Quantitative Analysis of Glycinergic Neurons Including Ia Inhibitory Interneurons in the Ventral Spinal Cord using a BAC-GlyT2-eGFP Transgenic Mouse Model

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Quantitative analysis of glycineergic neurons including Ia inhibitory interneurons in the ventral spinal cord using a BAC-GlyT2-eGFP transgenic mouse model

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

Palak Painter
B.S., De Montfort University 2006

2012
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Palak Painter ENTITLED Quantitative analysis of glycinergic neurons including Ia inhibitory interneurons in the ventral spinal cord using a BAC-GlyT2-eGFP transgenic mouse model BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Painter, Palak M.S., Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2012. Quantitative analysis of glycinergic neurons including Ia inhibitory interneurons in the ventral spinal cord using a BAC-GlyT2-eGFP transgenic mouse model.

We are interested in understanding the development of reciprocal inhibition, an important sensory-motor circuit that prevents co-contraction of antagonist muscles during locomotion. This effect is mediated by a subset of glycinergic neurons termed Ia inhibitory interneurons (IaINs). Transgenic mouse models are useful in helping to identify subsets of neurons in developing animals. In this thesis we first wanted to test the BAC-GlyT2-eGFP mice model by measuring GFP expression overlap in neurons with glycine in late-stage embryos (E17.5) and early postnatal animals (P0/P1). Our results suggest more than 98% of glycinergic neurons at these stages also express GFP in GlyT2-EGFP transgenic animals. Secondly, we quantitatively measured the number of IaINs at P0 and P1 using a combination of GFP expression in this transgenic model together with other immunohistochemical markers of IaINs. We found that IaINs account for 17% of glycinergic neurons found in lamina VII at this stage.
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I. INTRODUCTION

The main goals of this thesis are 1) to validate the BAC-GlyT2-eGFP mice model by carefully measuring the eGFP-Glycine expression in neurons from lamina VII at late embryonic and early postnatal stages in a quantitative manner and; 2) to identify Ia inhibitory interneurons (IaINs) and measure them in a quantitative manner at postnatal stages using the BAC-GlyT2-eGFP transgenic mice model. This study will build a strong foundation for the future work in our lab.

Our lab is involved in looking into mechanisms that guide the development of sensory-motor neuronal circuits, in particular the mechanisms involved in the maturation of the reciprocal inhibition circuit. Reciprocal inhibition (RI) acts to prevent co-contraction of antagonist flexor and extensor muscles to facilitate normal locomotion. A unique class of glycineergic interneuron, the Ia inhibitory neuron (IaIN), mediates this inhibition by integrating sensory feedback from the periphery and controlling the motor neuron (MN) firing during locomotion (Eccles and Lundberg, 1958). IaINs receive synaptic contacts from Ia afferents bringing signals from muscles in the limb and make synapses on the efferent MNs that project to limb muscles with antagonistic function at the same joints. Excitatory synaptic connections between extensor sensory afferents and extensor efferent MNs cause the extensor muscles to contract; at the same time, the IaIN interneurons activated by extensor Ia afferents diminishes electrical activity in flexor efferent MNs and cause
flexor muscles to relax (Figure 1). Increasing knowledge regarding the development of normal RI circuit function will shed some light on understanding the changes in RI seen in disease and injury state.

Locomotion is still under development at birth in humans, unlike some other mammals that quickly acquire motor skills of posture and the ability to walk at the time of birth. Therefore, one may speculate that the development of sensory motor circuits occurs postnatally based on experience dependent mechanisms. The assembly of sensory motor circuits during development is dependent on the close interaction between the experience-driven processes and programs of genetic predetermination. This makes motor circuits highly flexible, enabling reading, modifying and activating the motor output in response to the changing environment throughout life (Ladle et al., 2007). On the other hand, basic circuits of the sensory motor system seem to be pre-formed in embryos in many species. Reciprocal inhibition is present in newborn human infants (Mc Donough et al., 2001). Although humans undergo significant postnatal maturation in terms of locomotor skills, the presence of basic sensory-motor circuits at birth implies earlier specification of the basic elements that create these circuits.

RI is mediated by a single class of glycinergic neurons termed IaINs. The RI pathway includes many complex neuronal connections, but very little is known about the role and anatomical organization of IaINs in the early developmental stages of this circuit. It is known, however, that IaINs are glycinergic, and fortunately, we have a transgenic mouse model available in our lab that specifically labels glycinergic neurons. This model, generated by Zeilhofer and his team is a bacterial artificial chromosome (BAC) transgenic mouse,
**Figure 1: Reciprocal Inhibition Circuit.** The circuit mediating reciprocal inhibition is composed of Ia afferents (illustrated using red dashed lines) originating from muscle spindle primary endings and exerting monosynaptic excitatory actions on both synergist MNs (pink) and IaINs (yellow) which act to inhibit the MNs of antagonist muscle. The subset of IaINs contacted by flexor-Ia afferents is different than those contacted by extensor-Ia afferents. Hence, flexor Ia-afferents only evoke inhibition of extensor MNs, and extensor-Ia afferents only evoke inhibition of flexor MNs. Blue arrows indicate the direction of information flow in the circuit.
which specifically expresses enhanced green fluorescent protein (eGFP) under the control of the promoter of the glycine transporter (GlyT) 2 gene, which is a reliable marker for glycinergic neurons (Zeilhofer et al., 2005). Past studies have shown that BAC transgenic models can successfully be used to express specific proteins under the control of the promoter of a known gene to label cells of interest. (Heintz, 2001). Zeilhofer and colleagues showed that neurons expressing GlyT2-EGFP were intensely fluorescent, and their dendrites and axons could be visualized in great detail, but they did not justify their results in a quantitative manner. They simply commented that “the vast majority of eGFP-positive cells were strongly immunoreactive for glycine” (Zeilhofer et al., 2005). Also all the experiments were carried out using adult mice. As one of the focuses of this study, we wanted to quantify for the first time eGFP-glycine coexpression in the GlyT2-eGFP mouse model.

Another focus of our study was identifying inhibitory IaINs from among other inhibitory neurons in the ventral spinal cord, and focused on mapping and locating IaINs to further understand the anatomical organization of IaINs in this sensory motor circuit. Past studies have looked into the location of IaINs in adult animals (Jankowska, 1992), but we wanted to measure the IaINs located in the lamina VII in a quantitative way using the GlyT2-eGFP mice model at early postnatal stages which has not been done before.
II. BACKGROUND

Locomotion is fundamental to an animal’s life and is controlled by neuronal networks in the central nervous system (CNS). Coordinating complex movements involve multiple elements of the CNS, such as cortex, cerebellum, basal ganglia, brainstem, and spinal cord. Different motor control features are provided by each component. Neuronal circuits contained entirely within the spinal cord produce basic rhythmicity and patterning for stepping and other locomotion behaviors. There is limited knowledge of how spinal cord circuits develop and how different types of neurons are generated, specified, and incorporate themselves into these circuits. In order to understand the maturation of motor control, and potential abnormalities and dysfunctions that may occur during early development, it is very important to understand how and when initial connections are made in this pathway, and how and when their maturation occurs.

Past studies in the cat, by Eccles, Lundberg, Jankowska and others went a long way toward defining the spinal reflex circuitry in adult animals including the properties of the constituent interneurons and their actions on MNs. Most studies examining the neural circuits involved in quadripedal locomotion have been undertaken in adult cats and rodents (Eccles JC, 1968; Lundburg A, 1979; Jankowska E, 2001; Jankowska E. & Edgley S., 1993). Experiments in the cat, in particular, have helped in understanding pathways of spinal reflexes in terms of their organization and how sensory feedback is
involved in shaping movements. Sensory feedback is essential for starting and maintaining the correct locomotor rhythm. Recordings from the cat spinal cord have identified and characterized some of the interneuron cell types that are interposed in these spinal sensorimotor pathways. (Eccles JC, 1968, Lundburg A, 1979, Jankowska E, 2001, Jankowska E. & Edgley S., 1993). The analysis of motor behaviors has long been at the center of efforts to understand how the nervous system is organized and functions, with Sherrington’s studies providing important first insights into the integrative nature of neural pathways, the reflex arc, and the control of reciprocal motor actions by central inhibitory pathways (Sherrington, 1906).

This thesis revolved around identifying inhibitory IaINs from other neurons, and also focused on mapping and locating IaINs to set the stage for further experiments to understand the functionality of IaINs in sensory motor circuits. Inhibitory networks like recurrent and reciprocal inhibition control the activity of adult motoneurons (Pierrot-Deseilligny & Burke, 2005). Recurrent inhibition is a process of inhibition of motor neurons by particular interneurons termed Renshaw cells (RCs) that receive excitatory inputs from motor axons (Eccles et al., 1954). This is in contrast to reciprocal inhibition whereby inhibition of antagonist motor pools is evoked by stimulation of proprioceptive afferents (Eccles et al., 1956). Renshaw cells and IaINs have different functional roles and connections in an adult but they are both located in lamina VII (Jankowska, 1992) in the ventral horn of the spinal cord.
A. Reciprocal Inhibition (RI) in adults:

The demonstration that the contraction of a muscle is accompanied by the relaxation of its antagonist was shown by Sherrington in 1897 who named this effect “reciprocal inhibition”. The possibility that reciprocal inhibition was mediated by Ia afferents directly affecting MNs was postulated by Lloyd (1941). However, more than a decade later, Eccles demonstrated in 1956 that an interneuron (IN) was interpolated in the reciprocal inhibitory pathway. This interneuron was first called the “Ia intermediate interneuron”, which evolved into its current name, “Ia inhibitory interneurons (IaIN)”. The reciprocal inhibitory circuit as we know it today involves Ia afferents originating in the muscle spindle primary endings that exert monosynaptic excitatory action onto IaINs that act to inhibit motor neurons of the antagonistic muscle (Eccles and Lundberg, 1958).

B. Early Development of Reciprocal Inhibition:

Appropriate Ia afferent connections must be made with specific IaINs and those IaINs must make further contacts with their targeted MNs for the normal development of RI circuits. For example, Quadriceps (Quad) Ia afferents make connections with specific IaINs during development, such that when stimulated by Quad Ia afferents, these IaINs inhibit the MNs projecting to hamstrings muscles which act as antagonists to Quad at the knee joint. How and when these connections made at embryonic stages is still unknown. One can assume that initially IaINs may be contacted by Ia afferents from many, including inappropriate, muscle sources. During development, reinforcement of Ia-IaINs
connections that lead to appropriate inhibition might take place as the animal ages, whereas any inappropriate connections may be weakened or disappear.

C. **Role of IaINs in adults:**

The sensory-motor circuit that mediates RI acts to prevent the co-contraction of antagonist flexor and extensor muscles (Figure 1). IaINs receive inputs from Ia proprioceptive afferents and generate reciprocal inhibition by inhibiting motoneurons in motor pools innervating muscles that are antagonists of the muscle of origin of the Ia afferent (Eccles et al., 1956). Earlier studies have shown that IaINs are found in lamina VII of the ventral horn of the spinal cord. Subpopulations of interneurons with opposite actions, those mediating Ia reciprocal inhibition from flexors to extensors and from extensors to flexors of a given joint, have been found to inhibit each other (Hultborn et al., 1976) and their activity can be controlled by Renshaw cells (Hultborn et al., 1971).

D. **Early development of IaIN:**

Interneurons (INs) and MNs are the two general classes of neurons in the spinal cord. MNs generate the motor output to the muscle, while INs establish local circuits that control the activity of MNs. Initial developmental studies focused on MN development (Jessell, 2000). Recently, interest has turned to spinal cord INs. Previous studies have shown that IaINs, which mediate reciprocal inhibition, derive from V1 interneurons (Alvarez et al., 2005). The finding that all adult spinal INs develop from a few embryonic subclasses; six dorsal (dl1, dl2, dl3, dl4, dl5, and dl6) and four ventral (V0, V1, V2, and V3) was a major breakthrough (Goulding, 2009). Each of these interneuron
classes are derived from distinct progenitor domains located in different regions of the proliferative neuroepithelium in the early neural tube. The expression of the engrailed-1 (En-1) transcription factor was found to be a key marker for V1 interneurons (Sapier et al., 2004). Past studies undertaken using adult mice, has shown that out of the total V1 interneuronal population, 10% of interneurons were counted as RCs and 13% were counted as IaINs (Alvarez, 2005).

E. Location, anatomy and morphological identification:

IaIN’s position in the ventral horn was first determined from electrophysiological recordings (Hultborn et al., 1971) and they also showed that IaINs are directly inhibited by RCs located in the same spinal segment as the IaIN (Hultborn et al., 1971). More specific information on exact location came from intracellular labeling of IaINs. They were found in lamina VII (LVII) dorsal or medial to lamina IX (Burke et al., 1971, Jankowska 1992). IaINs are always located in the same spinal cord segment as the Ia afferents that excite them. Therefore, to exert reciprocal inhibition between pools of motoneurons located segments away, the axons of IaINs need to ascend or descend in the white matter surrounding the ventral horn and then send collaterals to target motor pools either locally or several segments away (Jankowska and Lindstrom, 1972). In our study, we identified IaINs by looking for eGFP positive neurons that received Ia afferent inputs, as well as inputs from RCs using a BAC-GlyT2-eGFP transgenic mice model.
F. GlyT2-GFP Transgenic Mice Model:

Biomedical research to study developmental biology has been enhanced by using transgenic mice as animal models. Bacterial Artificial Chromosome (BAC) DNA vectors have been developed to hold much larger pieces of DNA that serves as the promoter regions for specific genes than normal plasmid vectors. BAC transgenic mice models can be generated by microinjecting purified BAC DNA into the nucleus of fertilized mouse eggs.

To shed some light on the morphological and functional characteristics of glycinergic neurons, Zeilhofer and his team generated BAC transgenic mice. The BAC clone 365E4 (179,508 base pairs long) used by Zeilhofer and his team, contained the entire glycine transporter 2 (GlyT2) gene and an additional 105 kb DNA flanking the 5’ end of exon 1 and 21 kb DNA flanking the 3’ end of the last exon. The facilitation of eGFP expression was carried out by removal of exon 2 at the beginning of GlyT2 gene using a two-step homologous recombination procedure and inserting eGFP cDNA in the place of exon 2. Several homologous recombination and restriction digest steps were carried out to remove unwanted vector sequences and to achieve the final result: a transgenic mouse line which expressed enhanced GFP (eGFP) under the control of the promoter of the glycine transporter gene 2 (GlyT2).

For our experiments, an active breeding colony of BAC-GlyT2-eGFP mice had been established in our animal facility at Wright State University with Dr. Zeilhofer’s permission. Thus in the present study, we will demonstrate the eGFP-Glycine percentage
overlay in a quantitative way using BAC-GlyT2-eGFP transgenic mice model at embryonic day E17.5, and postnatal day P0 and P1. We will then locate and measure in a quantitative manner the percentage of IaINs in comparison to the other glycineergic neurons using BAC-GlyT2-eGFP mice model at postnatal day P0 & P1.
III. SPECIFIC AIMS

**Aim 1:** To test and validate in a quantitative manner the GlyT2-eGFP transgenic mice model at late embryonic and early postnatal stages. GlyT2-GFP is a transgenic mouse line in which green fluorescence protein (GFP) is expressed under control of the GlyT2 (neuronal glycine transporter) promoter (Zeilhofer et al., 2005). Previous studies have demonstrated expression of GFP in this mouse model in glycinergic neurons in the brain and spinal cord of an adult mouse, but did not quantify whether GFP was expressed in all glycinergic neurons. We are looking to quantify the percent eGFP-Glycine co-expression overlap by manually identifying GFP positive cells and then measuring the intensity glycine expression from confocal image stacks using Image-Pro software. Several sections from a single animal were averaged and then total average values (±SEM) from E17.5 (n=4) and P0/1 (n=5) were plotted in graphs to show the percent GFP-Glycine overlap.
**Aim 2**: To identify and measure in a quantitative manner immunohistochemically defined IaINs in the ventral horn of the spinal cord in GlyT2-eGFP postnatal mice. IaINs are glycineergic neurons that mediate reciprocal inhibition and previous studies have shown that these neurons are located in lamina VII of the ventral spinal cord. We quantified the abundance of IaINs in comparison to the total number of GlyT2-eGFP positive cells found in the lamina VII of postnatal spinal cord. Several sections from a single animal were scanned with confocal imaging and analyzed for IaINs and the total average values (±SEM) from 4 animals at P0/1 were determined.


IV. MATERIALS & METHODS

A. Animals used for the experiments:

All animal procedures were performed according to National Institutes of Health guidelines and were reviewed by the local Laboratory Animal Use Committees at Wright State University. For our experiments, an active breeding colony of BAC-GlyT2-eGFP mice had been established in our animal facility at Wright State University with Dr. Zeilhofer’s permission.

Time-pregnant females for GlyT2-eGFP transgenic mice model were created by mating GlyT2-eGFP positive males with C57/BL6 wild-type females. Once mated, regular vaginal plug checks were carried out every morning to record the time of the pregnancy. Once plugged, the females were separated into different cages and the time and date of the plug was recorded. The plugged females were then observed till embryonic day 17.5 and then euthanized to isolate the embryos. The embryos were genotyped using a fluorescent dissecting microscope to visualize the strong GFP signal through the skin of the embryo. Positive embryos were then further used for immunohistochemical experiments.

For P0/P1 pups, GlyT2-eGFP males were mated with C57/BL6 wild-type females as described for matings to generate embryos. On the day of birth, the litter was
genotyped using a fluorescent dissecting microscope to visualize the strong GFP signal through the skin of the pup. Positive pups were used for further immunohistochemical or retrograde labeling experiments.

B. **Tissue Preparation for analysis:**

**EGFP-Glycine overlay (E17.5):**

Time pregnant females were euthanized with Euthasol (pentobarbital) at E17.5 and embryos removed and transferred into an oxygenated ACSF solution. A ventral laminectomy was carried out on GFP positive embryos to expose the spinal cord and the spinal cord was submerged in tubes of paraformaldehyde for 2 hours (post fixed). Afterwards the spinal cord was washed with PBS (either 2-4 hours or overnight) and was then transferred into tubes of 30% sucrose solution (either 2-4 hours or overnight). A portion of the L4-L6 region of the spinal cord was cut and frozen onto a cutting block via Tissue Freezing Medium (OCT, Tissue Tek). The frozen tissue blocks were then cut into 12 micrometer sections using a cryostat and mounted directly on a slide for further immunohistochemistry.

**EGFP-Glycine overlay (P0/P1):**

Neonatal P0/P1 pups were anesthetized via exposure to ice cold water for approximately 2 minutes then transcardially perfused with 5ml of ice-cold paraformaldehyde (PFA). Pups were then eviscerated and decapitated. A ventral laminectomy was carried out and the spinal cord was isolated into tubes of
paraformaldehyde for 2 hours (post fixed). Afterwards the spinal cord was washed with PBS (either 2-4 hours or overnight) and was then transferred into tubes of 30% sucrose solution (either 2-4 hours or overnight). A portion of the L4-L6 region of the spinal cord was cut and frozen onto a cutting block via Tissue Freezing Medium. The frozen tissue blocks were then cut into transverse 12μm sections using the cryostat and mounted directly on a slide for further immunohistochemistry.

**Retrograde labeling of primary afferents:**

Retrograde labeling of primary afferents can be achieved by dorsal root fill technique. This strategy labels all types of afferents, but different types of afferents have different termination zones. Ia afferents extend their axons and make contacts with IaINs in lamina VII and MNs in lamina IX (Brown & Fyffe, 1978). Ib afferents, another proprioceptive afferent type, extends its axons and terminates in more dorsal lamina of the spinal cord (Brown & Fyffe, 1978). Group II muscle spindle secondary afferents, even though they terminate in the same lamina as Ia afferents, are not known to make contacts with IaINs (Jankowska, 1992). Thus, primary afferent terminals making contacts with IaINs can be identified by their unique termination zone following retrograde labeling through dorsal root-fill experiments.

The neonatal mice P0/P1 [n=4] GlyT2-eGFP transgenic mice utilized in this study were anesthetized via exposure to ice-cold water for approximately 2 minutes. The mice were then transcardially perfused with 5ml of ice-cold oxygenated artificial cerebrospinal fluid (ACSF; containing in mM: 127 NaCl, 1.9 KCl, 1.2 KH₂PO₄, 1 MgSO₄•7H₂O, 26
NaHCO$_3$, 16.9 D(+)−Glucose monohydrate, and 2 CaCl$_2$). Mice were decapitated and eviscerated. The vertebral column, including the spinal cord, was then transferred into a recirculating bath of ice-cold oxygenated ACSF. This was followed by a dorsal laminectomy of the spinal column and careful removal of the dura from the spinal cord. All the ventral roots on both the sides of the spinal cord as well as all the dorsal roots of lumbar segment were cut, except dorsal root L4 was kept intact on the side of interest. The hemisected spinal cord was then pinned down in a sylgard covered dish filled with recirculating oxygenated ACSF at room temperature while setting up the pipettes filled with Rhodamine dextran to label the primary afferents in dorsal root L4. The dye transportation takes place via active metabolic processes and requires overnight incubation (<18 hours). The next morning the spinal cord was isolated into a tube of paraformaldehyde for 2 hours (post fixed). Afterwards the spinal cord was washed with PBS (either 2-4 hours or overnight) and was then transferred into tubes of 30% sucrose solution (either 2-4 hours or overnight). A portion of L4-L6 segment of the spinal cord was then cut and frozen into a cutting block via Tissue Freezing Medium. The frozen tissue blocks were then cut into transverse 20µm sections using a cryostat and mounted directly on a slide for further immunohistochemistry.
Figure 2: Illustration of retrograde labeling of primary afferents via lumbar 4 dorsal root (DR4). Sensory axons in the dorsal root (red) are labeled via a rhodamine dextran filled micropipette. Sensory axon projections can be visualized in ventral spinal cord, including lamina VII and lamina IX. We focused our analysis on eGFP positive glycineric neurons (green pentagons and squares) are located in lamina VII, surrounding lamina IX in the ventral horn of the spinal cord. This technique allows labeling of all primary afferent classes projecting in DR4. The green square shaped cells labeled with eGFP illustrate the location of IaINs that are found in lamina VII, medial or dorsal to lamina IX.
C. **Immunohistochemistry:**

**EGFP-Glycine overlay:**

Slides of fixed E17.5 and P0/P1 tissue were taken through a step-by-step process of one PBS (0.1 M Phosphate Buffer Saline) wash for five minutes followed by immersion in a citrate buffer solution, which works as an antigen retrieval agent to enhance the glycine signal, for 20 mins at 95°C. Slides were then washed again in PBS. Next, the slides were incubated in Normal Donkey Serum (NDS) solution for an hour (Blocking Buffer (1X PBS + 1% Bovine Serum Albumin (BSA) + 10% Triton) + 1:100 NDS) to block the background. Primary antibodies (rat α glycine (Gly), 1:800 and mouse α Neuronal Nuclei (Neu N), 1:1000) were then added onto the slides and left on overnight at 4°C. The slides were then washed twice with PBS (5 mins each) before adding the secondary antibodies (Cy3 donkey α rat, 1:1000 and Cy5 donkey α mouse, 1:1000) for 2-4 hours at room temperature. The slides were then taken through three PBS washes (5 mins each) before being cover slipped using Vectashield mounting medium (Vector Laboratories).

**Retrograde labeling experiments:**

Slides with fixed P0/P1 tissue were first washed one time with PBS for five minutes. Next, the slides were incubated in NDS solution for an hour to reduce background signal. Primary antibodies (guinea pig α rhodamine (RHOD), 1:5000 and rabbit α calbindin (CB), 1:1000) were then added on to the slides and left on overnight at
4°C. The slides were then washed with PBS twice (5 mins each) before adding the secondary antibodies (Cy3 donkey α guinea pig, 1:100 and Cy5 donkey α rabbit, 1:1000) for 2-4 hours at room temperature. The slides were then taken through three PBS washes (5 mins each) before being cover slipped using Vectashield mounting medium.

**Imaging:**

The slides were imaged and analyzed using an Olympus FV1000 confocal microscope. Fluorophores were excited with line lasers of 405nm, 488nm, 568nm, and 633nm.

**Analysis:**

Images were composed using Fluoview (FV) software, Image Pro Plus 5.1, and CorelDraw 14. Brightness, contrast and sharpness in the images were enhanced without altering any content within the images.
Table 1: Antibodies used for immunohistochemistry

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<th>Antibody</th>
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V. RESULTS

**Aim 1:** To test and validate in a quantitative manner the GlyT2-eGFP transgenic mice model at late embryonic and early postnatal stages.

The work in our laboratory is looking to better understand the early development of the sensory motor circuit that mediates reciprocal inhibition. Reciprocal inhibition is achieved via a class of glycinergic interneurons that receive Ia afferent input and act to inhibit motor neuron targets. One tool that can be used to assist in identifying these specific interneurons is a transgenic mouse model wherein glycinergic neurons express GFP (Zeilhofer, 2005). To lay the foundation for future experiments, we needed first to validate expression of GFP in this mouse model in a quantitative manner.

BAC transgenic mice models can be generated to express a specific reported protein under the control of a promoter of a known gene in order to identify the cells of interest (Heintz, 2001). This technique was applied by Zeilhofer and colleagues to label glycinergic neurons, based on expression of GlyT2 in subsets of neurons (Zeilhofer et al., 2005). A statement that “the vast majority of eGFP-positive cells were strongly immunoreactive for glycine” was made by Zeilhofer and his team in 2005 (Zeilhofer et al., 2005). Yet, so far no one has carefully tried to measure the eGFP expression overlap with glycine immunoreactivity in a quantitative manner. In addition, no analysis of the model at earlier stages, including in embryos, has been performed. Therefore, our first aim was to test and validate the BAC-GlyT2-eGFP mouse model at early developmental stages in a quantitative manner.
A subset of neurons in the spinal cord use glycine as a neurotransmitter. Glycine is also a fundamental amino acid and must be packaged into synaptic vesicles for release as a neurotransmitter. The protein in neurons that is responsible for this is glycine transporter type 2 (GlyT2). The promoter of the GlyT2 gene was used to construct a BAC used in the transgenic animal model we are studying. We used immunoreactivity for GlyT2 and glycine as key markers to carefully quantify the percentage overlap with GFP expression in GlyT2-EGFP animals. We collected lumbar spinal cord tissue from GlyT2-eGFP positive animals at embryonic day 17.5 (E17.5; n=4) and postnatal day 0 and 1 (P0/P1; (n=5). Previous studies have shown that sensory-motor connectivity begins at E17.5 and one of the focuses of our study in future would be to understand the function of RI at birth, hence the reason we chose these two time points for our current study.

Genotyping of embryos was facilitated by strong GFP fluorescence signal in the spinal cord that was visible through the skin using an epifluorescence dissecting microscope. Genotyping was confirmed by post hoc PCR.

First, we determined if the native eGFP signal in GlyT2-eGFP animals was strong enough after fixation to allow for analysis, or whether signal enhancement with antibodies against GFP was required to detect weakly labeled cell structures. We performed this analysis using both 50µm floating sections as well as 12µm tissue sections fixed to slides. Selected GFP+ cells were visualized by confocal laser scanning microscopy with or without antibody staining against GFP in different tissue sections. We found even the finest neuronal processes could be seen without antibody staining, and that sections stained with the antibody showed poor penetration. This experiment
revealed that anti-eGFP antibodies did not penetrate more than 6-8µm from the surface using either 50µm floating sections or 12µm tissue sections fixed to slides. In addition, anti-GFP staining did not label more neuronal structures than seen with native eGFP fluorescence after fixation (data now shown). Therefore, all following experiments were performed without further amplifying the eGFP fluorescence (see Figures 3A, 4A for examples).

Initially we attempted to validate the faithfulness of GFP expression in GlyT2-eGFP mice using an antibody against the membrane-bound glycine transporter 2 (GlyT2). Previous studies have shown that immunoreactivity (IR) for GlyT2 is a reliable marker for glycineric neurons in the CNS (Poyatos et al., 1997; Liu et al., 1993). Only axon terminals are labeled by GlyT2 antibodies (Spike et al., 1997) as this transporter is concentrated at axon terminals in order to load synaptic vesicles with glycine. In our hands, although the GlyT2 antibody was able to label the axon terminals in adults, GlyT2 IR was not distinct in E17.5 embryos and P0-P1 pups (data not shown). The GlyT2 signal in the axon terminals of E17.5, P0 and P1 tissue sections was very weak compared to the background, which made it impossible to accurately measure the GlyT2-eGFP expressing cells in a quantitative manner.

To show the general positioning of all neurons in spinal cord, we used the pan-neuronal marker NeuN (Figure 3C, 4C). This allowed us to identify the pools of large motor neurons in lamina IX of the ventral horn. Visualizing the border of lamina IX was important for our study as we were focusing on studying the glycineric neurons in the lamina VII right adjacent to the lamina IX (Figure 3A, C; Figure 4A). Even after the
Figure 3: Representative section illustrating glycine in a transgenic GlyT2-eGFP mouse at E17.5 A) Transverse section of L4 ventral horn showing eGFP expression in a GlyT2-eGFP embryo. B) Immunoreactivity for glycine on the same section. C) Immunoreactivity for NeuN on the same section. A pool of large neurons (motor neurons) is clearly seen to be labeled with pan-Neu N in lamina IX. D) Overlay of A and B showing co-localization of eGFP (green)-glycine (red) in neuron somata. E) Merged view of A, B and C showing eGFP-glycine overlay in yellow and NeuN labeled cell bodies in blue.
Figure 4: Representative section illustrating glycine in a transgenic GlyT2-eGFP mouse at P0/P1. A) Transverse section of L4 ventral horn showing eGFP expression in a GlyT2-eGFP mouse at P0/P1. B) Immunoreactivity for glycine on the same section. C) Immunoreactivity for NeuN on the same section. A pool of large neurons (motor neurons) is clearly seen to be labeled with pan-NeuN in lamina IX. D) Overlay of A and B showing co-localization of eGFP (green)-glycine (red) in neuron somata. E) Merged view of A, B and C showing eGFP-glycine overlay in yellow and NeuN labeled cell bodies in blue.
paraformaldehyde fixation, strong eGFP fluorescence was observed in both embryonic and postnatal spinal cords tissue sections (Figure 3 A, F & 4 A, F) and hence further amplification of the signal was not needed. The eGFP fluorescence was readily seen in subsets of neurons throughout the spinal cord with the exception in lamina IX, where MN cell bodies are located.

Visual inspection of the staining patterns of glycine and NeuN using the GlyT2-eGFP mice model at embryonic and postnatal stages, revealed that at E17.5 neurons were found to be generally more densely packed in the ventral horn of spinal cord (Figure 3A, C) in comparison to the more evenly distributed band of eGFP-Glycine expressing neurons seen adjacent to lamina IX at P0 and P1 (Figure 4A, C). By eye one can easily see a degree of overlap between the eGFP and glycine (Figures 3&4) but further analysis was carried out to quantify the eGFP-Glycine overlay.

From figure 3 and 4, we can say that there was a good degree of overlay between eGFP and glycine expression; however after taking a closer look by looking at the images taken at higher magnification using confocal microscopy, we observed that there were a few cells which strongly expressed eGFP but had either weak or no expression of glycine (Figure 5). Similarly, it was also observed that there were couple cells in each section which displayed strong glycine signal, but very weak EGFP signal. The fact that we noticed some cells were weakly expressing eGFP but strongly expressed glycine or vice versa when visualized by confocal laser scanning microscopy, did not imply that the eGFP or glycine expression was completely absent in them, the reason being that the expression might still be there but may have overwhelmed by the strong expression of
one or the other channel and therefore not visible at a glance. Hence to measure the degree GFP-Glycine overlap we used a software called Image Pro, which allowed us to manually map out the eGFP positive cells in the lamina VII by drawing a trace around the cell and then objectively measure the intensities for eGFP and glycine (Figure 6).

Image-Pro software measures the highest pixel intensity of multiple channels of the traced cell derived from a three dimensional image. After identifying GFP positive cells and drawing regions of interest around the cells using one of the drawing tools in an Image Pro, we limited the intensity measurement to those particular regions. Background intensities were then measured by separately drawing regions of interest in the neuropil to decide on the cut-off point for both eGFP and glycine channels for every single image analyzed. Intensities of as many GFP positive cells as could be found in lamina VII in each image were manually traced and measured. In addition, five background intensities for EGFP–Glycine overlay were measured by tracing the background signal for every single image. The average of background intensities of eGFP and glycine were then calculated separately and a 10% cut off point intensity was selected, i.e. a cell expressing 10% higher signal than the background cut off point was counted as a positive cell and cells with fluorescent intensity within or below the 10% background cut off point were discarded from the final result count for each channel. The rationale for selecting a 10% cut off point for both the channels was due to the higher background signal for glycine in comparison to the actual glycine positive cells. It was noticed that by raising the cutoff point to more than 10%, we were discarding some actual glycine positive cells labeled in the lamina VII of the spinal cord. Figure 6 is an example of this analysis showing five
Figure 5: Demonstration of immunoreactivity of an antibody against glycine in a transgenic GlyT2-eGFP mouse spinal cord. A) View of a transverse section of L4 ventral horn showing eGFP expression (green) in a GlyT2-eGFP mouse. The arrow labeled in yellow points at a cell which is eGFP positive but was found to be negative for glycine IR. The blue arrows are pointed towards the cells which are IR for glycine, but lacked in expressing eGFP. The white arrows represent eGFP positive neurons which are also IR for glycine. B) Transverse section of L4 ventral immunostained with an antibody against glycine (red) in a GlyT2-eGFP mouse. C) Overlay of A and B showing co-localization of eGFP (green)-Glycine (red) in neuron somata and dendrites. The white arrows represent the eGFP positive neurons which are also IR for glycine.
Figure 6: Representation of eGFP-Glycine overlay analysis in a GlyT2-eGFP mouse. A) Regions of interest (numbered 1-5) drawn around strong eGFP expressing cells (green). Letter B represents similar sized regions which were manually mapped to measure GFP and glycine signal intensity in background to decide the cutoff point. B) View of eGFP-Glycine expressing neurons showing almost perfect overlap. The cells 1, 2 and 3 were eGFP expressing cells which were also strongly IR for glycine. Cells 4 and 5 were observed to be eGFP positive but were weakly labeled with glycine.
eGFP positive cells mapped manually (figure 6A) using a drawing tool to measure the eGFP and Glycine intensities and three background regions mapped to work out the 10% cut off point. After the analysis, cells 1, 2 and 3 were counted as eGFP-Glycine positive cells (figure 6B). Due to glycine intensity below the cutoff point, cells 4 and 5 (figure 6B) were counted as glycine negative cells.

Data from a total of four animals for E17.5 and a total of five animals for P0/P1 were collected and are displayed in Tables 2 and 3. Several sections from each animal were analyzed and averaged. Then the total average values (±SEM) from E17.5 (n=4) and P0/1 (n=5) were plotted into graphs (Figures 7 and 8). These results showed that at E17.5, 90.7% eGFP positive cells were also positive for glycine and 98.1% of glycine positive cells were eGFP positive (Figure 7). At P0/P1, 90.3% of eGFP positive cells were glycine positive and 98.9% glycine positive cells were also eGFP positive (Figure 8).
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Figure 7

Percent (%) GFP-Glycine overlay

E17.5 Glycine also GFP+  E17.5 GFP also Glycine+
Figure 7: Graphical representation of the percent eGFP-Glycine overlay at E17.5 in the lamina VII of a GlyT2-eGFP mouse model. The green bar shows the percentage of eGFP expressing cells which were also found to be positive for glycine. The percent GFP-Glycine overlap was analyzed manually, by identifying GFP positive cells and then measuring the intensity of the glycine channel using Image-Pro software. Several sections from a single animal were averaged and then average values (±SEM) from E17.5 (n=4) is plotted in this graph. The red bar represents the percentage of all the glycine positive cells which were observed to be strongly labeled with GFP.
Figure 8

P0/P1, GFP also Glycine

Percent (%) GFP-Glycine overlap
Figure 8: Graphical representation of the percent eGFP-Glycine overlay at P0/P1 in the lamina VII of a GlyT2-eGFP mouse model. The percent GFP-Glycine overlap was analyzed manually, by identifying GFP positive cells and then measuring the intensity of the glycine channel using Image-Pro software. Several sections from a single animal were averaged and then average values (±SEM) from P0/1 (n=5) is plotted in this graph. The green bar represents the percentage of all GFP positive cells which were observed to be strongly labeled with glycine. The red bar represents the percentage of all the glycine positive cells which were observed to be strongly labeled with GFP.
These results confirmed that the BAC-transgenic mice model expresses intense and stable green fluorescent protein (eGFP) under the control of the GlyT2 promoter, specifically in glycineric neurons and can help to identify numerous eGFP-expressing neurons throughout the spinal cord at as early as E17.5 time point. Further it was observed that the EGFP-Glycine expression and distribution was consistent at both the time points making this model reliable for use in future experiments.
Aim 2: To identify and measure in a quantitative manner, immunohistochemically defined IaINs in the ventral horn of the spinal cord of early postnatal mice.

In this study, Ia inhibitory interneurons (IaINs) were identified using immunohistochemical markers. Having validated the GlyT2-eGFP mouse model as faithfully expressing GFP in glycinergic neurons, we were able to use this model in our analysis of IaINs. Our criteria for identification of IaINs included: eGFP expression in neurons receiving synaptic contacts from CB-IR Renshaw cell axons as well as from Ia afferents. The relative abundance of IaINs among all glycinergic neurons located in the ventral horn is unknown, and estimating the number of IaINs among all GlyT2-eGFP neurons will set the stage for future studies investigating the properties of these neurons. Because these future functional studies will focus on properties of IaINs in postnatal animals, we made our analysis of IaINs in lumbar spinal cords from P0/P1 mice. Numbers of IaINs were quantified by manual analysis of confocal image stacks.

An important identifier an IaIN is that these interneurons are contacted by Ia primary afferents. After testing several anatomical methods to label primary afferents in postnatal mice, we used retrograde tracing with Rhodamine-conjugated (RHOD) dextran from the lumbar 4 dorsal root to label primary afferents in early postnatal spinal cords. Several attempts were made and the four experiments that showed the greatest degree of primary afferent labeling were selected to carry out further analysis. In order to enhance the signal in primary afferents after fixation following retrograde transport, the RHOD signal was further amplified using an antibody against RHOD. Initially, we wanted to combine labeling of primary afferent axons with a specific presynaptic marker indicating
the presence of a synapse. For this purpose, we attempted to use an antibody against vesicular glutamate transporter isoform 1 (VGLUT1). VGLUT1-IR has been proved to be a specific marker of sensory afferent terminals in postnatal and developing mice in previous studies. Interestingly, VGLUT1-IR in our study was found to be noticeably weaker at P0/P1 than at later postnatal stages (data not shown), probably because the protein has not been sufficiently enriched in synaptic terminals at that stage. Consequently, we labeled primary afferent terminals only with RHOD dextran.

As mentioned before, a classic property of IaINs is direct inhibition from RCs (Hultborn et al., 1971; Todd et al., 2003; Alvarez et al., 2004) Anatomical studies have found that some INs in the ventral horn receive a dense innervation on the cell body and proximal dendrites from Renshaw cells (labeled with calbindin antibodies). In parallel with electrophysiological criteria (Alvarez & Fyffe, 2007) these INs were identified as IaINs (Alvarez et al. 2005; Siembab et al., 2010). In neonatal mice, RCs can be uniquely identified by high expression levels of the calcium binding protein, calbindin (CB) (Geiman et al., 2000). Synaptic terminals of RCs on MNs and spinal interneurons are first observed at E15.5 and are abundant by E17.5 (Siembab-Neff, 2009).

Figure 9 shows a low magnification (20X) image of the ventral horn of the spinal cord taken using a confocal microscope. Retrograde tracing of dorsal root L4 afferents produced dense primary afferent labeling in the ventral horn of the postnatal P0/P1 spinal cord. As shown earlier in this study, there was a clear band of eGFP labeled cells in lamina VII surrounding IX (Figure 9). The primary afferent labeling clearly showed
axons approaching and making contacts with different neurons in the ventral horn of the spinal cord including MNs (Figure 9). CB-IR was also very strong in some interneurons.

Viewed at higher magnification (60X), eGFP expressing cells in the ventral horn of the spinal cord could be divided into several categories. Some eGFP positive neurons received clear contacts from RHOD-IR primary afferents, but did not receive contacts from CB positive terminals (Figure 10D). Others received CB-IR contacts, but not primary afferent contacts (Figure 11D). Some received both types of contacts (Figure 12D), and still others received no discernible contacts from primary afferents or CB-IR fibers (Figure 13).
Figure 9: Illustration of retrograde labeling of primary afferents at low magnification in a GlyT2-eGFP mouse spinal cord. A) Confocal image of a transverse section of L4 showing brightly labeled eGFP cells in the ventral horn of the spinal cord. B) Primary afferent projections approaching from dorsal into the ventral horn of the spinal cord. C) Brightly labeled calbindin contacts (puncta) and strongly labeled calbindin cell somata shown in a confocal image of a transverse section of L4 in the ventral horn of the spinal cord. D) Merge view of a confocal image showing eGFP positive cells (green), primary afferent labeling (red), and calbindin immunoreactivity (blue) in the ventral horn of the spinal cord.
Figure 10
Figure 10: Demonstration of retrograde labeling of primary afferents at higher magnification in lamina VII of a GlyT2-eGFP mouse spinal cord. A) 60X confocal image of brightly labeled eGFP neurons. B) Rhodamine-dextran labeled primary afferents viewed at 60X. C) Merged view at 60X of eGFP positive cells (green) receiving primary afferents contacts (puncta in red) surrounding the cells. D) Zoomed view of a single eGFP positive cell. The arrows point towards the primary afferent contacts at the cell surface.
Figure 11: Illustration of calbindin IR at a higher magnification in the lamina VII of a GlyT2-eGFP mouse spinal cord. A) 60X confocal image of brightly labeled eGFP neurons. B) Image of strongly labeled calbindin contacts (puncta) and some neurons that are strongly positive for calbindin in the cell soma viewed at 60X. C) Merged view at 60X of eGFP positive cells (green) receiving calbindin contacts (puncta in magenta) surrounding the cells. D) Zoomed view of a single eGFP positive cell. The arrows point towards the calbindin contacts along the cell surface.
Figure 12: Illustration of identification of IaINs in the lamina VII of the ventral horn of a GlyT2-eGFP mouse spinal cord. A) A single eGFP positive cell imaged at 60X. B) Image showing retrograde labeled afferents (red) making contacts with the eGFP positive cell. The arrows point to primary afferent contacts surrounding the cell. C) The same eGFP positive cell was observed to receive calbindin contacts. Blue arrowheads point to calbindin contacts (magenta puncta) found surrounding the cell. D) Merged view representing eGFP positive cells (green) receiving primary afferent contacts (red) and strong calbindin contacts (magenta puncta). Arrows and arrowheads as in B and C.
Quantitative analysis of these cell populations was carried out by taking several overlapping 60X images from a single section and reassembling the images to create a high magnification montage of the area surrounding lamina IX using CorelDraw software. A lower magnification image (20X) of the same area was used as a guide in assembling the montage (Figure 13). Once the pool was mapped out in CorelDraw, each GFP positive cell in lamina VII of the individual 60X images was analyzed for the above mentioned criteria to identify and characterize IaINs. This was performed with the original three dimensional image stacks using Imaris software and required analysis of possible synaptic contacts on a given neuron in all the individual optical sections in an image. Several sections from total of four animals at P0/P1 (n=4) were analyzed and data for the different types of neurons identified is presented in Table 3. After averaging the data from different sections of the same animal, total average values (±SEM) for P0/P1 (n=4) were calculated (Table 4) and the percent count of different neurons including IaIN was plotted into a graph (Figure 14).

Our results show that on average, , 16 percent of eGFP expressing cells in the lamina VII of ventral horn of the spinal cord did not receive any primary afferent contacts or RC inputs and were characterized as GFP-only neurons. eGFP expressing cells receiving only inputs from RHOD-IR primary afferents accounted for 22 percent of the total population. In contrast, more than one-fourth of the total population (29%) of eGFP positive cells received brightly labeled CB-IR RC contacts. IaINs were carefully identified as eGFP expressing cells receiving inputs from RHOD-IR primary afferents.
Figure 13: Illustration of technique used to carry out the quantitative analysis of IaINs found in lamina VII of a GlyT2-eGFP mouse. A) Merged 20X confocal image showing the retrograde labeled primary afferents (red), eGFP positive cells (green), and calbindin signals. Some cells strongly expressing calbindin can also be viewed in this image. B) Montage of several 60X images assembled in CorelDraw using the 20X confocal image of the same section as a guiding tool. This map was further used to locate different subsets of cells including IaINs and measure them in a quantitative manner.
Figure 14: Graphical representation identified subsets of eGFP neurons found in lamina VII of GlyT2-eGFP P0/P1 mice. eGFP expressing cells in lamina VII were divided into several groups based on contact status from primary afferents and calbindin IR terminals. Average values for each group (±SEM) derived from P0/1 animals (n=4) are plotted in this graph.
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and CB-IR RC inputs measured in quantitative manner and accounted for 17 percent of
the glycinergic neurons in lamina VII. Lastly, there was a subset of eGFP positive
neurons (16%) that were observed to be strongly labeled with Calbindin in their soma but
lacked primary afferent contacts (Table 4). After careful consideration, we came to a
conclusion that they do meet the criteria of being a RC hence might account for a subset
of RCs located in lamina VII of a spinal cord. Altogether, our study proved that GlyT2-
eGFP transgenic mice model can be successfully used to identify and quantify the IaINs
at as early as postnatal day 0.
Conclusion:

Our analysis has shown that GlyT2-eGFP mice model is a most useful and powerful tool for immunohistochemically labeling glycinergic neurons at early developmental stages like embryonic day 17.5, postnatal day 0 and day 1.

Based on the consistency of eGFP-Glycine overlay results between E17.5 and P0/P1, we can suggest that the model is as accurate in identifying IaINs at E17.5 as it is for postnatal day 0 and 1. Thus we are confident we can reliably use the GlyT2-eGFP model in our future experiments.
VI. DISCUSSION

Our results suggest that BAC-GlyT2-EGFP mice model allows labeling of more than 98% of glycinergic neurons by eGFP-Glycine overlay at late embryonic E17.5 and postnatal P0/P1 stage. Further our results confirmed that IaINs are found in the lamina VII, adjacent to lamina IX and suggests that IaINs accounts for 17% of the total eGFP expressing cell population in the lamina VII of ventral horn of postnatal mice spinal cord.

Previous studies have utilized the BAC-GlyT2-eGFP transgenic mouse model for studying glycinergic neurons. One study concluded that the “vast majority of either glycine or eGFP positive neurons were doubly labeled” for GFP and glycine (Zeilhofer et al., 2005). A second study used the model as a part of their study to show that putative glycinergic, GABAergic, and glutamatergic commissural interneurons are expressed in almost equal numbers in mice spinal cord (Restrepo et al., 2009). Restrepo and colleagues did not justify exactly how efficient in quantitative manner the BAC-eGFP-GlyT2 model was in identifying glycinergic neurons at P0/P1. Quantitative answers to several questions were not provided in these studies. Were there any false positive or false negative labeled cells? False positives are defined as cells which were eGFP positive but not glycine positive. In contrast, false negatives are defined as cells which were seen to be glycine positive but not eGFP positive. Thus it was important to carry out this study to determine quantitatively how faithful the GFP expression replicated the distribution of glycinergic neurons in the spinal cord. We found that exactly 1.8% and 1.1% of glycine positive cells did not express eGFP in E17.5 and P0/P1 stages.
respectively. These cells can be counted as false negatives. The eGFP may well be expressed by those cells, but because the eGFP intensities in those cells were below the background cut off points they were counted as negative for eGFP. Whereas, we found 9.2% at E17.5 and 9.7% at P0/P1 of eGFP positive cells which did not express glycine, again those can be counted as false positives. The glycine may well be expressed by those 9.2% cells at E17.5 or 9.7% cells at P0/P1, but because the glycine intensities were below the background cut off points they were counted as negative for glycine. This suggests that the BAC-eGFP-GlyT2 mice model either lacked to express eGFP in 1-2% of the glycinergic neurons found in the lamina VII at E17.5 and P0/P1 or those cells were “false negative cells” which were observed to strongly labeled for glycine but weakly (below background cut off point) labeled for eGFP.

The past studies which Zeilhofer and Restropo conducted using BAC-GlyT2-eGFP mice model lacked to show the identification of glycinergic neurons at an embryonic stage E17.5. The eGFP expression is known to be already very high in GlyT2-eGFP mice embryos at E13 or E14, the rationale for using E17.5, P0 and P1 for our studies was based on the Ia afferent to motoneuron connectivity study carried out by Mears and Frank in 1997. Frank observed that axons of Ia afferents were found to be overlapping with the dendrites of motorneurons as early as E15.5, but did not record any sensory motor connections until E17.5 (Mears and Frank 1997). Even at this early stage the Ia afferent connectivity with homonymous motorneurons in mice was shown to be highly specific and remained that way throughout the first postnatal week (Mears & Frank, 1997). But no knowledge of the onset of Ia afferent connectivity to IaINs is gained
in last several years as till date. Several studies using BAC-GlyT2-eGFP mice models have shown to be successfully labeling glycinergic neurons including IaINs in mice (Zeilhofer et al., 2005; Alvarez et al., 2005; Restrepo 2009), but no one has tested the eGFP expression at embryonic stage, hence there was a need for testing and validating the model at embryonic stage before it could be further used for studying early developmental stages of RI circuit.

Our results show that at E17.5, the labeling of glycinergic neurons using BAC-eGFP-GlyT2 is consistent with that shown at P0/P1 (<98%). However, the BAC-eGFP-GlyT2 model lacked labeling 1-2% of glycinergic cells in our study. Again those 1-2% cells can be counted as false positives i.e. the glycine may well be expressed by those 1-2% eGFP cells but the intensity of the glycine channel in those particular cells were below the background cutoff point and hence those cells were counted as negative for glycine. Altogether from our study we can say that the BAC-eGFP-GlyT2 transgenic mouse model is efficient in labeling more than 98% (98.2% at E17.5 & 98.8% at P0/P1) of glycinergic neurons in the spinal cord at as early as embryonic day 17.5.

Past studies have proved that different markers including neuropeptides or calcium-binding proteins like Calbindin expressed in neurons such as GABAergic neurons helps in defining its anatomical organization (Demeulemeester et al., 1991). The generation of BAC-eGFP-GlyT2 transgenic mice, which drives the eGFP expression under the control on GlyT2 promoter, represents additional powerful tool for studying functional role of glycinergic projections in the CNS (Zeilhofer et al., 2005). Although GlyT2 is proved to be a valuable tool for labeling glycinergic neurons in past (Poyatos et
al., 1997; Spike et al., 1997) it immunoreactivity is restricted to only axon terminals, using eGFP-GlyT2 transgenic mice model combined with immunohistochemical immunostaining with Glycine and GlyT2 considerably extends past observations notably by labeling the glycinergic neurons including their soma, dendrites and axon terminals (Zeilhofer et al, 2005).

One of the limitations for using this technique is relying on a protein expression driven under the promoter GlyT2 gene to label the glycinergic neurons. Glycine antibody labels the cell body and dendrites but sometimes there can be variability in the labeling of the neurons depending on the expression of the protein present in one neuron to other. In contrast GlyT2 is a unique gene marker for labeling glycinergic neurons. Ideally immunohistochemical labeling using glycine in combination with GlyT2 would be optimal. An alternative technique that could have been used to confirm the glycinergic nature of GFP+ cells, would have combined with in situ hybridization of mRNA encoding glycine transporter 2 (GlyT2) with GFP expression in tissue slices. (Restrepo et al, 2009). In situ hybridization technique is a powerful method to identify glycinergic neurons as it only labels cell bodies of the neurons that almost certainly use glycine as their neurotransmitter. Due to the limited resources available for our present study the demonstration of this technique was not possible in given time, but it is something our lab is considering for future studies.

Having demonstrated the usefulness of the GlyT2-GFP model, the second focus of this thesis was to map, locate and identify in a quantitative manner the IaINs located in the lamina VII early postnatal stages using BAC-GlyT2-eGFP mice.
Mears and Frank in 1997 using electrophysiology demonstrated that Ia afferent connectivity with motoneurons were observed to be highly specific from embryonic stages (E17.5) till the first postnatal week (Mears & Frank, 1997). More than a decade later using electrophysiology Wang and his team showed that RI circuit is functional even at P0 in mice (Wang et al. 2008). Above mentioned studies suggest that the Ia afferent connectivity to motoneurons is highly specific even at embryonic stage but no one looked into how specific the Ia afferent connectivity with IaINs is? What anatomical or functional change takes place if any during the embryonic development of RI pathway? From our study we have validated that GlyT2-eGFP mice model is an accurate model to study the IaIN location, function and Ia afferent to IaIN connectivity in a late embryonic developmental stage till postnatal day P1.

In mature spinal cords, IaINs were previously identified histologically by Parvalbumin (PV)/ RC expression and proprioceptive inputs, labeled respectively by CB and VGLUT1 (Alvarez et al., 2005). Parvalbumin is calcium binding albumin protein which is found to be expressed in abundance in GABAergic neurons. Identification of RCs can be carried out using calcium binding protein, Calbindin (Geiman et al., 2000). Previous studies have shown that RC and Ia afferent inputs are a unique criteria for identification of interneurons mediating reciprocal inhibition in lumbar spinal cord (Hultborn et al., 1971; Jankowska and Lindstrom, 1972; Alvarez et al., 1997). Ia afferents enter the spinal cord dorsally and make contacts with the IaINs and motoneurons to mediate the process of RI in the ventral horn of the spinal cord (Eccles et al. 1956). This anatomical arrangement gives an opportunity to investigate the location of the Ia
interneurons in relation to the primary afferents which excites them and to other interneurons or motorneurons which they are supposed to inhibit.

In the present study anatomical tracing was carried out on L4 spinal segment by filling dorsal root 4 with RHOD dextran to search for Ia inhibitory interneurons. Characteristics of interneurons in the Ia inhibitory pathway were used as a key marker to identify them in the lamina VII of ventral horn of postnatal GlyT2-eGFP mice. Although studies in past have focused on the identification of IaINs, no one has looked into carefully identifying number of IaINs compare to other neurons in a quantitative way at neonatal stage. This quantification will allow researchers to have an idea of exactly how carefully one needs to look into the lamina VII to identify IaINs via electrophysiological assays. By knowing that out of the total cell population in the lamina VII of the spinal cord at postnatal stages P0 or P1, IaINs accounts for only 17% percent, sheds some light on probability of locating an IaIN in the lamina VII, while doing electrophysiological experiments for measuring their functionality in the RI circuit.

This study is the first to carry out identification of IaINs using GlyT2-eGFP mice model. One advantage using this technique is that we were able to identify the IaINs at postnatal day P0 and P1. The studies in past used PV as a marker to label IaINs in combination with Calbindin and primary afferent inputs in adult mice, and demonstrated that in adults IaINs accounts for 13% of the total V1 derived interneurons in the spinal cord (Alvarez et al, 2005). Our analysis at early postnatal stages shows that approximately 17% of total eGFP expressing cell population found in the lamina VII, dorsal and medial to lamina IX, were found to be IaINs. Therefore one could assume that
the number to IaINs do not change much as the animal develops but instead may be the Ia afferent connections with IaINs strengthens in the circuit making the circuit more specific as the animal develops. One step ahead from here would be identifying a specific protein, enzyme or neuropeptide which is restricted to only IaINs, and would efficiently label all the IaINs found in the ventral horn of the spinal cord.

Together with the identification of IaINs, the GlyT2-eGFP also demonstrated subset of neurons which received bright Calbindin contacts but no primary afferent inputs or strong primary afferents but no Calbindin contacts, those neurons may account for other important and interesting neurons but for now we are not aware of their functions in the lamina VII of postnatal mice spinal cord.

**Future studies:**

For RI circuit to be functionally normal, appropriate Ia afferent connections must be made with specific IaINs and those IaINs must make further contacts with their targeted MNs. Quadriceps (Quad) and Adductors (Add) afferents make connections with specific IaINs in the RI pathway. For example stimulation of Quad muscle group (knee extensors) results in disynaptic inhibition of MNs projecting to posterior biceps / semitendinosus muscles (PBST, knee flexors) (Eccles and Lundberg, 1958). But stimulation of Add muscle group does not inhibit the PBST MNs, even though they both enter the spinal cord via the same dorsal root and terminate in the same area where IaINs are located. This suggests that the subsets of IaINs that project to and inhibit PBST MNs
are contacted by Quad afferents only, and not Add afferents. The RI circuit is known to be functional at P0 (Wang et al., 2008).

For future studies we want to determine the anatomical organization and functionality of RI circuit in the late embryonic stages to see how early in the development of RI circuit are those connections first made and how appropriate are those connections. We would like to test the hypothesis that more inappropriate Ia afferent connections exists prenatally than postnatally in the RI circuit. The study would be carried out using GlyT2-eGFP mice at embryonic stage E17.5, E18.5 and postnatal stages P0 and P1. We would look at the status of afferent connections with IaINs in embryos up till postnatal day P1, by labeling the afferents projections to specific muscles with strategies similar to what used in present study.
VI. REFERENCES


