in the forebrain. Scale bar: 1000  $\mu\text{m}$ . Images reproduced with the permission of Miryam Pando.

The glycine transporters share some similarities. These proteins share 48% identity at the amino acid sequence (Liu et al., 1993), present in a common protein structure with 12 transmembrane domains, and an intracellular amino and carboxyl-terminal (Olivares et al., 1994; Zafra et al., 1995). However, they also have their differences. For instance, they have different distribution **[Figure 2]** and cellular expression patterns in the CNS, function (Eulenburg et al., 2005) and stoichiometry (Roux and Supplisson, 2000; Scopelliti et al, 2016).

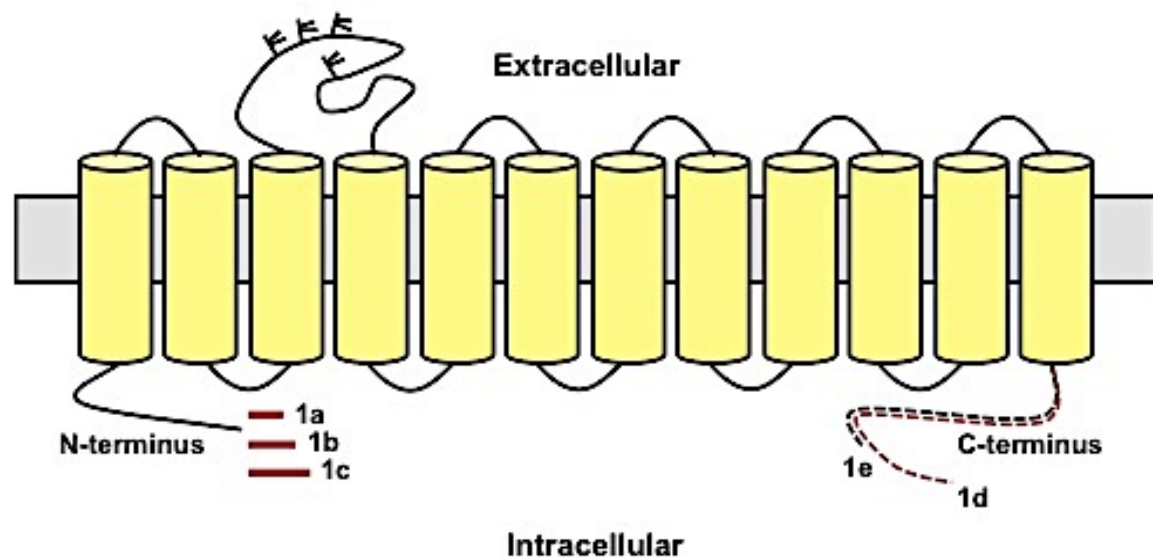
The function of glycinergic neurotransmitter transporters will be described in the following section.

### ***Glycine transporter 1 (GlyT1)***

For decades, the function of GlyT1 has been studied from different perspectives; nevertheless, its full characterization has not been accomplished yet. In the CNS, GlyT1 is expressed at its highest concentration in the caudal areas of the brain, in the forebrain (figure 2) and in retina. In the caudal structures, GlyT1 is expressed in glial cells (Cubelos, 2005). It is involved in the reuptake of glycine into the presynaptic terminal or surrounding glia cell (Bennet et al., 1974) where it is associated with the inhibitory glycinergic neurotransmission. Previous investigations have performed immunochemistry technologies and confirmed expression of GlyT1 in neurons and glial cells in the forebrain areas such as the neocortex and the hippocampus (Cubelos, 2005), but not in the thalamus. This neurotransmitter transporter is also expressed in amacrine neurons (Pow and Hendrickson, 2000) where it combines functions covered by neuronal GlyT2 and glial GlyT1 (from caudal glycinergic synapses) (Eulenburg et al., 2018). In the forebrain, it has also been suggested the double role of this transporter (Zafra, et al., 1995).

Experimental evidence showed that GlyT1 is essential for mouse survival. Studies from knockout mouse model demonstrate that the lack of GlyT1  $-/-$  is lethal in early postnatal life but not during perinatal life (Gomez et al., 2003). The absence of GlyT1 expressed on glial cells disrupts the clearance of glycine released from the presynaptic neuron at the synaptic cleft, leading to excessive glycinergic inhibition of the respiratory function in the brainstem (Eulenburg et al., 2010). GlyT1 is crucial for

maintaining a low concentration of extracellular glycine levels near the excitatory synapse. Its main role is to prevent binding and saturation of the glycine-B site on NMDA receptors. In other words, GlyT1-mediated glycine reuptake regulates the activation of NMDA receptor under physiological conditions.



**Illustration 2. Glycine Transporter 1 topology and isoforms.**

Glycine transporter 1 with its 12 transmembrane domains connected by six extracellular and five intracellular loops. The three GlyT1-isoforms differing in the N-terminus region (1a, 1b, and 1c), and two in the C-terminus (1e and 1d). Figure adapted from Adams et al., 1995.

In some areas of the brain, the presence of glycine transporters overlaps with the NMDA receptors. Electrophysiological (Chen et al., 2003) and pharmacological (Tsai et al., 2004) experiments demonstrate that the inhibition or deletion of GlyT1 increases the extracellular levels of glycine, thereby increasing the activation of the NMDA receptor. NMDA activation could be beneficial for alleviating neurological disorders such as the schizophrenia (Harvey and Yee, 2013). Individuals that have been diagnosed with schizophrenia present a deficit of the NMDA receptor signaling.

GlyT1 exist in five different isoforms; three of them, GlyT1 a, b, and c have a different amino terminal and the other two, GlyT1 e and d, differ in their carboxyl terminal **[Illustration 2]** (Eulenburg et al., 2005). These variants occur because of alternative promoter usage, but the distribution of these different isoforms has not been fully addressed yet (Harvey and Yee, 2013).

### ***Glycine transporter 2 (GlyT2)***

GlyT2 is expressed in the presynaptic glycinergic neurons, principally in caudal regions of the brain such as spinal cord, brainstem and cerebellum (Jursky and Nelson, 2002). It is also present with very low levels in the forebrain **[Figure 2]**. GlyT2 is essential for glycine reuptake into the presynaptic neuron, and the recycling of glycine for vesicular release (Eulenburg et al., 2005). Through the action of GlyT2, the vesicular amino acid transporter known as VIAAT/VGAT, can refill and store glycine. (Zafra and Giménez, 2008).

The absence of GlyT2 leads to a deficit of glycinergic inhibition, meaning that the disruption of GlyT2 is lethal. Experiments performed on knockout mouse reveals that the loss of GlyT2 located at the presynaptic neuron extremely deprives the refilling of glycine from the presynaptic vesicles and disrupts neurotransmission (Gomez et al., 2003). An important point to consider is that genetic defects in human-GlyT2 have associated to cause startle disease, also known as hyperekplexia (Rees, 2006; Harvey and Yee, 2013). This neurological disorder refers to the involuntary movement of parts of the body triggered by a specific stimulus such as surprise, fear or acute pain among others. The most noticeable features of reflex are closure the eyes with force,

movement of arms and neck, and flexion of trunk, elbows, hips and knees after auditory or tactile startling stimuli. The startling stimuli can also induce responses other than startle reflexes, such as startle-induced epilepsy (Bakker et al., 2006).

## **1. 4 The Forebrain**

### **1. 4. 1 The thalamus and the *Ventral posterior medial thalamic nuclei***

The thalamus is an area located in the forebrain between the cerebral cortex and the midbrain, surrounding the third ventricle. This important area is a hub of cortical-subcortical connections and a regulator of cortical activity (Giraldo-Chica and Woodward, 2017). The thalamus was considered and referred as the relay station of the brain between sensory organs and the cerebral cortex. Recent studies have demonstrated that this area is involved in more functions. The thalamus plays a central role in regulating arousal, level of awareness and activity. Malfunction and abnormal thalamic structure have been implicated in schizophrenia and other neurological diseases such as mood disorders (Penner et al., 2017). Damage to this area can lead to permanent coma (Laureys et al., 2016).

A very important pathway where the thalamus is involved is the spinothalamic tract. It is a sensory pathway from the skin, which carries information about pain, temperature, pressure, itch, and crude touch. This pathway decussates in the spinal cord and continues to the somatosensory areas of the thalamus (Al-Chalabi and Gupta, 2018). Then the information ascend to the cerebral cortex (Sherman, 2016).

The ventral posterior medial thalamic nuclei is a small area of the thalamus. Temperature and pain sensations from the skin of the face, neck and oral cavity



(trigeminal system) are carried to the VPM. This pathway is called the trigeminothalamic tract and is known as the trigeminal lemniscus (Anthoney, 1993).

### **1. 5 Overarching Goals and Aims of the Thesis**

The overarching goal of this work is to identify the cells expressing the neurotransmitter transporter GlyT1 in the mouse VPM and to characterize these cells. It has been described that GlyT1 is mainly expressed in glial cells and only a few reports describe that GlyT1 is also expressed in small populations of glutamatergic neurons (Cubelos, 2005). Based on previous experimental results, this study provides foundation to suggest that GlyT1 is expressed in neurons in the forebrain, specifically in the VPM.

The laboratory mouse model has been used for this study. Different mouse strains were used since some lines have been genetically modified. These strains allows identifying the expression of specific genes and proteins of interest. Moreover, histological experiments and intracranial injections of fluorescent tracers provide evidence that suggest novel concepts regarding the cell type expressing GlyT1 in the VPM.

Experimental studies have demonstrated that inhibition of glycine transporters in the forebrain has shown beneficial effects in patients with neurological disorders such as schizophrenia (Chue and Baker, 2013). It has been proposed that the negative and cognitive symptoms of schizophrenia originate from the hypo-function of the NMDA receptors (Gray and Roth, 2007; Hashimoto, 2014; Umbricht et al., 2014). Current

GlyT1-inhibitors have not improved completely the negative symptoms from schizophrenia such as withdrawal and lack of motivation (Goff, 2014).

It is imperative to determine whether GlyT1 is expressed in neurons or/and glial cells because depending on its location, it will be possible to understand the overall kinetics of the glycinergic neurotransmission. In other words, if GlyT1 is expressed in neurons, then it can be suggested that this protein has a similar function as GlyT2 when this one is not present in the cell. Therefore, it could be suggested that GlyT1 would have a dual function and mechanism in the CNS as it has been proposed in the amacrine neurons (Eulenburg, 2018).

After understanding how glycinergic homeostasis is achieved, these insights will be an important step towards future drug development for alleviating neurological disorders in the thalamus and other areas of the brain.

## Chapter 2

### Materials and Methods

#### 2.1 Animals Strains

Wild type and transgenic C57BL/6 and FVB/N strains were used for the stereotaxic surgeries and brain collection. Animals were housed in the vivarium facility located in the University of Texas at El Paso (UTEP) with controlled temperature, oxygen and a 12-hour light/dark cycle. Transgenic animals arrived to UTEP in the spring of 2018 from The Jackson laboratory. Experimental procedures were conducted under Dr. Miranda's protocols (A-201303-1) in compliance of the UTEP Institutional Animal Care and Use Committee (IACUC).

**Table 1. Animal Strains**

Strain	Age	Weight	Procedures	JAX stock #
C57BL/6 – wild type	3 – 4 months	28 – 33 grams	Stereotaxic surgery and perfusion	n/a
FVB/N-Tg 14Mes/J	3 – 4 months	28 – 33 grams	Stereotaxic surgery and perfusion	003257
B6.Cg-Tg(Slc32a1-COP4*H134R/EYFP)8Gfng	3 – 4 months	28 – 33 grams	Stereotaxic surgery and perfusion	014548
CB6-Tg(GAD67-EGFP)G42Zjh/J	3 – 4 months	28 – 33 grams	Stereotaxic surgery and perfusion	007677

#### 2.2 Stereotaxic Surgery and Tracers Injection

A group of wild type-male mice (3.5 months old) was injected unilaterally in the VPM (Illustration 3). The Picospritzer III apparatus (Parker Hannifin Corporation, Precision Fluidics Division) and the stereotaxic injector were used to inject 100 nL of the tracers. These tracers were the retrograde tracer Fluoro-Gold (Invitrogen), the anterograde adeno-associated viral particles expressing the reporter gene mCherry

and GFP under control of the synapsin and the GFAP promoter (University of North Carolina at Chapel Hill, Vector Core).

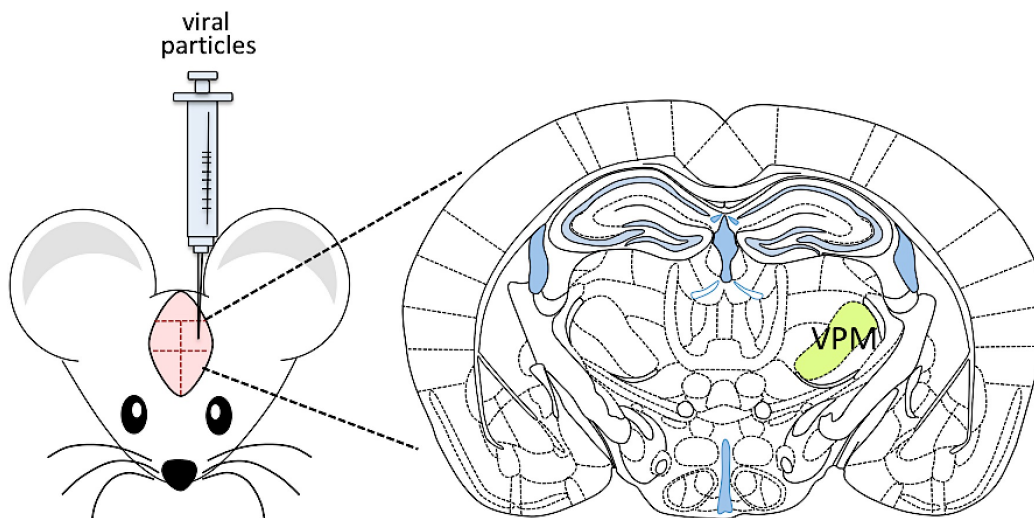
Mice were weighed, shaved and anesthetized with isoflurane, USP (Piramal Healthcare) administered followed by inhalation. Mice were properly placed on the mouse stereotaxic instrument (Stoelting). Eyes remained open during the time of the surgery; therefore, lubricant eye ointment (Akorn) was applied on them to prevent corneal drying. Surgical, utensils and solutions used for the surgery were autoclaved.

The coordinates of the site of the injection used for each animal were obtained from the Mouse Brain atlas. According to the Paxinos and Franklin, section 46 indicates that the VPM area is located in the following coordinates (table 5).

**Table 2. Stereotaxic coordinates**

<b>Coordinates</b>	<b>millimeters</b>
Anterior Posterior (AP)	1.79
Medial Lateral (ML)	1.50
Dorsal Ventral (DV)	3.50

The micro syringe was inserted into the desired area for five minutes before and after the injection. After tracer delivery, the micro syringe and the ear bars were removed. The open skin was sealed with mouse clips using a clip applier (Reflex 7) and triple antibiotic ointment (Curad, Bacitracin Zinc, Neomycin Sulfate, and Polymyxin B Sulfate) was applied on the wound. Mice were monitored for three hours right after the surgery and received the post-procedural treatment. The post-procedure supervision continued for the next five days as the IACUC protocol states.



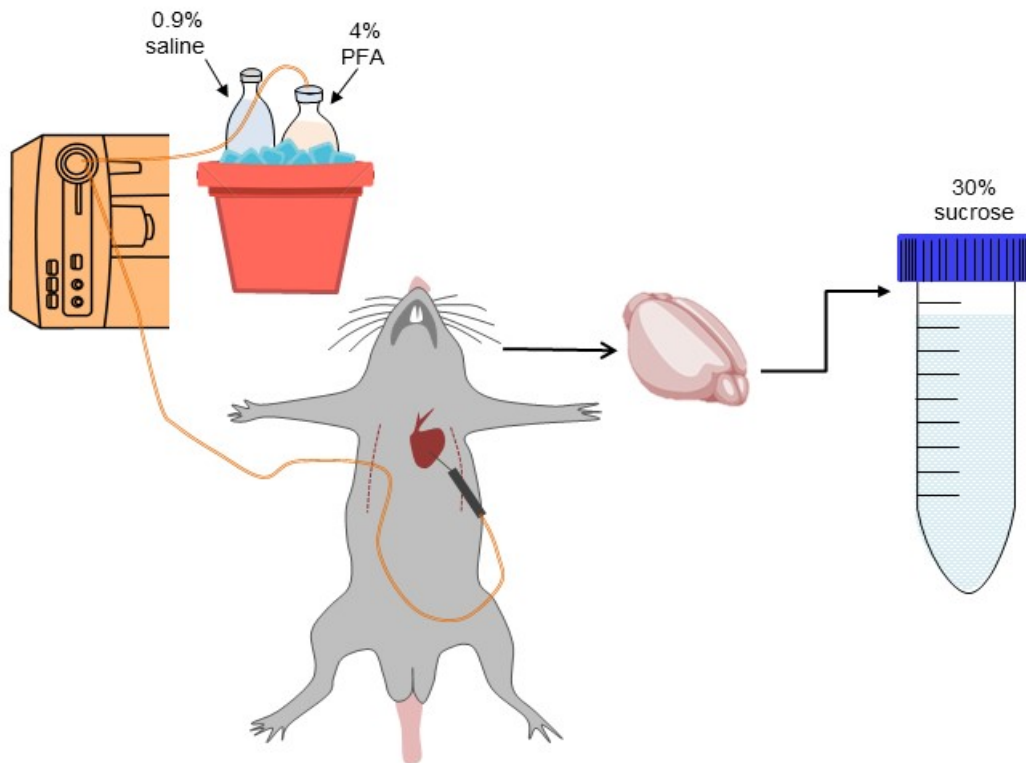
**Illustration 3. Schematic representation of the injection site.**

Mouse head with an incision showing the bregma and lambda lines on the skull, and the injector on the site of injection. Coronal brain section showing the desired area of injection (VPM in green).

## 2. 3 Perfusion

Animals were perfused after two weeks of the stereotactic surgery (Illustration 4). This time allowed tracers to travel within the brain. First, the mice were deeply anesthetized with isoflurane and they were intraperitoneal injected with a lethal dosage of sodium pentobarbital (50 mg/ml stock) at a concentration of 80 mg/kg. Mice were transcardially perfused (left ventricle of the heart) with about 300 mL of 0.9% saline solution followed by 300 mL of cold 4% paraformaldehyde (Paraformaldehyde Granular, Electron Microscopy Sciences, Cat. No. 19210) in 1x of phosphate buffered solution (PBS, 137 mM sodium chloride (Sigma Aldrich, LOT No. 081M0051V), 2.7 mM potassium chloride (Sigma Aldrich, LOT No. 077k0024), 8.1 mM sodium phosphate dibasic (Sigma Aldrich, LOT No. 096K0141), 1.47 mM potassium phosphate monobasic (Mallinckrodt Chemicals, LOT E18478), pH 7.4) using a peristaltic pump (MasterFlex

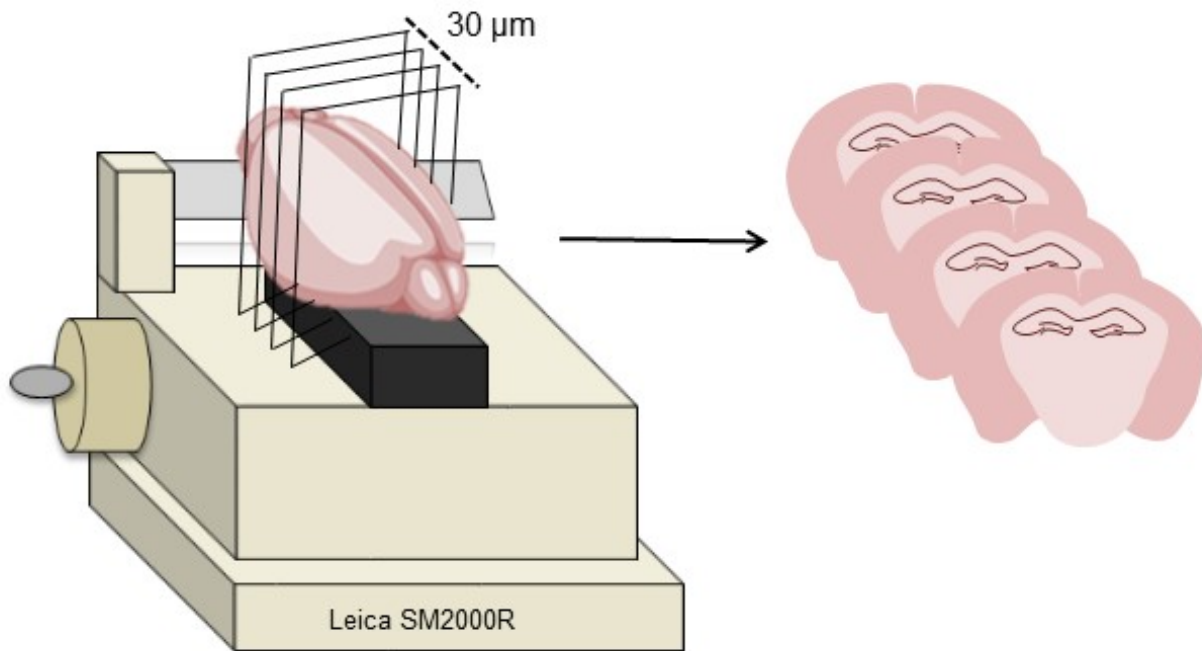
console drive, Cole-Parmer Instrument Company from Dr. Kristin Gosselink's lab). The animals were decapitated and the brain was taken out from the skull and placed it into 50 mL of 4% PFA in 1x PBS at 4 °C. Post-fixation will be overnight followed by cryprotection using 30% sucrose (Sigma, Lot. No. SLBP5260V) in 1x PBS for at least 24 hours at 4 °C.



**Illustration 4. Schematic representation of perfusion and brain collection.** Each animal was transcardially perfused through the ventricular catheter with saline solution followed by 4% paraformaldehyde. After removal of the brain, it was placed in the fixative solution, followed by 30% sucrose.

## 2. 4 Tissue collection

After fixation and cryoprotection of the collected brains, they were sectioned using a Leica SM200R sliding microtome (Illustration 5). The 30  $\mu\text{m}$ -thick- coronally sections were obtained. Five adjacent sections of brain tissue series were distributed per well in a 24 well cell culture plate (Cellstar, cat. No: 662160). They were incubated with 500 mL of antifreeze solution (6.58 sodium phosphate monobasic, 19.23 mM sodium phosphate dibasic, 30% ethylene glycol, 20% glycerol (Sigma, Lot # BCBQ5800V) and stored at -20 °C.



**Illustration 5. Schematic representation of the brain tissue collection.**

Each brain was sectioned coronally using a sliding microtome (Leica SM2000R), starting anteriorly and continuing on the posteriorly part of the brain. Brain tissues were stored at -20 °C in an antifreeze solution until further use.

## **2. 5 Histology**

### **2. 5. 1 Nissl Staining**

Nissl staining is an imperative technique needed for the comparison of the collected brain sections with The Mouse Brain Atlas (Paxinos and Franklin, 2012) sections. In this way it is possible to confirm the collected sections include the region of interest. The first part of this technique dehydrates the tissue allowing it to be compatible with the xylenes, which remove the fat from the tissue. The second part allows the tissue to be rehydrated making it able to stain with the thionine solution. The Nissl stain (thionine) stains the ribosomal RNA. Finally, the tissue is dehydrated allowing the mounting medium DPX to be compatible with the tissue. One of the five sections previously collected was used and stained utilizing this technique.

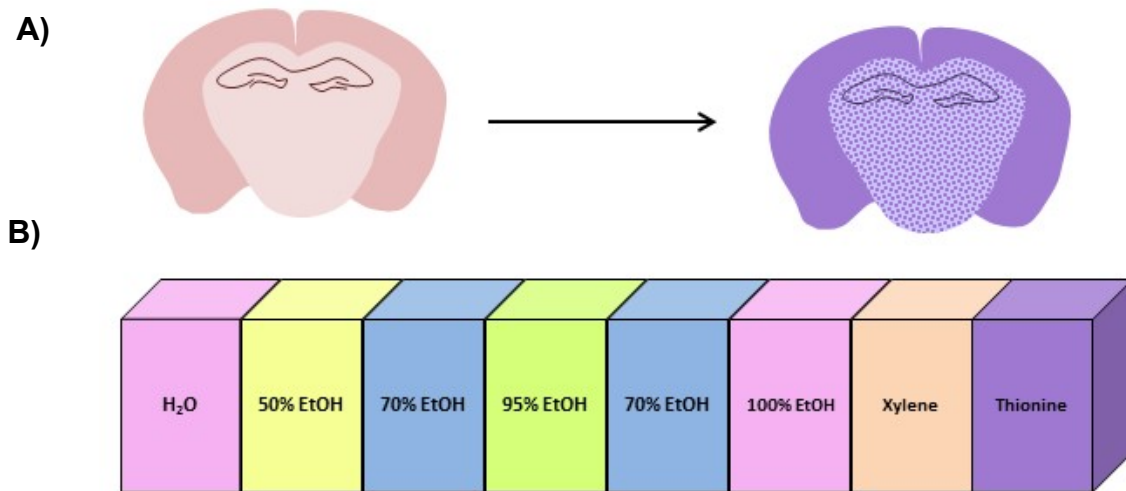
For the Nissl staining, gelatin-coated slides were used to mount the brain tissue. In order to coat the slides, VWR Micro slides (Fisherbrand Superfrost, Microscope Slides, white and pre cleaned, size 25 x 75 x 0.1 mm) were used. They were subbed in 1% Knox Original Gelatin Unflavored (Kraft Foods Global INC) and chromium potassium sulfate (Acros Organics, Lot: A0343854) solution in water at 45 °C for 5 minutes. The slides were placed into the oven at 65 °C for 48 hours to let them dry. In the meantime, the solutions were prepared and poured into small containers. Therefore, reagent alcohol for histological grade (Fisher Chemical, A962P-4, LOT 165021) was diluted in deionized water at increasing concentrations: 50%, 70%, 95%, 95%, 95%, 100%, 100%, 100%. Xylenes for histological grade (Fisher Chemical, X3P-1GAL, LOT 165460) were used at 100% for two containers (xylenes 1 and xylenes 2) (Illustration 6). The Nissl stain was prepared with 5.84% glacial acetic acid (Sigma Aldrich, L



OT 04896DMV), 1 M sodium hydroxide, and 0.5% Thionine acetate salt (Sigma, LOT No. BCBR1862V). The solution was filtered and stored in the dark at 65 °C. Once the slides, solutions and stain were ready to use them, the tissue was mounted. The following day, the slides with the tissues were passed through the Nissl procedure as shown in Table 2. Slides with brain tissue sections and with no residuals, were poured with 300 µL of DPX Mountant for histology (Sigma, Lot # BCBH3443V). Coverslips (Fisherbrand, Microscope Cover Glass, size 24 x 60 mm) were used to seal the slides. In order to analyze the brain sections, pictures were taken in the microscope and compared with The Mouse Brain Atlas (Paxinos and Franklin, 2012). As a result, each section was assigned to a corresponding atlas level.

**Table 2. Nissl Procedure**

<b>Duration</b>	<b>Solution</b>
3 minutes per solution	DI Water, 50%, 70%, 95%, 95%, 100%, 100%, 100% EtOH
5 minutes	Xylenes 1
30 minutes	Xylenes 2
2 minutes per solution	Xylenes 1, 100%, 100%, 100%, 95%, 95%, 70%, 50% EtOH, DI Water
15 dips	Nissl Stain: Thionine
20 dips	DI Water, 50%, 70% EtOH
3 minutes per solution	95%, 95%, 100%, 100%, 100% EtOH, xylenes 1, xylenes 2



**Illustration 6. Nissl Staining Procedure.**

Schematic representation of **A)** the pink tissue symbolizes the tissue before the staining process and the purple tissue represents the appearance of the section after the Nissl staining. **B)** Each color box represents the order of the solutions in this technique.

### 2. 5. 2 Tissue Immunofluorescence

The sections that have the areas of interest were used for immunohistochemistry. First, the desired tissue was transferred into a net well plate filled with 1X TBS. Five washes with 1X for five minutes each were required. The plate was on the belly dancer with a speed of four at room temperature. Tissues were transferred to a new 24- well plate. They were incubated for two hours with 300  $\mu$ L of blocking solution (2% normal goat serum, 0.1% triton (Triton X-100, Sigma. Lot # 031M0301V), 1X TBS)) per section. The sections were transferred to a new well with 300  $\mu$ L of primary antibody (see table 3) depending on the protein of interest and diluted in blocking solution. This incubation took place on the belly dancer in the cold room (4°C) for 18 to 24 hours. The plate was covered with aluminum foil to prevent evaporation of the solutions. Prior to and after the incubation with the specific secondary

antibody, washes for five times with 1X TBS for five minutes were done. The required secondary antibody (see table 4) was diluted in 1X TBS. Brain sections were incubated for two hours at room temperature and in the dark. The plate was covered with aluminum foil, but this time to prevent quenching of the fluorescent dye. Lastly, sections were mounted on a normal glass slide (VWR Micro slides Fisherbrand Superfrost, Microscope Slides, white a precleaned, size 25 x 75 x 0.1 mm). A petri dish was filled with 1X TBS and the tissue was placed into the buffer. A fine paintbrush was used for tissue handling and to guide it on the glass slide. After the tissues were onto the slide, 300  $\mu$ L of sodium bicarbonate glycerol (0.1 M NaHCO<sub>3</sub>, pH 8.62) were poured on top. The slide was covered with the coverslip (Fisherbrand, Microscope Cover Glass, size 24 x 60 mm). Clear nail polish was used to seal the edges of the slide and to prevent any tissue damage. Slides were kept overnight in the dark at room temperature, and then stored at 4°C. Finally, slides were ready to be analyzed by the fluorescent and confocal microscope.

**Table 3. Primary Antibodies**

Reagent	Host Species	Dilution	Source	Lot Number
GlyT1	Rabbit	1:500	Dr. Manuel Miranda	In house
Mouse Anti-MAP2 a&b Monoclonal antibody	Mouse	1:500	Millipore	LV1486527
Mouse X Neuronal Nuclei (NeUN)	Mouse	1:500	Millipore	NG1876252
Purified Mouse Anti-rat TAU	Mouse	1:1000	BD	12951
Mouse monoclonal (GF5) to GFAP (Glial Fibrillary Acidic Protein)	Mouse	1:500	Abcam	Ab10062

**Table 4. Secondary Antibodies**

Reagent	Anti-Species	Dilution	Source	Lot Number
Alexa 488 – conjugated AffiniPure Donkey Anti-mouse igG	Mouse	1:2500	Jackson ImmunoResearch Laboratories	104332
Alexa 488 – conjugated AffiniPure Donkey Anti-Rabbit igG	Rabbit	1:2000	Jackson ImmunoResearch Laboratories	126883
Cy3 – conjugated AffiniPure Donkey Anti-Mouse igG	Mouse	1:2000	Jackson ImmunoResearch Laboratories	124774
Cy3 – conjugated AffiniPure Donkey Anti-Rabbit igG	Rabbit	1:2000	Jackson ImmunoResearch Laboratories	76381

## **2. 6 Microscopy and Confocal Microscopy Analysis**

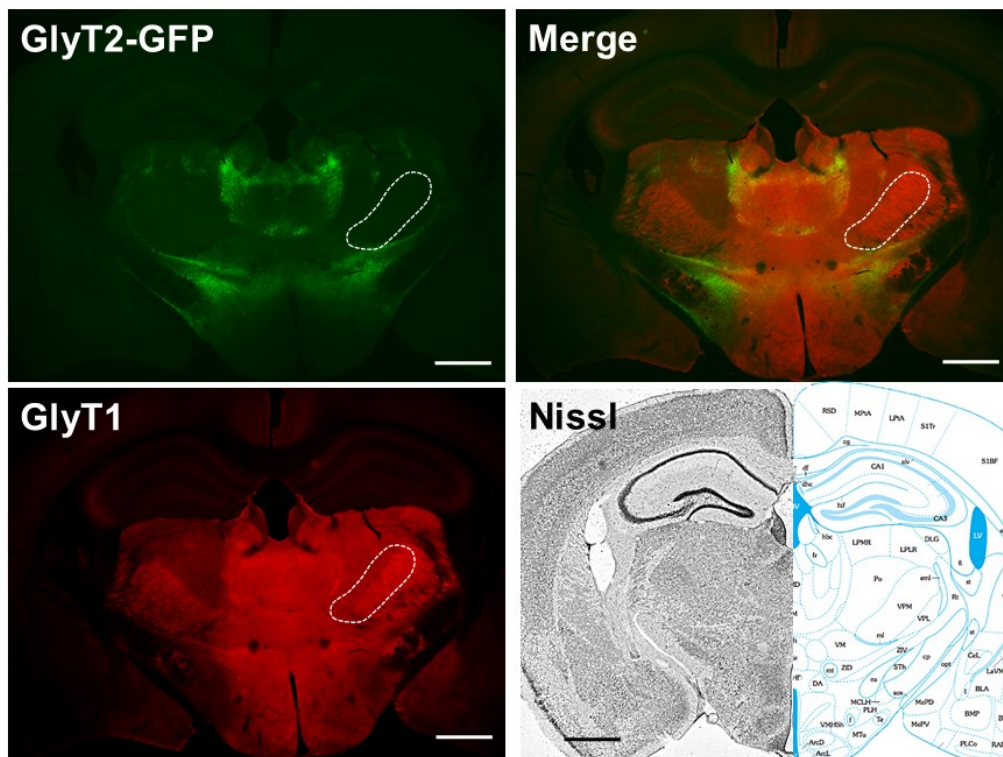
Nissl images were taken using a Zeiss Microscope, AxioCam MRm, software AxioVision Rel. 4.8, (SN: 3834002557), 10X objective, white light and MosaiX command. The brain sections were analyzed using a fluorescent microscope and a confocal microscope. The required filter sets of DAPI, Alexa 488, and Cy3 were used to visualize the fluorescent dyes in the mouse brain tissue. High-resolution digital fluorescent images were captured using a Zeiss LSM 700 confocal microscope (Cytometry, Screening and Imaging Core Facility). The microscope is equipped with a 40 and 63 immersion oil objective. ZEN 2009 software (Zeiss, New York, NY) was used for acquisition and processing of confocal images. Single-plane images were sequentially scanned using specific settings for each channel and acquired at the highest pixel resolution. The same software was used to visualize localization of the red and green signals from the stained neural tissue.

## Chapter 3

### Results

#### 3.1 GlyT1 and GlyT2-GFP Distribution in the Forebrain

The immunoreactivity of GlyT1 and GlyT2-GFP exhibit different distribution throughout the forebrain (figure 2). GlyT1 has a widespread distribution throughout the thalamus, whereas GlyT2 is present only in areas such as the paraventricular thalamic nucleus and the optic tract. The distribution of GlyT1 and GlyT2 overlaps with a higher concentration in some areas of the forebrain such as the lateral habenular nucleus medial and lateral part, the central thalamic nucleus, the medial thalamic nucleus, and



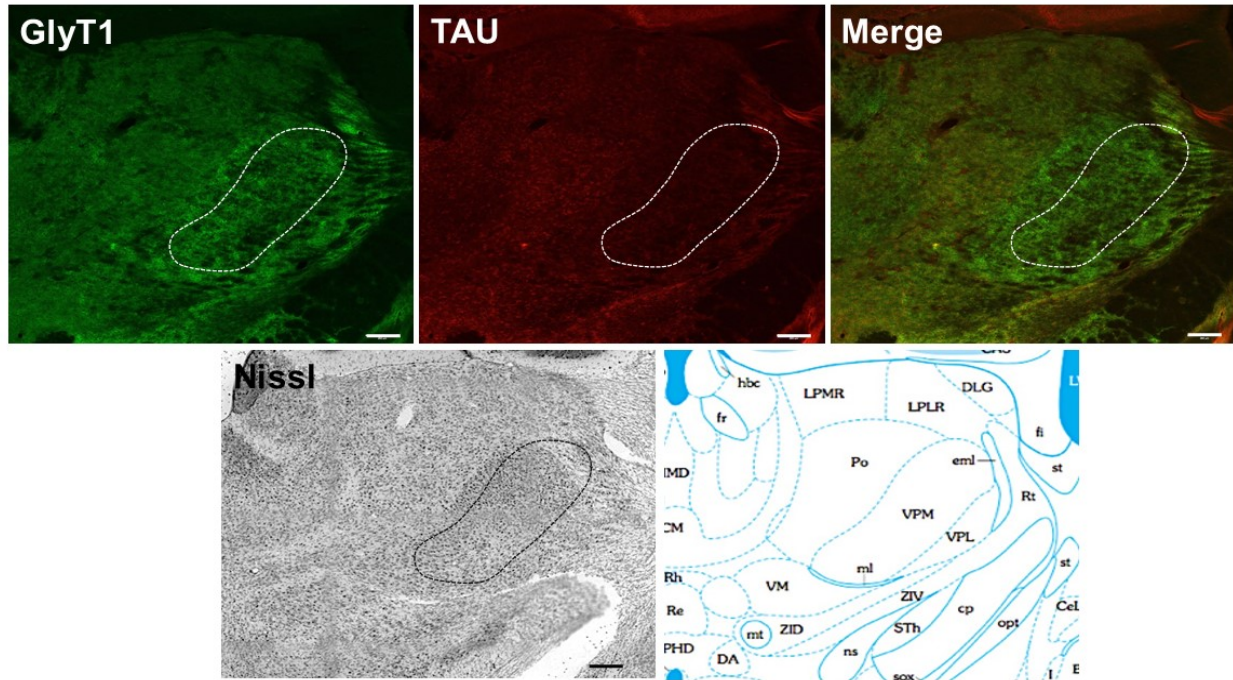
**Figure 2. Expression pattern of GlyT2-GFP and GlyT1 in the mouse forebrain.**

A comparative distribution of the patterns of the GlyT1 (red) and GlyT2 (green) in a coronal section from a transgenic mouse that expresses GFP under the control of GlyT2 (Zeilhofer et al., 2005). Scale bar: 1000  $\mu$ m.

the dorsal and lateral zona incerta. The distribution of both transporters overlaps with a lower concentration in the posterior thalamic nuclear group, dorsal hypothalamic area and in the nigrostriatal tract. It is worth mentioning that neither GlyT1 nor GlyT2 are present in the external medullary lamina, mammillothalamic tract and cerebral peduncle. GlyT1 is highly expressed and well distributed in the VPM. Even though there are many other areas of the mouse brain that have been labeled with GlyT1, this study focuses in the VPM. This area attracted our attention because of the high density of glycinergic fibers and the ease of analysis. These results might suggest that GlyT1 has a different role and may be located and function in putative glycinergic fibers in the VPM .

### **3. 2 GlyT1 and Neuronal Markers**

After finding the expression of GlyT1 in a such important area of the thalamus, it was not clear whether neurons or glial cells express this transporter. Therefore, neuronal and glial biomarkers along with the highly-specific GlyT1 antibody were used to identify the cell type expressing GlyT1 by histological techniques.



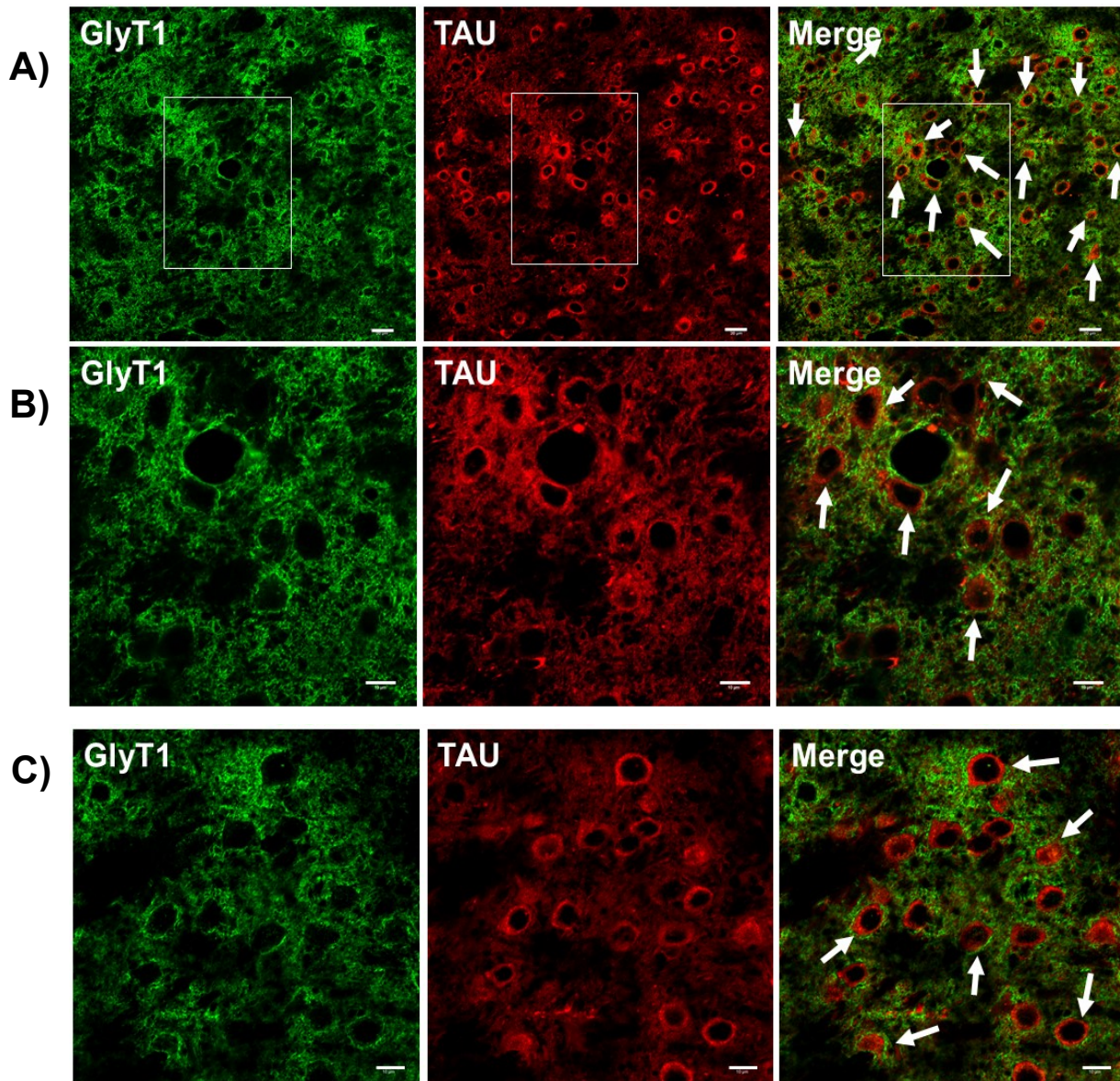
**Figure 3. GlyT1 and TAU immunoreactivity in a coronal section of the mouse thalamus.**

Immunoreactivity from GlyT1 (green) and TAU (red) in the thalamus. Encircled region represents the VPM area. Both proteins of interest are highly expressed in the VPM. Nissl image represents figure 46 from The Mouse Brain Atlas (Paxinos and Franklins, 2012). Scale bar: 200  $\mu$ m

The antibodies TAU, MAP2 and NeuN were used as neuronal markers. The target proteins of interest are microtubule-associated protein tau (TAU), microtubule-associated protein 2 (MAP2), and neuronal nuclei (NeuN). Axons and dendrites contain microtubules and microtubule-associated proteins (MAPs) with characteristic structures. Among MAPs, MAP2 is specifically expressed in dendrites whereas tau proteins are abundant in the axons (Chen, 1992). Both proteins are also expressed in cell bodies. In figure 3, both proteins of interest, TAU in red and GlyT1 in green are expressed in the VPM. The VPM at a higher magnification (figure 4) allows visualizing the immunoreactivity from both proteins in a detailed way.



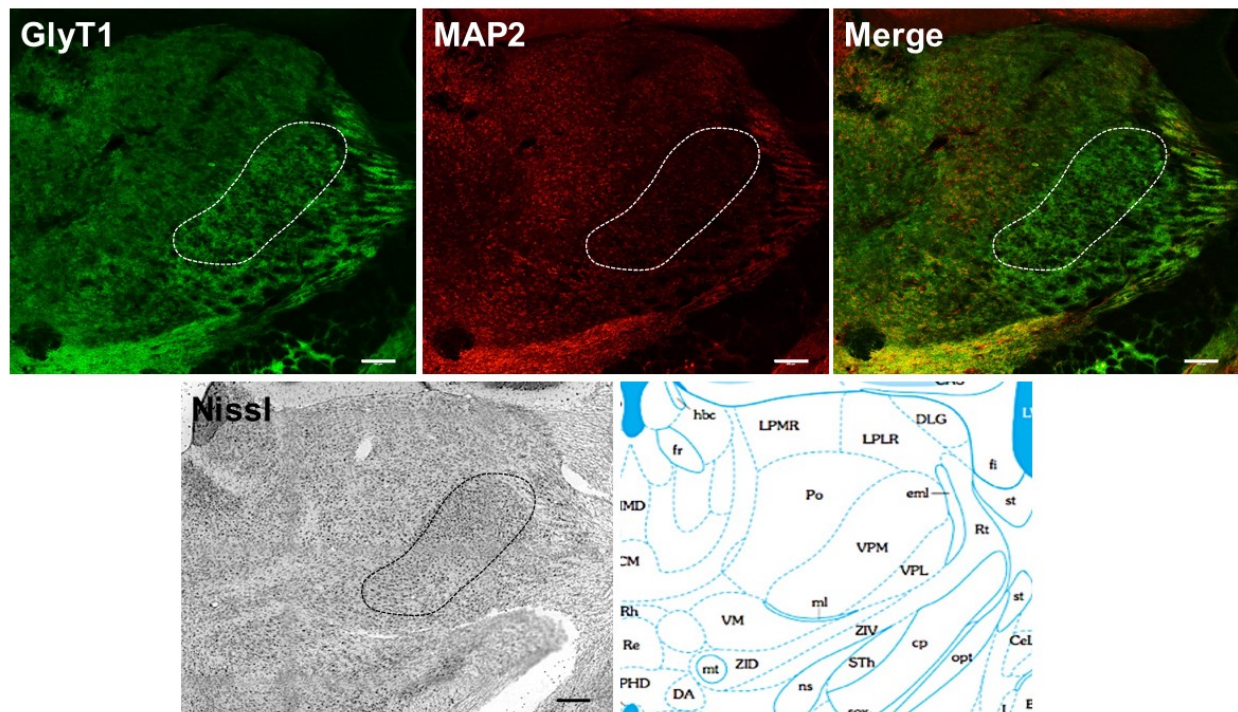
Moreover, the immunofluorescence labeling of TAU aids to identify the cell type expressing GlyT1. In figure 4 (panel A-C), the white arrows indicate the neurons that are surrounded by the GlyT1 immunoreactivity.



**Figure 4. Immunofluorescence labeling GlyT1 and TAU proteins in the VPM.** Confocal images from the VPM stained with GlyT1 (green) and TAU (red) antibodies. White arrows in (A, B and C) represent the neurons labeled with TAU and surrounded by GlyT1. (B) In the higher-magnification image from (A), the TAU expression surrounded by the GlyT1 is observed in a detailed manner. Scale bar: (A) 20 and (B) 10  $\mu$ m.



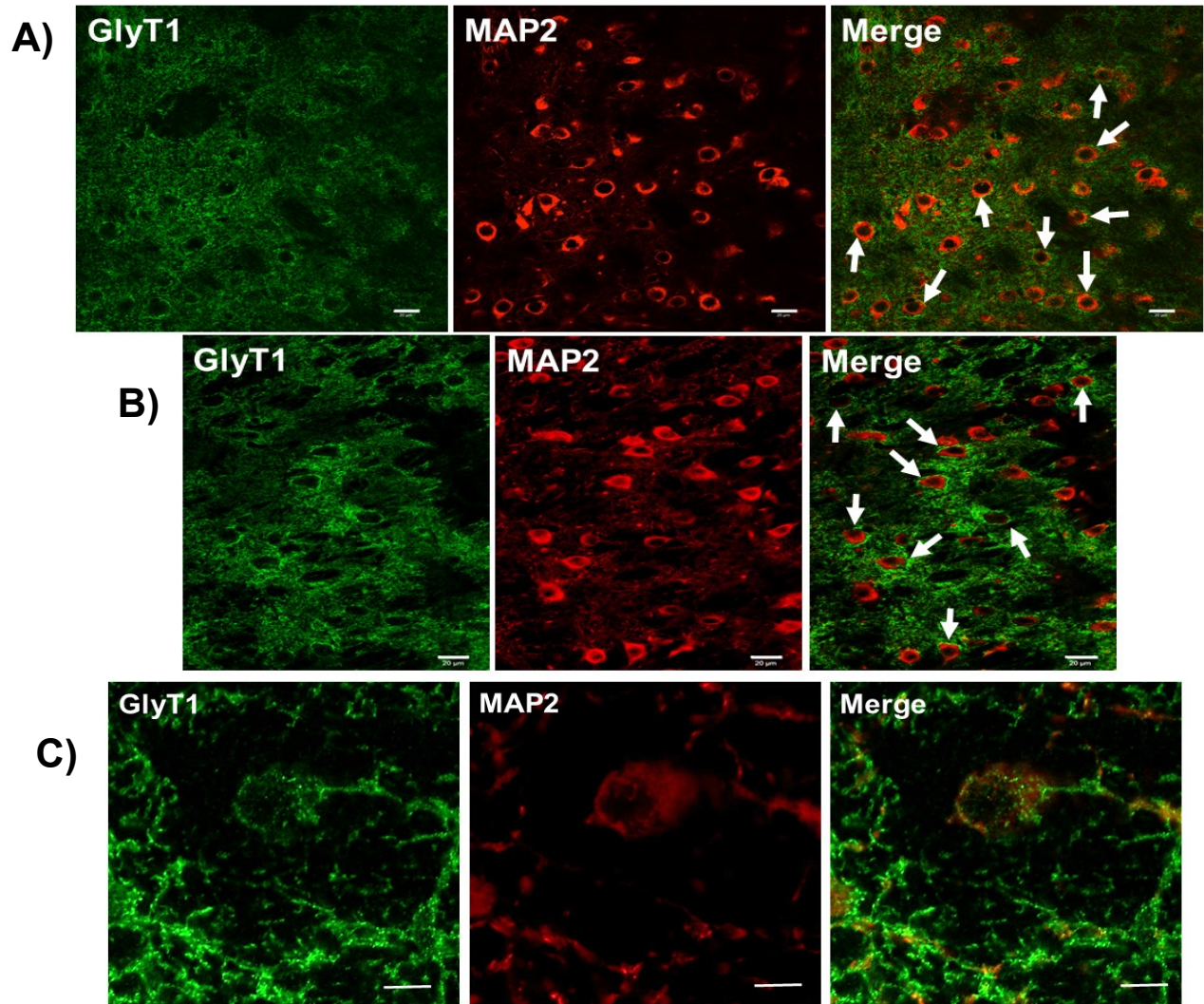
The MAP2 and NeuN cell markers were used to confirm the findings of the GlyT1 and TAU immunoreactivity in the same cell. MAP2 (figure 5) and NeuN (figure 7) antibodies were successfully expressed in the thalamus. Images at a higher magnification from both sections, indeed, confirm the expression of GlyT1 in neurons labeled with MAP2 (figure 6) and NeuN (figure 8).



**Figure 5. GlyT1 and MAP2 immunoreactivity in a coronal section of the mouse thalamus.**

Immunoreactivity from GlyT1 (green) and MAP2 (red) in the thalamus. Encircled region represents the VPM area. Both proteins of interest are highly expressed in the VPM. Nissl image represents figure 46 from The Mouse Brain Atlas (Paxinos and Franklins, 2012). Scale bar: 200  $\mu$ m.

Higher magnification images from GlyT1 and MAP2 immunoreactivity (figure 6) allow visualizing more cells bodies labeled compared to the dendrites of the cell.

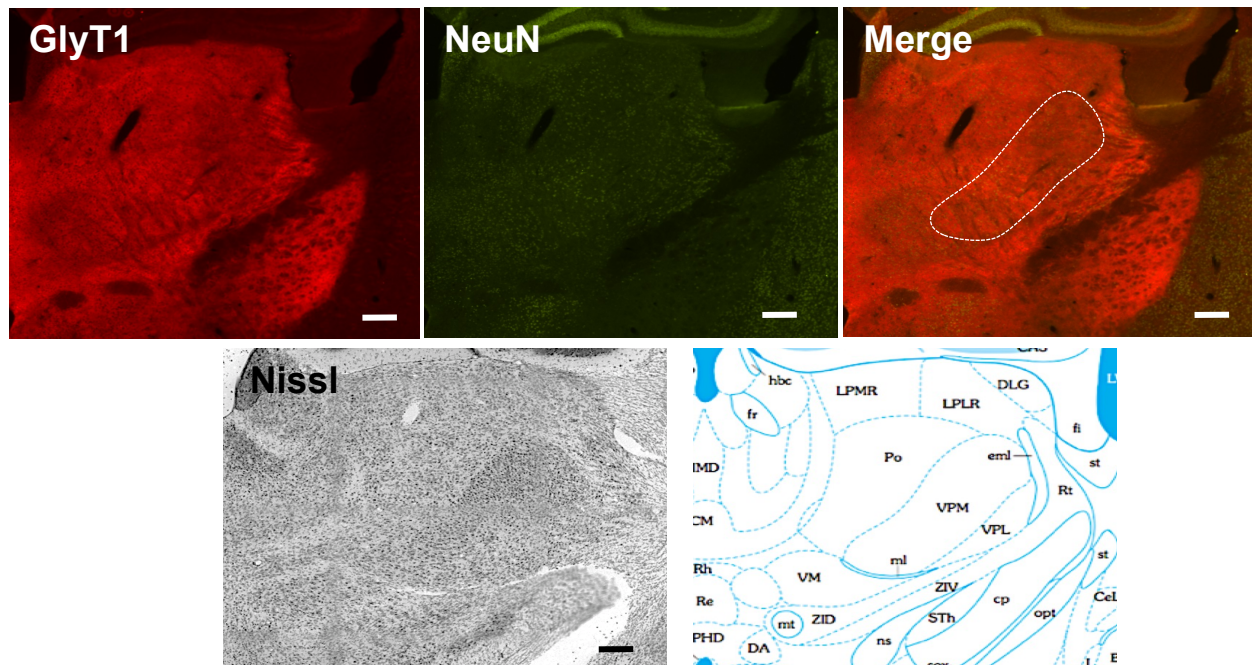


**Figure 6. Immunofluorescence labeling GlyT1 and MAP2 in the VPM.** Confocal images from the VPM stained with GlyT1 (green) and MAP2 (red) antibodies. White arrows in the merge figure of **(A)** and **(B)** show the neurons labeled by MAP2 and expressing GlyT1. **(C)** A single neuron where its dendrites and cell bod are labeled with MAP2 and co-localizing with GlyT1. Scale bar: **(A)** and **(B)** 20  $\mu\text{m}$  and **(C)** 10  $\mu\text{m}$ .

A reason of the partial absence of the stained dendrites could be that the section is not thick enough to show the complete neuron. However, in panel C it is possible to observe a single neuron labeled with MAP2 and the GlyT1 expression by the plasma membrane of the cell.

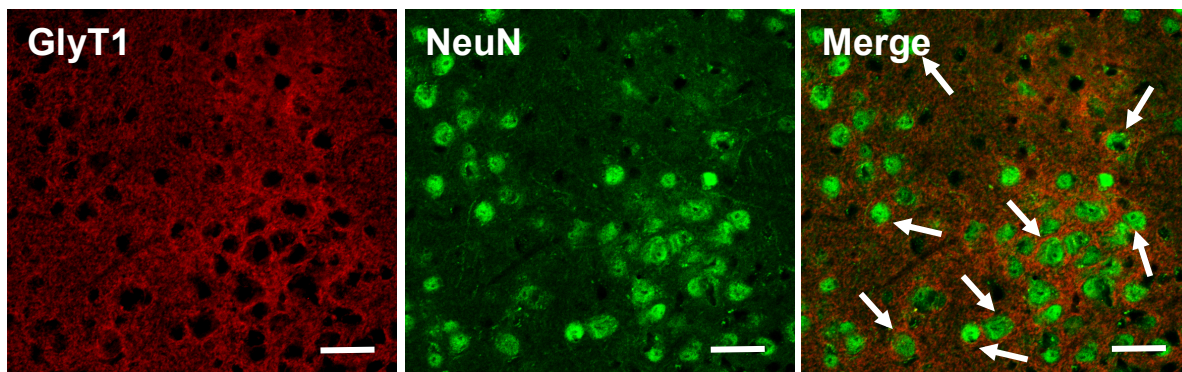


The neuronal biomarker NeuN was also used to confirm the cell type expressing GlyT1 (figure 7). As expected, neurons stained with NeuN also express GlyT1 (figure 8).



**Figure 7. GlyT1 and NeuN immunoreactivity in a coronal section of the mouse thalamus**

Immunoreactivity from GlyT1 (red) and NeuN (green) in the thalamus. Encircled region represents the VPM area. Nissl image represents figure 46 from The Mouse Brain Atlas (Paxinos and Franklins, 2012). Scale bar: 200  $\mu$ m.



**Figure 8. Neuron cells stained with NeuN express GlyT1.**

Confocal images from the immunofluorescence labeling GlyT1 (red) and NeuN (green) in the VPM. White arrows in the merge figure depict the neurons with their stained nucleus and expressing GlyT1. Scale bar: 20  $\mu$ m.

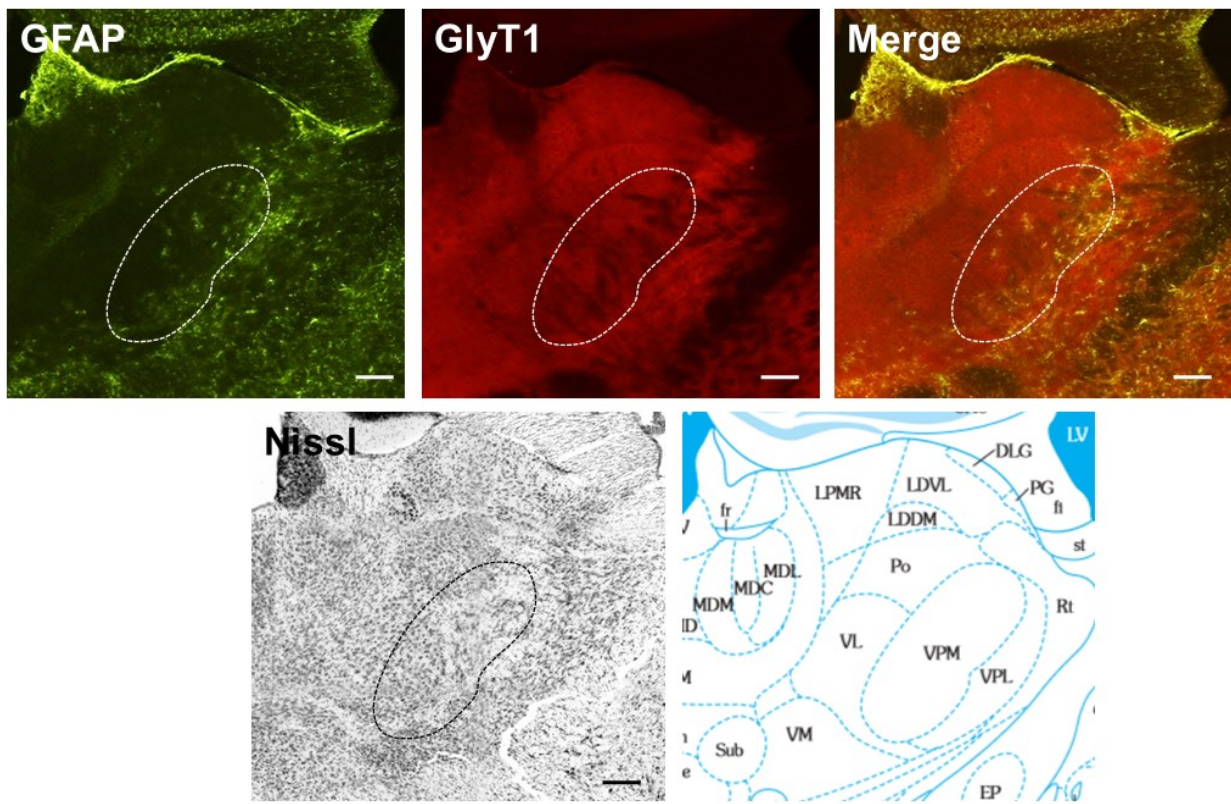
### 3.3 GlyT1 and Glial Marker

Glial cells are essential for neuronal function and viability such as structural support, neurotransmitter removal, glial scar formation and metabolic support to neurons (Nishiyama et al., 2005). These cells are further involved in the formation and stabilization of synapses and the modulation of synaptic efficacy (Doetsch, 2003).

Astrocytes are stellate cells that contain glial fibrillary acidic protein, the molecule constituent of glial filaments (Eng, Ghirnikar. and Lee, 200). The astrocyte-specific glial fibrillary acidic protein (GFAP) antibody served as a glial marker that allowed the identification of the glial cells through the brain tissue.

This antibody was used for labeling the glial cells through the thalamus (figure 9). Although the major glial types expressed in the CNS are astrocytes and oligodendrocytes (Jessen, 2004), the thalamus has not been stained with the GFAP antibody. This might be because of several reasons. One of them is because the

antibody is not as specific as expected or the protocol is not the optimal for the antibody incubation.

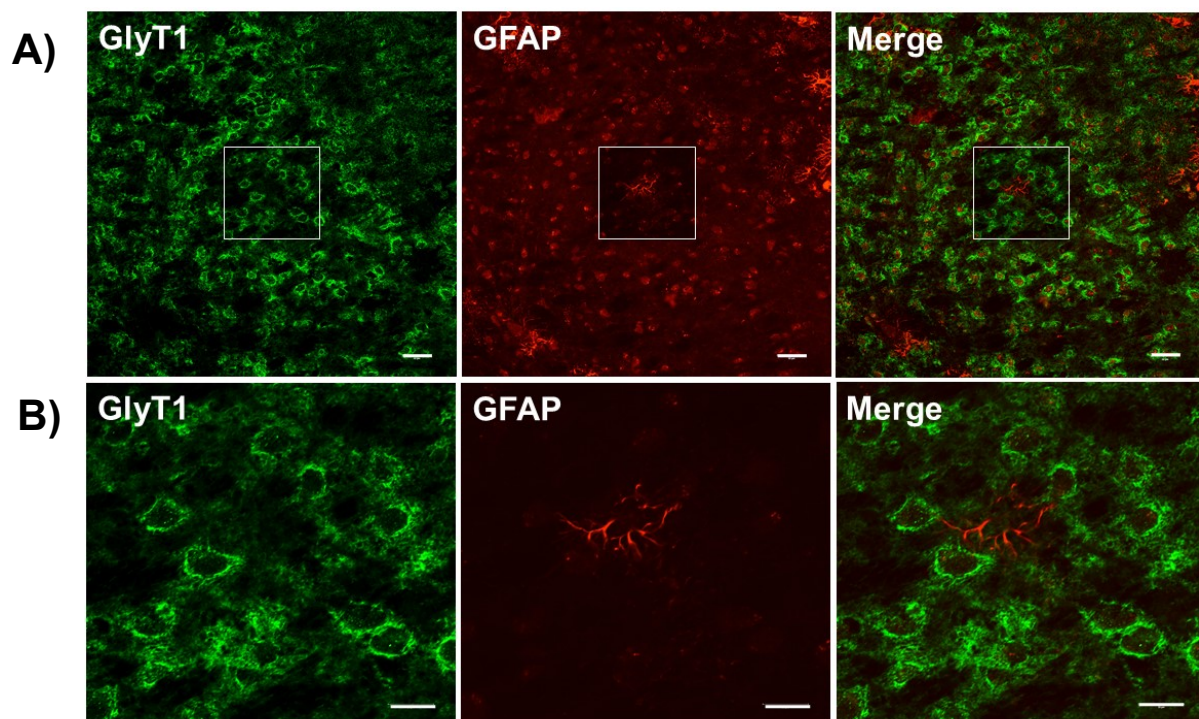


**Figure 9. GlyT1 and GFAP immunoreactivity in a coronal section of the mouse thalamus.**

Immunoreactivity from GFAP (green) and GlyT1 (red antibodies). GFAP is not highly expressed in the VPM as it is in dorsal areas of the thalamus. Encircled region represents the VPM. Nissl image represents figure 44 from The Mouse Brain Atlas (Paxinos and Franklins, 2012). Scale bar: 200  $\mu$ m.



A higher magnification of immunoreactivity from GFAP in the VPM (figure 10), show a small population of glial cells labeled with the antibody. However, the single cell is excluded from GlyT1 immunoreactivity. Based on these findings, we can suggest that GlyT1 is not expressed in astrocytes. However, further immunohistochemical experiments and evidence will have a solid answer whether GlyT1 is expressed in other glial cells (such as microglia) or it is only expressed in neurons.

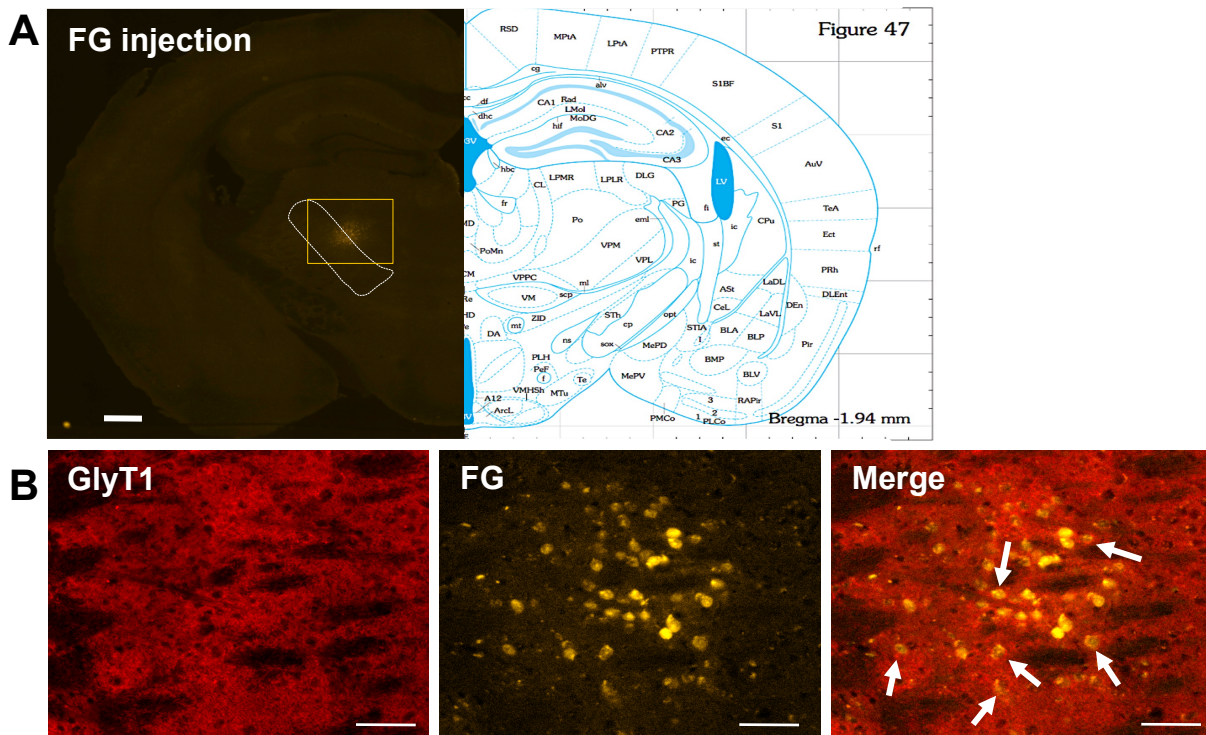


**Figure 10. Glial cells are devoid of GlyT1 immunoreactivity in the VPM.**

Confocal images from the VPM stained with GlyT1 (green) and GFAP (red) antibodies. **(A)** GlyT1 do not co-localize with GFAP in the VPM, **(B)** shows a higher-magnification from figure **(A)**, where shows that the single glial cell is not surrounded by GlyT1. Scale bar: **(A)** 50  $\mu\text{m}$  and **(B)** 20  $\mu\text{m}$ .

### 3.4 Stereotaxic Surgeries and Injection of Fluorescent Tracers GlyT1 and Fluoro-Gold

To better visualize and confirm the previous results, *In vivo* intracranial injections on mice were performed. Three different tracers were used for this study. The retrograde tracer



**Figure 11. Fluoro-Gold injection delivered into the mouse thalamus and GlyT1 immunoreactivity.** (A) The injection site of the tracer into the VPM. The site of the injections is enclosed by the yellow square, whereas the enclosed region represents the VPM. Nissl image from the coronal section represents level 52 from The Mouse Brain Atlas (Paxinos and Franklins, 2012). (B) Confocal images from the VPM stained with GlyT1 (red) and FG (yellow). Arrows show the neurons expressing GlyT1. Scale bar: (A) 500  $\mu$ m and (B) 50  $\mu$ m.

Fluoro-Gold (Figure 11- A) and adeno-associated viral (AAV) particles that express the mCherry protein under the promoter of synapsin (Figure 12- A) and GFAP.

Retrograde tracers traced neural connections from their synapse to their cell body. In contrast, anterograde tracers traced axonal projections from the cell body to the synapse (Purves, 2008).

### **GlyT1 and Neuronal Fluorescent Tracer**

Tissues from these brain injections were stained with the GlyT1 antibody in order to observe the transporter and learn whether it is expressed in neurons or glial cells.

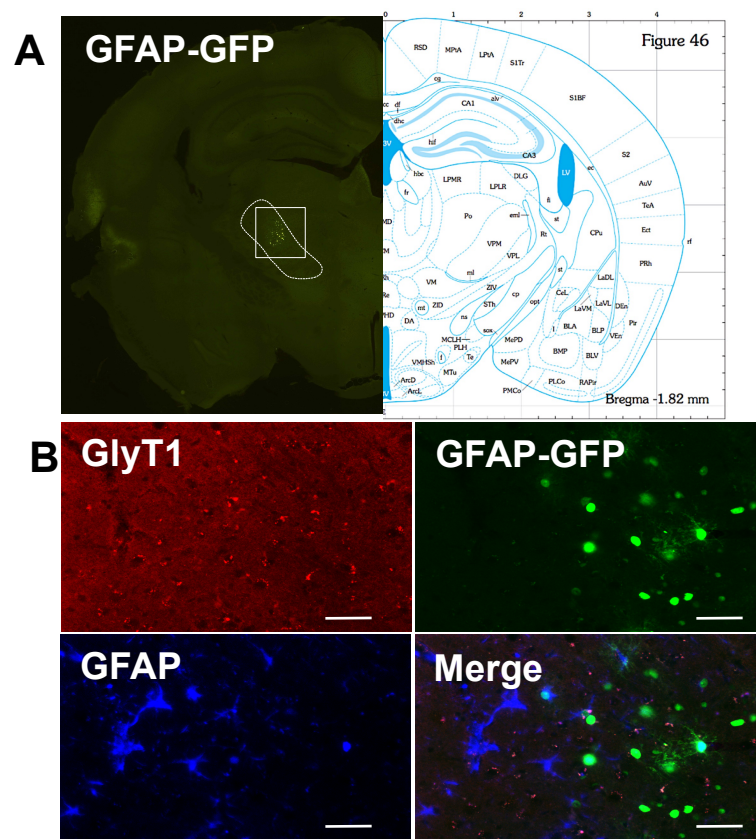
As shown in figure 12-A, the injection site of the AAV particles was into the VPM and the particles were deposited in this region. Higher resolution images from figure 12-B and 12-C show that GlyT1 staining of mCherry-expressing tissue demonstrate that GlyT1 is expressed in neurons (white arrows). Indeed in figure 12-C, it is possible to observe the co-location of GlyT1 in the cytoplasmic membrane of the labeled-neuron.





## GlyT1 and Glial Fluorescent Tracer

Moreover, the AAV expressing the reporter gene GFP under GFAP promoter was delivered into the same manner as the neuronal tracers in the VPM (Figure 13 – A). These viral-fluorescent particles were used as a glial marker. It was expected glial cells were going to encode and express the GFP protein. Using the GFAP antibody along the tracing aimed to identify and confirm whether these viral particles were expressed as expected. Unfortunately,



**Figure 13. Glial fluorescent tracer is not expressed in all glial cells.**

(A) Injection site of the glial tracer (green). The site of the viral particles concentration is surrounded by the square and the enclosed region represents the VPM. Nissl image represents level 46 from The Mouse Brain Atlas (Paxinos and Franklins, 2012). (B) histology experiment stained with GlyT1 (red) and the glial antibody GFAP (blue) along the glial tracer GFP-GFAP. The glial tracer does not co-localize with the antibody and is over expressed in the cells. Scale bar (A) 500  $\mu$ m (B) 20  $\mu$ m

when the high-resolution images were taken under the confocal microscope it was possible to observe the GFAP antibody and the glial tracer did not co-localize and the fluorescent tracer was overexpressed in the infected cells. As a result, this brain tissue could not reveal either suggest clearly whether GlyT1 is also expressed in glial cells besides neurons in the VPM. However, if the fluorescent tracers is excluded and only the GFAP antibody is taking into account, it is possible to suggest that GlyT1 is excluded from glial cells.

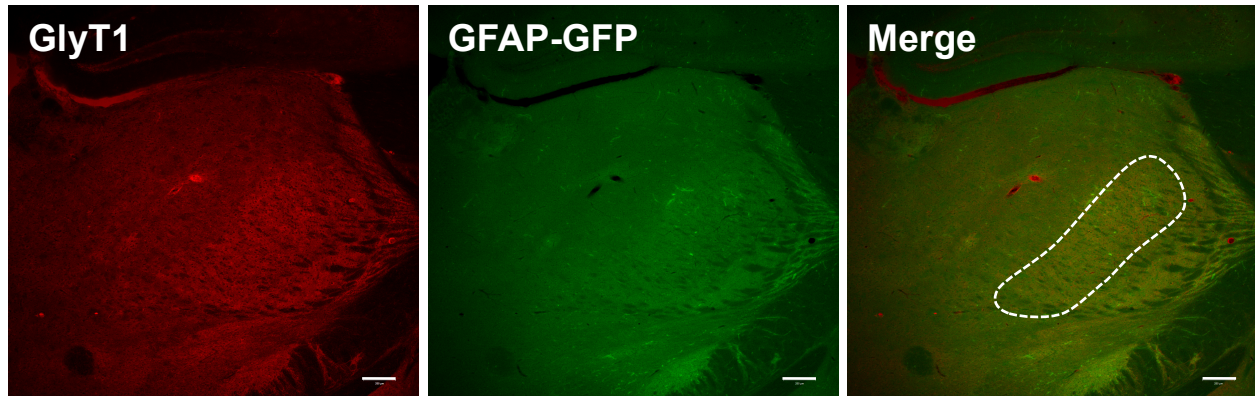
### **3.5 Transgenic Brain Tissue**

Brain tissues obtained from the transgenic mice were stained with the GlyT1 antibody to detect its presence throughout the thalamus. These sections expressed specific fluorescence protein on the tissue depending on their strain. Three different strains of transgenic mice were used for this study: **FVB/N-Tg 14Mes/J**, **B6.Cg-Tg(Slc32a1-COP4\*H134R/EYFP)8Gfng/**, and **CB6-Tg(GAD67-EGFP) G42Z -jh/J**. The green fluorescent protein (GFP) is expressed under the control of the GFAP, VGAT and GAD67 promoters, respectively.

#### **GlyT1 and GFAP-GFP**

The strain FVB/N-Tg 14Mes/J expresses GFP under the control of the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter. Brain tissue from these animals were used with the purpose of confirming our previous findings where the monoclonal GFAP antibody was used. However, only a small portion of glial cells were identified in

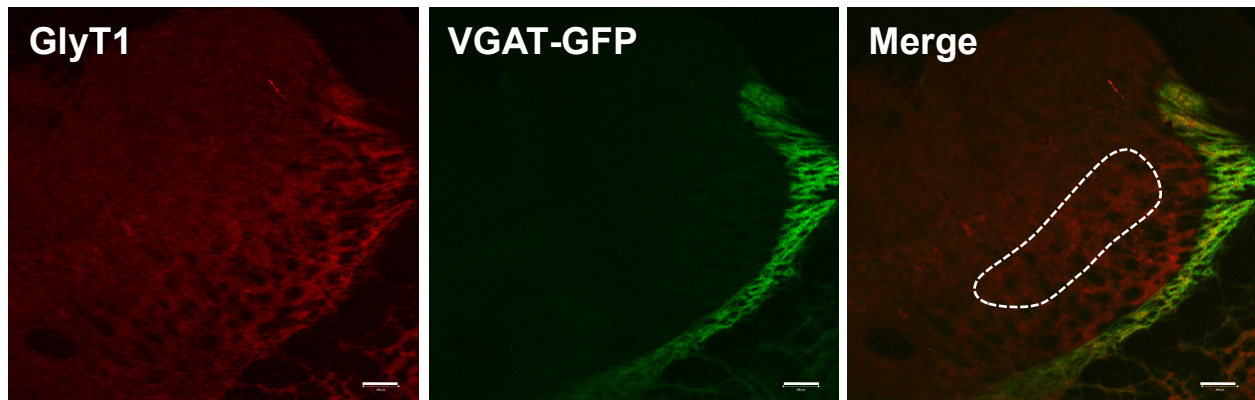
the VPM (figure 14) and transduced cells showed high expression of GFP, preventing the observation of cells by microscopy.



**Figure 14. GFAP-GFP brain tissue from a transgenic mouse and GlyT1 staining.** Histology experiment stained with GlyT1 (red) in brain tissue from a transgenic mouse, which express GFP under the glial promoter (green). A small portion of glial cells is expressed in the VPM (depicted in the merge figure). This section represents level 48 from The Mouse Brain Atlas (Paxinos and Franklins, 2012) (not shown). Scale bar is 200  $\mu$ m.

### **GlyT1 and VGAT-GFP**

On the other hand, our ultimate goal was to characterize the neurons expressing GlyT1 and learn whether GABA and GlyT1 markers were located in the same cell. Therefore, two transgenic mouse lines were used, the VGAT and GAD67 lines that use the corresponding promoters to express the reporter protein GFP. The B6.Cg-Tg(Slc32a1-COP4\*H134R/EYFP)8Gfng/J expresses GFP under the control of the vesicular GABA transporter (VGAT or *Slc32a1*) promoter. VGAT is specifically expressed in GABAergic and glycinergic neurons (Gasnier, 2000). VGAT is highly



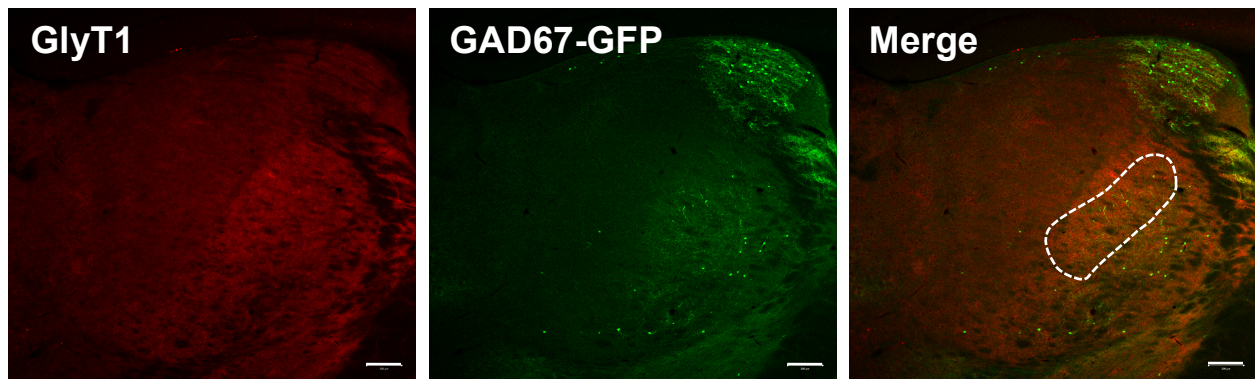
**Figure 15. VGAT-GFP transgenic mouse does not express VGAT in all the thalamus.** Histology experiment stained with GlyT1 (red) in brain tissue from a transgenic mouse, which express GFP under the vesicular GABA transporter, VGAT (green). The greatest population of glutamatergic cells are expressed in the lateral margin of the ventricular surface of the thalamus. This section represents level 48 from The Mouse Brain Atlas (Paxinos and Franklins, 2012) (not shown). Scale bar is 200  $\mu$ m.

concentrated in the nerve endings of GABAergic neurons in the brain and spinal cord but also in glycinergic nerve endings (Legendre, 2001). GABA is an inhibitory neurotransmitter that was studied before discovering glycine. Glycine and GABA mediate inhibitory neurotransmission in the spinal cord and in different areas in the central nervous system (Muller et al., 2008). The co-release of both neurotransmitters by the same presynaptic terminal was first described in cerebellar Golgi cells, in the spinal cord and in the superior olivary complex (Buras et al., 2015). This mouse line was used to look for the presence of GlyT1 in the VPM. In figure 15, it is possible to observe that VGAT is expressed in the lateral margin of the ventricular surface of the thalamus and not in all areas. However, higher-resolution image from the tissue stained with GlyT1 was not taken because the VGAT expression was completely absent from the area of interest.



## GlyT1 and GABA-GFP

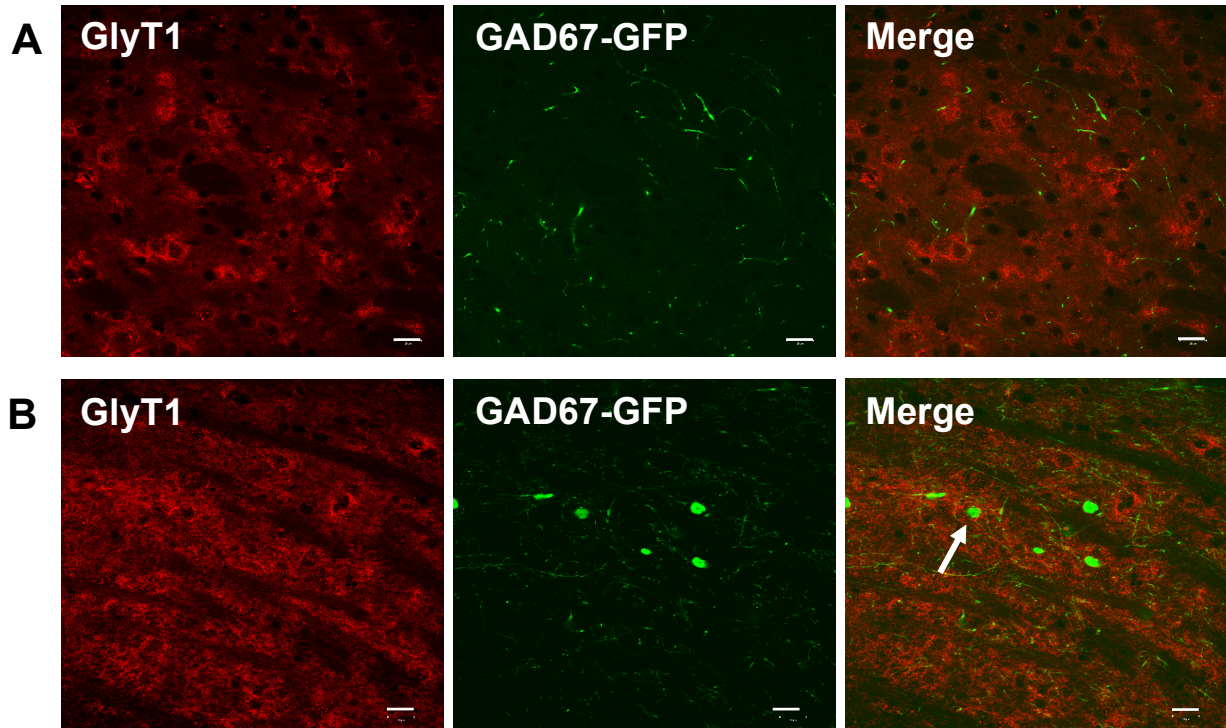
The strain CB6-Tg(GAD67-EGFP)G42Zjh/J also known as the G42 line, expresses GFP under the control of the GAD67 promoter. The glutamic acid decarboxylase (GAD) is an enzyme required for the conversion of L-glutamate to GABA and only labels pre-synaptic neurons producing GABA. This transgenic mouse tissue was used as another way to see if the presence of GlyT1 in the VPM.



**Figure 16. GAD67-GFP transgenic mouse and GlyT1 staining.** Histology experiment stained with GlyT1 (red) in brain tissue from a transgenic mouse, which express GFP under the glutamic acid decarboxylase, GAD 67 (green). Glutamatergic neurons are well expressed in the dorsal and lateral margins of the thalamus. This section represents level 46 from The Mouse Brain Atlas (Paxinos and Franklins, 2012) (not shown). Scale bar is 200  $\mu$ m.

In figure 16, the expression of GAD67 is more abundant in the thalamus compared to the VGAT-reactivity from the transgenic mouse tissue. GAD67 is highly expressed in areas of the thalamus such as the dorsal lateral geniculate nucleus (DLG) and the lateral posterior thalamic nucleus (LPLR). Both nuclei are important for the visual pathway; meanwhile the DLG is a relay center in the thalamus which It receives a major sensory input from the retina. GAD67 is also present in the VPM but not as much as the previous areas (figure 16). Indeed, GAD67 and GlyT1 do not show co-expression from the same neuron in the VPM (figure 17- A), but they might be shared by a small

number of neurons in the DLG (Figure 17- B). The white arrow depicts a neuron that has been labeled with VGAT and GlyT1.



**Figure 17. GAD67-GFP transgenic mouse does not express GAD67 in all the thalamus.** Confocal images from the immunostaining with GlyT1 (red) along the GAD6 expression (green). **(A)** GAD67 is partially expressed in the VPM and does not co-localize with GlyT1, whereas it does in the **(B)** dorsal lateral geniculate nucleus and the neuron is represented with the white arrow. Scale bar: **(A, B)** 20  $\mu\text{m}$ .20  $\mu\text{m}$ .

## **Chapter 4**

### **Discussion and Future Directions**

#### **4.1 Discussion**

The major goal of this research study was to elucidate the identification of the cell type expressing GlyT1 and to characterize these cells. To answer this question, we performed different approaches to differentiate whether this neurotransmitter transporter is expressed by neurons or glial cells. These approaches include immunohistochemistry, intracranial injections of retrograde and anterograde tracers and transgenic mice models.

The thalamus is an important area of the brain, which is involved in sensory and motor signal relay. We observed that this region is enriched in GlyT1 and not in GlyT2. It is interesting that the role of GlyT1 has been well studied in areas of the brain where GlyT1 and GlyT2 are co-expressed; since both are required for the glycine homeostasis. However, in areas of the brain where GlyT1 is expressed, it is suggested that it plays an important role for the NMDA receptor function. Research studies describe that GlyT1 is expressed mainly in glial cells in the caudal regions of the brain, but the cell expression GlyT1 in the forebrain is poorly understood. To determine the identification of the cell type, we stained brain sections of mouse and used different neuronal and glial markers. Interestingly, we found that GlyT1 is located in neurons and it appears always surrounding the expression of a cytosolic protein; by contrast, we did not see glial cells expressing GlyT1.



The intracranial injections of the fluorescent tracers and the AAV particles were delivered into the VPM, where GlyT1 is highly expressed. The accumulation of the fluorescent particles were deposited on neurons or glial cells. The results gathered from the neuronal tracers show a clear view where the cells bodies are surrounded by GlyT1 immunoreactivity. However, although the glial tracer was delivered and the particles were deposited in the VPM, it did not provide a clear view of the dye in the cells. Hence, these sections were not carried out by GlyT1 immunofluorescence because of the lack of the GFAP-specificity.

In addition, we wanted to study if the neurons expressing GlyT1 were also GABAergic. GAD and VGAT are essential components of GABAergic neurons and the neurotransmitter GABA is present in the thalamus. It is worth mentioning that brain tissue from the transgenic mice was expected to show more GFP expression throughout the brain. Hence, we cannot conclude that the cells expressing GlyT1 are GABAergic neurons because of the lack of expression. A possible cause from these enigmatic features could be due to the different isoforms that characterize the VGAT and GAD67 proteins. Another possible reason of the absence of both GABAergic proteins in the tissue can be that these mice only express neuronal populations in restricted areas of the brain.

## **4.2 Future Directions**

Future studies should confirm the presence of neurons expressing GlyT1 in GABAergic neurons. This can be accomplished using additional cellular markers targeting neuronal cytoskeleton or cell membrane to verify the presence of the cytoplasmic

GlyT1. Moreover, additional and different astrocytic cell markers should be used in order to add or exclude the presence of GlyT1 in these cells.

In additional experiments, using viral injections for the transfection of neuronal cells expressing the GlyT1 transporter would be an ultimate project to detect both, neurons and GlyT1, in a very precise way.

Moreover, it will be important to further clarify whether the neurons that express GlyT1 represent a new subtype of glycinergic neuron.

Furthermore, additional studies will increase the understanding of a role for GlyT1 in neurons. For this objective, optogenetics would help to elucidate the function of glycinergic neurons in the thalamus.

The knowledge obtained from this study will advance our understanding of the importance of the glycinergic homeostasis in the CNS.

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## **Curriculum Vitae**

Patricia A. Lozano was born and raised in Cd. Cuauhtémoc, Chihuahua, México. In the fall of 2011, she moved to El Paso, Texas and started her college education at The University of Texas at El Paso (UTEP). Ms. Lozano obtained a Bachelor's degree in Cellular and Molecular Biochemistry with cum laude honors in the fall of 2016, where she also received the Academic and Research Excellence Awards in Cellular and Molecular Biochemistry. In the spring of 2013, she started as a volunteer- research assistant in Dr. Manuel Miranda's laboratory where she stayed for six years. During the summer, she became part of the Vulnerability Issues in Drug Abuse (VIDA) project and she received the Campus Office of Undergraduate Research Initiatives (COURI) award that supported her for three years. In addition, she was selected to spend the summer of 2016 at the Mayo Clinic in Jacksonville, Florida for a research project focused on the Alzheimer's disease. In August of 2017, her contributions were published in the scientific journal PNAS. In the spring of 2017, she was admitted into the Biology Master's program at UTEP. During her graduate education, her research focused on the neurotransmitter glycine transporter 1. In addition, during her graduate studies she presented her research project and findings at the international Society for Neuroscience annual conference in 2018 in Washington, D.C. Moreover, she received the UTEP Graduate School Travel Grant in May of 2018, which allowed her to attend the Workshop on Assisted Reproductive Technologies in the Laboratory Mouse at the Jackson Laboratory Facilities in Bar Harbor, Maine.

This thesis was typed by Patricia Lozano, [palozano3@miners.utep.edu](mailto:palozano3@miners.utep.edu).