2014-01-01

Glycine Transporter 2: Expression and Interactions in the Central Nervous System.

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DEDICATION

Dedicated to my Parents and Saurabh, my husband.

GLYCINE TRANSPORTER 2: EXPRESSION AND INTERACTIONS IN THE CENTRAL NERVOUS SYSTEM.

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SHWETA LAVANIA, B.Sc., M.Sc.

THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Biological Science

THE UNIVERSITY OF TEXAS AT EL PASO

August 2014

ACKNOWLEDGEMENTS

There is no greater pursuit than the pursuit of knowledge and I consider myself extremely fortunate to have been given this opportunity to study Biology at the University of Texas at El Paso.

A Graduate degree in Biology has not just provided me the opportunity to seek a career in Science but it has helped me to become a better person. Sometimes, peering inside a living cell, working endless hours in the lab perfecting your skills, mentoring young students etc. can teach you a lot more about yourself than you would ever know.

For all this and so much more, I'd like to begin by thanking my mentor Dr. Manuel Miranda- Arango for giving me a chance to work and to learn. The research at UTEP has always been a multi- disciplinary effort and I am eternally grateful to all the professors who have trained me and guided me all these years.

I shall forever be grateful for all the help and support that the members of the Miranda laboratory, past and present, extended to me during the course of my studies. I cherish the friendship and am grateful to all my friends and colleagues in the Biological sciences.

I would like to express my gratitude towards my family and my husband for their unstinted and tireless support of my academic pursuits. I hope that I get a chance in life to make all my family, the professors and friends at UTEP proud of me and contribute to Biology in some meaningful way.

ABSTRACT

Glycine is an important inhibitory neurotransmitter that is localized in the caudal areas of the nervous system and plays an important role in mediating many important functions related to breathing, spinal reflexes, nociception etc. Termination of neurotransmission is achieved by the re-uptake of glycine back into the pre- synaptic neuron and neighboring glial cells by the two glycine transporters namely Glycine transporter 1 (GlyT1) and Glycine transporter 2 (GlyT2). These transporters play an essential role in regulating glycinergic inhibition in the central nervous system by controlling the duration and intensity of neurotransmission and therefore the efficacy of synaptic inhibition as well.

The GlyTs share 50 % amino acid sequence identity and similar topology; however, unique to GlyT2 is the presence of a 200-amino acid long N-terminal tail which is not present in any other related transporter such as the monoamine or GABA transporters, and its functional aspects have not been explored to date. The importance of this N- terminal domain can be gauged from the fact that many individuals diagnosed with sporadic Hyperekplexia, a neurological disorder characterized by excessive startle response, carry point, nonsense and frameshift mutations along the GlyT2 sequence, including the N-terminal domain (Davies, Chung et al. 2010). The aim of this research is to understand the role of this N-terminal domain of GlyT2 in regulating the function and anchorage of the transporter and to study its expression in the glycine- rich areas of the nervous system like the brainstem, cerebellum and the spinal cord. This is accomplished by performing Yeast Two Hybrid assay in order to identify proteins that could interact with the N- terminal domain of GlyT2. Out of the 74 clones identified in the screen, the first 26 proteins have been identified and are mainly cytoskeletal proteins as well as some involved in signaling cascades, thus hinting at the important role of the N- terminal domain

of GlyT2 in anchoring the protein at the plasma membrane and perhaps, influencing its functioning at the glycinergic synapse.

Although it is well known that GlyT2 is abundant in the spinal cord and caudal regions of the CNS, the location of glycinergic nuclei, axonal projections and connections have not been explored in detail. The efforts to study these neuronal circuits have been limited, due in part, to the essential function of GlyT2 for survival and the lack of reagents such as highly specific antibodies. To map the neuronal pathways containing GlyT2, a transgenic mouse was developed and kindly provided by Dr. H. U. Zeilhofer, University of Erlangen-Nurnberg, Germany (Zeilhofer et al. (2005) J. Comp. Neurol. 482, 123-41) which expresses the green fluorescent protein (GFP) under the control of the GlyT2 promoter. Coronal and sagittal sections of the transgenic mice brains reveal that the majority of GFP signal is localized to the medulla, pons and midbrain. Several nuclei containing GFP are restricted to the Colliculi and several areas of the Pons in the brain stem. Consistent with these findings, we have identified several GFP-, GlyT2- positive neurons in the Central nucleus if the Inferior Colliculus (CIC) and several areas of the brainstem such as the ventral cochlear nuclei (VCA), and several nuclei which make up the Superior Olivary Complex (SOC). The existence of GlyT2 in the areas involved in audition could provide important insights into the importance of glycinergic inhibition in mediating audition and could possible lead to further research in understanding the important sense of audition and treat maladies associated with it.

The work described in this thesis has provided insights into the importance of GlyT2 in mediating glycinergic inhibition and could serve as a foundation to identify glycinergic circuits in the CNS, which can be manipulated by optogenetic techniques to gain a better understanding of glycinergic neurotransmission.

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CHAPTER 1: INTRODUCTION.

1.1: Glycine as a neurotransmitter:

Glycine, a non- essential amino acid is an essential inhibitory neurotransmitter in the central nervous system (CNS). Along with gamma amino butyric acid (GABA), glycine plays an important role in the maturation of the developing nervous system as well. While GABA is ubiquitously expressed in the CNS, glycine is predominately localized in the caudal areas of the nervous system such as brainstem, cerebellum and the spinal cord. During fetal development, both neurotransmitters are excitatory in nature due to the absence of the Cl-/K+ antiport system (Eulenberg V, 2005) (Zafra F, 2005). However, after birth, they resume their inhibitory function with glycine providing immediate inhibition as compared to prolonged inhibitory action by GABA (Eulenburg V, 2010). Glycine plays an important role in regulating a variety of motor and sensory functions such as vision, audition, respiration, and nociception and influencing neural development (Aragón and López-Corcuera 2003; Aragon and Lopez-Corcuera 2005; Coleman, Fischl et al. 2011) In addition to its inhibitory functions, glycine also plays an important role in excitatory glutamatergic neurotransmission. It acts as an obligatory co-agonist for the activation of N-methyl-D-aspartate (NMDA) receptors, which play an important role in synaptic plasticity and learning and memory (Johnson and Ascher, 1987).

1.2 Role of Neurotransmitter Transporters:

Once a neurotransmitter has been released into the synapse, it needs to be cleared away rapidly in order to end the signal and to start a new wave of neurotransmission. This is accomplished by a) the rapid breakdown and recycling of the neurotransmitter into its

components (e.g. breakdown of acetylcholine by acetylcholinesterase) or b) by mediating the clearance and reuptake of the neurotransmitter by a special family of proteins known as 'neurotransmitter transporters'. (Eulenberg V, 2005). These transporters are responsible for the high affinity uptake of neurotransmitter from the synapse back into the pre- synaptic neurons and the neighboring glial cells. Based on their primary structure and the site of action, neurotransmitter transporters are classified into two super-families namely, the plasma membrane transporters and the vesicular membrane transporters. The plasma membrane transporters superfamily can be further divided into two families depending on their ionic dependence: 1) the Na^+/Cl_2^-dependent transporters and 2) the Na^+/ K^+-dependent transporters (Masson, Sagne et al. 1999). Plasma membrane neurotransmitter transporters, as the name suggests, are localized exclusively at the plasma membrane. DAT, SERT, NET, GAT1–4, GlyT1 and GlyT2 neurotransmitters transporters belong to the SLC6 gene family transporters (Gether, Andersen et al. 2006). Several members of the SLC6 transporter family have been implicated in the development of neurological and psychiatric diseases like drug addiction, depression, schizophrenia, hyperekplexia etc. (Gether, Andersen et al. 2006), thus making them attractive targets for the development of novel therapeutic options for the treatment of the above mentioned complications. Some of the well-known members of the SLC6 superfamily of neurotransmitter transporters are mentioned in the illustration below:

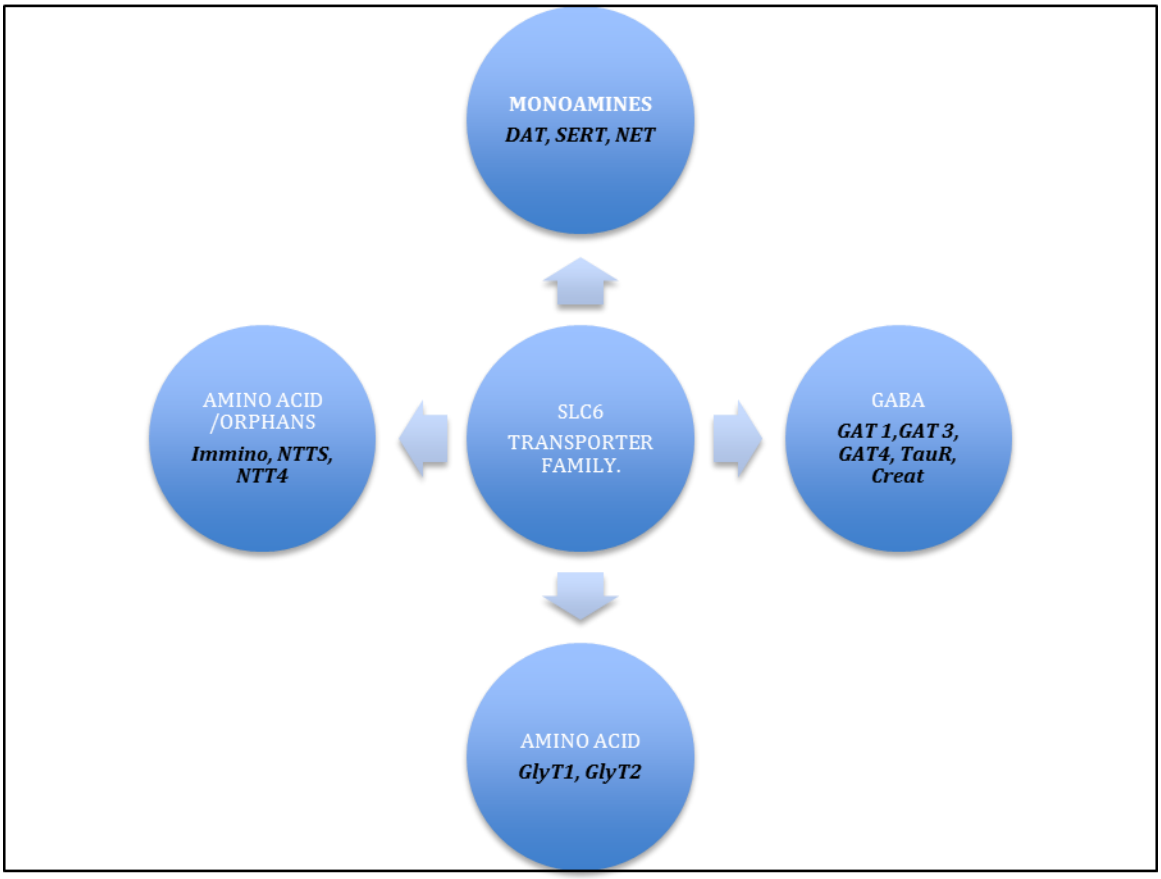

Illustration 1: SLC6 family of Transporters: SLC6 family of transporters is divided into 4 sub families with Glycine transporters 1 and 2 found in the amino acid sub family.

1.3 Glycine transporters:

The intensity and duration of glycinergic neurotransmission is terminated by the re-uptake of glycine back into the presynaptic neuron through the action of two transporters, glycine transporters 1 and 2 (GlyT1 and GlyT2 respectively), encoded by different genes. These high-affinity transporters for glycine belong to the Na^+/Cl^- dependent transporter family SCL6, and share many structural characteristics found across various members of the SCL 6 family. (Aragon C, 2003)

Based on the homology modeling and hydropathy analysis, the GlyTs consists of 12 transmembrane domains, a large glycosylated extracellular loop between TM3 and 4, and intracellular N- and C-terminal tails. Both GlyTs exist in different isoforms: three GlyT2 a, b, and c, which differ in their amino terminal, and five GlyT1 a, b, and c differing in their amino terminal and GlyT1d, and e, with differences in the carboxyl terminal (Eulenburg, Armsen et al. 2005). Both transporters are glycosylated at four asparagine (Asn) residues located in the second large extracellular loop, which triggers the transporter to its cellular location, the plasma membrane (Gether, Andersen et al. 2006). Figure 1 depicts the membrane topology of glycine transporters.

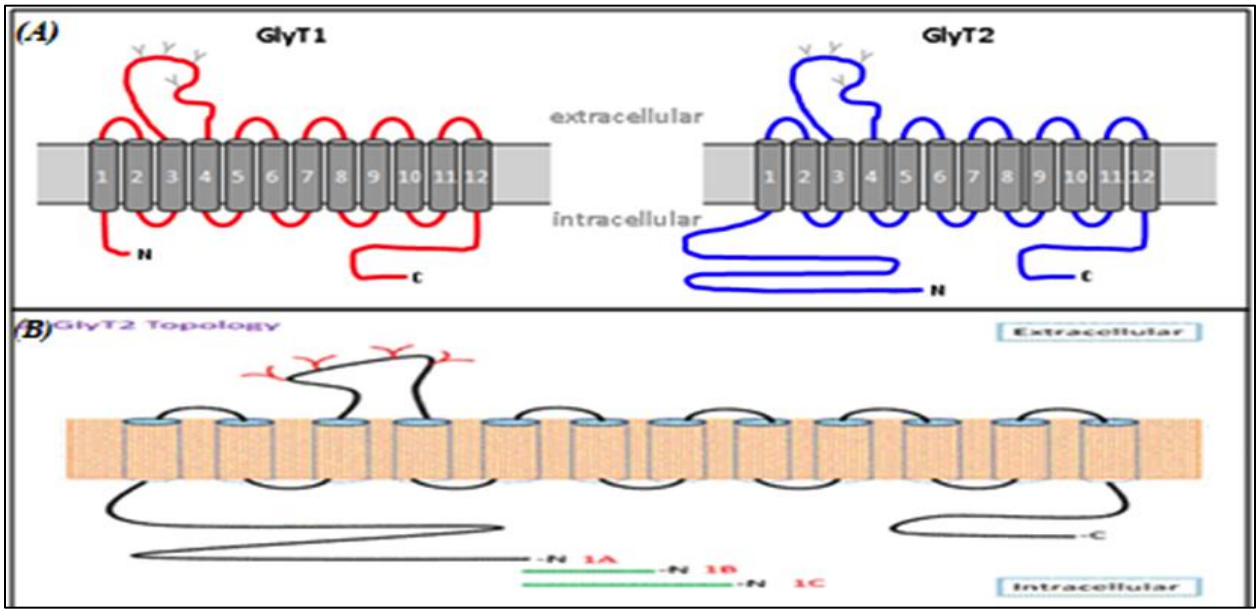

Figure 1: A) Membrane toplogy of Glycine Transporters (GlyTs).
Both transporters show the following common characteristics namely, 12 Transmembrane domains, 6 extra- cellular loops and 5 intracellular loops,
intracellular N & C termini and 4 extracellular glycosylation sites on the second extra- cellular loop.
B) GlyT2 unique aspects: 3 N – terminus isoforms a, b &c have been identifies. The N- terminus shows presence of extra 201 amino acid, long tail.

Although GlyT1 and GlyT2 share a 50% sequence identity, they differ in their N-terminus sequence, with GlyT2 having a 200 amino acid long N-terminus of unknown function (Eulenburg, Armsen et al. 2005).

1.4 Distribution and function of Glycine transporters:

GlyT1 is predominately localized in the glial cells of the brain, with some recent evidence suggesting that it is also expressed neuronally in certain areas of the brain. GlyT2 on the other hand, is exclusively neuronal and hence considered to be a marker for glycinergic synapses. (Eulenberg V, 2005). GlyT1 is predominantly localized in areas such as olfactory bulbs, cortex, hippocampus, septum and thalamus and hindbrain areas such as brainstem, cerebellum and spinal cord where GlyT2 is exclusively found as well. This expression profile indicates that despite the complementary role of both transporters, in removing glycine, there are subtle differences in the way they achieve this goal. GlyT2 is predominantly responsible for glycine uptake at glycinergic synapses, whereas GlyT1 is involved in clearance of glycine from the synapse into adjacent glial cells and monitoring glycine concentration in the vicinity of NMDA receptor expressing synapses.

1.5 Glycine transporter 2:

GlyT2 is found to be expressed exclusively in glycinergic neurons of the brain stem and spinal cord where it is chiefly responsible for mediating the efficient removal of extracellular glycine from the synaptic cleft and its repackaging into the glycinergic synaptic vesicles. Thus

GlyT2 is considered as a marker for glycinergic inhibitory circuits. (Eulenburg, Armsen et al. 2005).

Despite widespread structural homology between GlyT2 and GlyT1 and other members of the Na+/Cl- dependent transporter family, a unique characteristic of the GlyT2 is the presence of a long N- terminal tail consisting of about 201 amino acids, not found in any other neuronal transporter protein. BLAST analysis of the GlyT2 N-terminal domain or shorter amino acid segments against the Swiss-Prot database did not identify any related sequence published to date, thus confirming that this domain is unique to GlyT2 and might play an important role in its function/or structure. (See Figure 2)

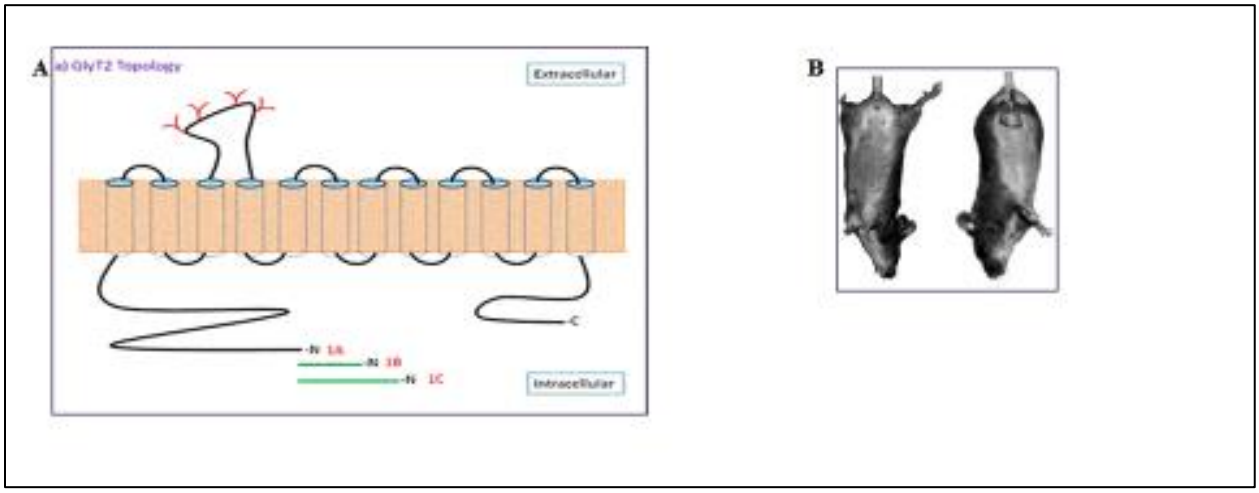

Figure 2: A) GlyT2 topology: The N and C- termini are intracellular, The N- terminus shows an extra 201 amino acid tails.
Differential splicing of GlyT2 gene gives rise to 3 known isoforms- GlyT2 a, GlyT2 b and GlyT2 C.
Mutations in the N- terminus tail amino acids is linked to Sporadic Hyperkplexia in humans.
B) GlyT2 deficient mice show poor growth and reflexes.

The importance of GlyT2 can be validated by the fact that frameshift mutations and micro-deletions along the GlyT2 sequence, including in the N-terminus of the GlyT2 gene results in the development of Hyperekplexia or Startle Disease, a neurological disorder characterized by exaggerated startle response to noise or touch stimuli, non-epileptic seizures, muscle weakness and stiffening (Davies, Chung et al. 2010) (Harvey R, 2008). Deletion of GlyT2 in neonatal mice leads to symptoms like difficulty in breathing, muscle stiffness, and an exaggerated startle response to stimuli followed by death within two weeks (Gomeza. J, 2003). All these features highlight the importance of GlyT2 in maintaining important sensory and motor functions by influencing glycinergic neutransmission. Interestingly, GlyT2 appears to be unique to chordates whereas GlyT1 is present in non-chordates as well, suggesting that GlyT2 is required for regulation of functions that evolved quite later in evolution.

Although GlyT2 structure and function are critical for animal survival and proper neurotransmission, very little effort has been dedicated to understanding its unique aspects or function at the pre-synaptic neuronal plasma membrane.

It has been reported that GlyT2 is predominantly localized in glycinergic neurons which are abundantly found in the caudal areas of the central nervous system like the brainstem, cerebellum and the spinal cord ((Zafra, Aragon et al. 1995). However, there are no studies which conclusively reveal the location of glycinergic cell bodies in the caudal areas of the brain nor is there any mention of the axonal projections of these glycinergic neurons and the connections they make throughout the brain to modulate important sensory and motor functions. Unlike the well-established neuronal projections of the dopaminergic and serotonergic cell bodies in the substantia niagra, ventral tegmental area (VTA) and the brainstem respectively, there seems to be

no mention of a glycinergic circuit and its projections. The efforts to study these glycinergic circuits have been limited by technical issues such as a lack of reagents including highly specific antibodies against glycinergic markers. The identification of these glycinergic circuits in the brain would pave the way in understanding the role of glycine and GlyT2 in mediating important sensory and motor functions like breathing, pain perception, and spinal reflexes.

1.6 Specific Aim of the Research:

The overall aim of this research is to develop a better understanding of GlyT2 and in order to accomplish this objective I have chosen to focus on the following two aspects:

Specific Aim 1: To identify proteins interacting with the N-terminal domain of the GlyT2.: The working hypothesis of this project is that the unusually long tail of N-terminus could play a crucial role in regulating the function and anchorage of the transporter by serving as a binding site for a number of cytoskeletal proteins or proteins involved in signaling pathways. A large number of proteins have been identified that interact with the N-terminus of DAT, NET or SERT including different kinases, receptor and scaffolding proteins either to modulate the activity or regulate their trafficking and degradation (Lee, Kim et al. 2004; Eriksen, Jørgensen et al. 2010; Sager and Torres 2011). Studies have been published that reveal the presence of certain proteins like syntaxin-1 that interact with the carboxy terminus of the GlyT2 and facilitate its localization at the pre-synaptic membrane (Ohno, Koroll et al. 2004; Armsen, Himmel et al. 2007). In contrast, to date, the only protein so far identified and reported to interact with the GlyT2 N-terminal domain is the Ulip6, a member of the collapsin response mediator protein family with unknown function. (Horiuchi, El Far et al. 2000; Horiuchi, Loebrich et al. 2005). Although Ulip6 was shown to co-immunoprecipitate with GlyT2, immunocytochemical analysis failed to show co-localization of Ulip6 with GlyT2 in neuronal primary cultures or shed any light on its possible role in modulating GlyT2 function or anchorage at the presynaptic membrane. Moreover, there seems to be no information about the exact role of Ulip6 in mediating GlyT2 activity.

Hence, the objective of this project is to identify a wide variety of proteins interacting with the N-terminus of GlyT2 which would provide new insights into the role played by

these interacting proteins in the anchorage of the transporter and any possible role in influencing its functioning at the presynaptic membrane.

Specific Aim 2: To identify GlyT2 expression in the glycine- rich areas of the central nervous system and glycinergic cell bodies and their projections: While it has been known that GlyT2 is found exclusively in the presynaptic nerve terminals of glycinergic neurons, there have been no studies done in order to identify the location of these glycinergic nuclei, axonal projections and connections in the brain. The identification of these glycinergic circuits in the brain would pave the way in understanding the role of glycine and GlyT2 in mediating important sensory and motor functions.

Therefore, the objective of this aim is to map the glycinergic circuits in mouse brain, to identify the cell bodies and their projections in the glycine-rich areas of the brain such as the brainstem, cerebellum and the spinal cord and to confirm the localization of GlyT2 in these glycinergic circuits.

The forthcoming chapters elaborate upon the experimental methodology adopted to accomplish these research aims, the results of the experiments, and a detailed discussion about the findings of this research as a conclusion.

CHAPTER 2: RESULTS

2.1: Results for Specific aim 1:

In order to accomplish the first specific aim of the project, I decided to adopt a genetic approach by performing the Yeast Two- Hybrid screening protocol to identify potential interacting partners of the N- terminus tail of GlyT2.

2.1.1 Yeast two hybrid assay: Yeast two-hybrid (Y2H) system provides a sensitive method for detecting relatively weak and transient protein interactions. The Y2H is a sensitive genetic *in vivo* assay that allows for identification of novel interactions from complex libraries and analysis of interaction between known proteins. The assay was developed by Fields and Song (1989) by exploiting the modular nature of the eukaryotic transcription factors, specifically the yeast Gal4 protein and the relative ease in using a simple eukaryote like Saccharomyces *cerevesiae* as a model system.

Principle of Y2H screens:

Briefly, the binding domain is a portion (domain) of the transcription factor that binds a specific sequence of DNA whereas the activation domain (AD) acts as a point of contact for the transcription machinery to attach and initiate transcription. Hence, transcription of a gene downstream is initiated only if the binding domain and the activation domain physically interact with each other. Illustration 2 outlines the basic principle of Yeast Two Hybrid assay.

In a typical Y2H screen, the protein of interest termed as 'bait' is fused with the binding domain of Gal4 while the prey is fused to the activation domain of Gal4 such that the interaction of the bait-prey would result in reconstitution of the transcription factor thus leading to the expression of the reporter genes located downstream. The interaction is monitored by either observing colorimetric reaction with the *lac Z* gene or by the use of auxotrophic markers (usually

essential amino acids tryptophan, leucine, histidine, adenine) that allow the growth of yeast in minimal media. Y2H can be easily automated for high throughput studies of protein interactions on a genome-wide scale be it simple organism like yeast, phage or even complex mammalian genomes.

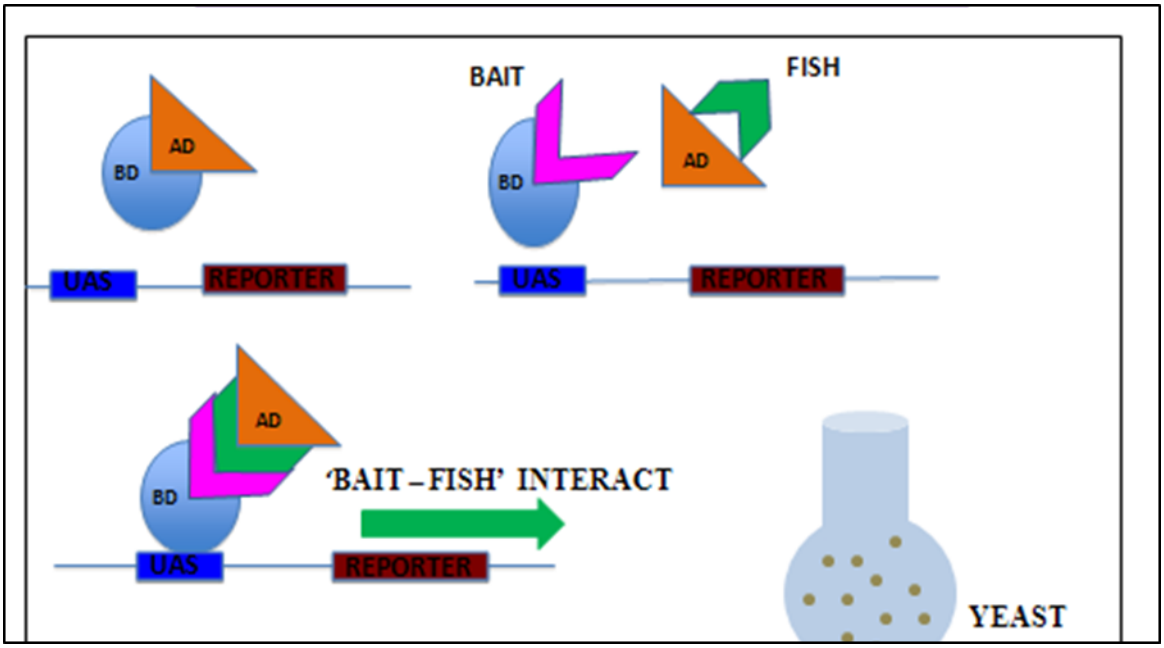

Illustration 2: Yeast Two- Hybrid assay- an overview.
Abbreviations: AD: activation domain, BD: Binding domain.

2.1.2 Methodology:

For the experiments, I used the Y2H system to screen a normalized human brain cDNA library (Clontech Inc.) using the large cytoplasmic N-terminal region of GlyT2 as bait. The DNA sequence encoding the unique 201 amino acids of the amino terminal of GlyT2 (1- 201) was amplified by PCR from a plasmid encoding the cDNA sequence. The amplified fragment was subcloned in frame into the inducible vector pGBKT7 vector and verified by DNA sequencing. The bait, GlyT2 N terminus amino acids 1-201 was used to screen 3 X 10^6 normalized clones of the human brain cDNA library in pGADT7T transformed into the yeast strain AH109.

2.1.3 Control experiments:

Before the actual screening of the library is initiated, it is important check the expression of the bait protein in the yeast and to ensure that the fusion protein isn't toxic to the yeast strain. Accordingly, a series of control experiments were devised to ensure accurate results and to reduce the number of false positives. As a first step, yeast strain AH109 was transformed with the bait vector, pGBKT7 + N-terminus of GlyT2 and the empty vector pGBKT7, both of which contain the C-myc tag. In order to verify the expression of the bait vector a western blot was performed.

Yeast Protein extraction: The colonies of yeast cells transformed with empty bait vector and fusion protein (transformed with empty vector were used as negative controls) were picked from the petri dish and grown overnight in 10 ml of minimal nutrient synthetic media (SD) overnight until an O.D. of 0.6 to 0.8 was reached. The cells are vortexed and transferred to 50 ml Yeast Peptone Dextrose media (YPD) and grown overnight at 30^0C shaking at 220-250 rpm until

the OD reaches 0.4 to 0.6. The cells are transferred to pre-chilled falcon tubes, centrifuged at 1000 X g, washed and the pellet is re-suspended in TE buffer and placed on ice.

The cells were transferred to a new centrifuge tube to which add 500 µl of lysis buffer fortified with protease inhibitor cocktail (cracking buffer) and 300 µl of glass beads and vortexed for 15 minutes. The debris is separated from the pellet by subjecting the centrifuge tubes to centrifugation at 14000 rpm for 5 minutes at 4^0C. The supernatant is transferred to a new centrifuge tube and the process is repeated, if necessary. The protein extract is kept at 4^0C. The protein content of the sample is quantified using the Bradford method of protein estimation. The negative control and the fusion protein extract samples are mixed with loading dye, briefly boiled for 5 minutes and loaded onto an agarose gel, which is run at a 100 V for 3-4 hours in order to separate the proteins. The proteins are transferred to a nitrocellulose membrane, blocked with milk in TBST for an hour and incubated with the C- myc antibody and GlyT2 antibody that recognizes the N- terminus of GlyT2. Shown in Figure 3 is the expression of the bait protein containing the N-terminus of GlyT2 in the yeast protein extract (~37kDa). This transformed strain was then used to screen the normalized human brain cDNA library (Clontech Inc.)

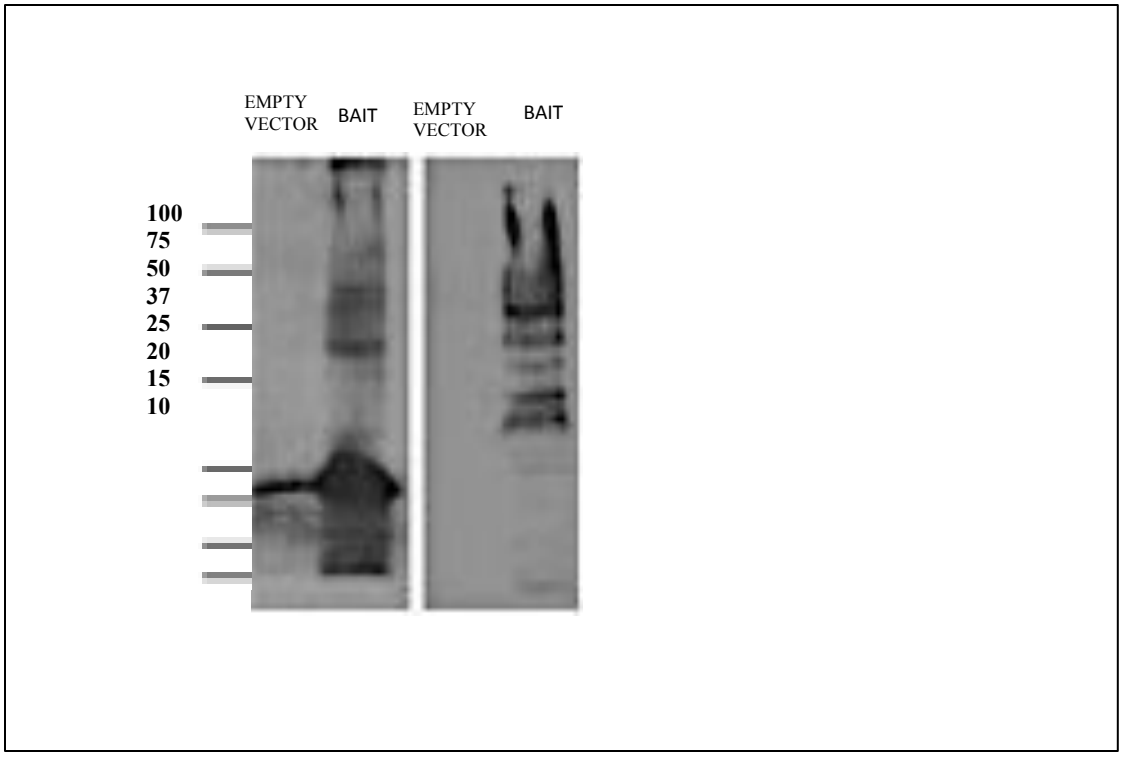

Figure 3: western Blot to detect expression of the bait fusion protein. Yeast strain AH 109 was transformed with a)Plasmid pGBKT7 (Empty vector with C- myc tag) and b) pGBKT753+ N-GlyT2 and the yeast protein extract was used to perform western blot using C-myc and GlyT2 antibodies to check for the expression of the bait fusion protein.

2.1.4 Screening of the cDNA library: As shown in illustration 3, the yeast strain AH 109 is co-transformed with the bait (N-terminus +pGBKT53) along with the prey vector (normalized cDNA of human brain proteins expressed in pGADT7T). The transformed yeast strain was plated on low stringency (-Leu/-Trp) plates and high stringency (-Ade/-leu/-Trp/-His) plates to identify true positive interactions.

Screening Results: Out of the 210 positive colonies obtained on the low stringency (*-Leu/-Trp)* plates, 74 positive clones were obtained on high stringency plates *(-Ade/-His/-Leu/-Trp).* Since the colonies obtained on the high stringency plates have a higher probability of interacting strongly with the N-terminus of GlyT2, we decided to focus on identifying these clones. The colonies were picked and allowed to grow on appropriate SD medium (-Ade/-His/-Leu/-Trp) and the plasmid was extracted by using the yeast plasmid extraction kit (Clontech). Each of the purified plasmid was transformed in competent E.coli bacterial cells and recovered using the Mini- prep plasmid extraction kit (Qiagen). The recovered purified plasmids were sent for sequencing to decipher their identities. The results obtained for clones 1 to 26 are summarized in table 1 on page 21:

Library screening protocol.

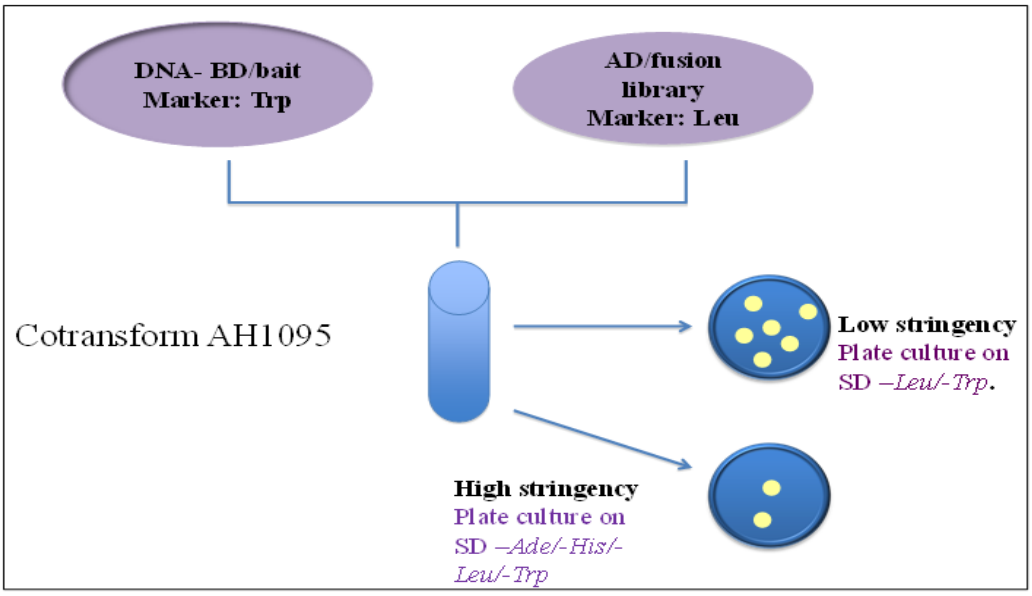

Illustration 3: Yeast Two- Hybrid- Library Screening Protocol- a brief outline.

Table 1. List of proteins identified in the Y2H screen using N- terminus of GlyT2 as the bait for screening normalized adult human brain library (*Clontech Inc.)*

No.	Ascension Number	Description	Repetition of Clones
1.	Unknown proteins		4 (clones: 7, 9, 10, 15)
2.	NM_014756.2	*Homo sapiens* cytoskeleton associated protein 5 (CKAP5).	3 (clones: 6, 11, 13).
3.	NM_016841.4	*Homo sapiens* microtubule-associated protein tau (MAPT).	3 (clones: 3,4, 8)
4.	NM_001143973.1	*Homo sapiens* tectonic family member 3 (TCTN3) isoform.	1(clones:1)
5.	NM_001172818.1	*Homo sapiens* phosphoglucomutase-1 (PGM1).	1 (clone: 12)
6.	NM_020839.2	WD repeat-containing protein 48 isoform 3 Hypothetical protein (*Homo sapiens*).	2 (clones: 2, 14)

7.	NM_032972.1	*Homo sapiens* protocadherin 11- Y (PCDH 11- Y).	1 (clone: 5)
8.	NM_002828.3	*Homo sapiens* protein tyrosine phosphatase, non-receptor type 2 (PTPN2).	1 (clone: 16)
9.	NM_005875.2	Homo sapiens eukaryotic translation initiation factor 1b (eIF1b).	3 (clone: 19, 20, 24)
10.	NM_006837.2	PREDICTED: COP9 signalosome complex subunit 5.	4 (clone: 21, 23, 25, 26)
11.	NM_001100603.1	Homo sapiens KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2 (KDELR2), transcript variant 2, mRNA.	1 (clone: 22)

The plasmids from clones 1 to 26 were recovered from these transformed yeasts by the yeast plasmid recovery kit protocol (Clontech Inc). These recovered plasmids were then transformed in competent E.*coli* bacterial cells (Agilent technologies, US) and the purified DNA was sequenced to reveal their identity (Table 1). A BLAST search has revealed that the 26 clones sequenced so far are mostly cytoskeletal proteins found abundantly in neurons especially in the presynaptic terminals. Out of the 26, the identity of 4 is not known as no matches were found in the BLAST database. The remaining 22 proteins remain promising targets for further studies.

As a further step in checking the validity of our results and to identify clones that grow better (hence indicating better chances of positive interactions), a drop test was performed. Serial dilutions of clones 1 to 24 were prepared at the following concentrations: $10^{1,}$ 10^{0}, 10^{-1}, 10^{-2} and plated (10µls) on the low stringency *(-Leu/-Trp)* and high stringency *(-Ade/-His/-Leu/-*Trp*).* The positive control is the fusion protein of bait pGBKT7 53 and prey pGADT7-T plasmids co-transformed in the yeast AH109 while the negative control is the empty bait vector pGBKT7 53 transformed in yeast AH109. Figure 4 shows the results of drop test for clones 1 to 24. As expected all the clones grew well on the low stringency plates, but only a few clones grew well on both low and high stringency plates (2, 6 and 9 to 16).

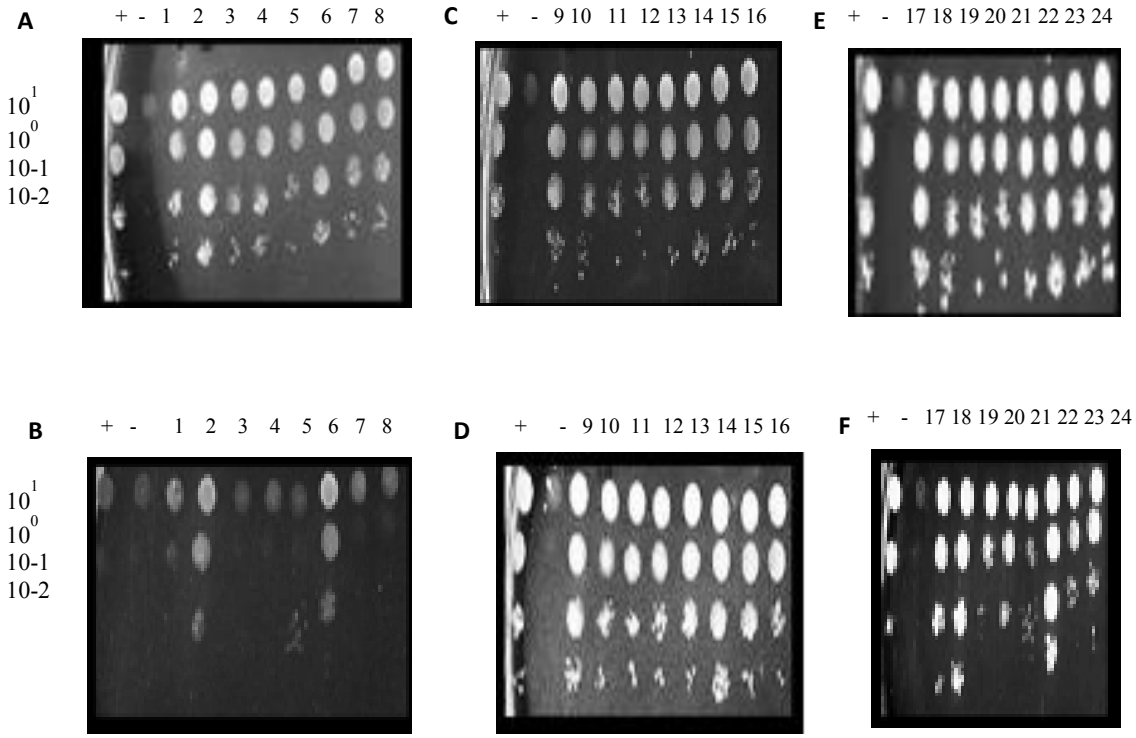

Figure 4: Drop Test: Clones 1 to 24 were diluted at the following dilutions: 10^{1} 10^{0} 10^{-1} 10^{-2} and plated at a volume of 100 µl on Low stringency (- Leu/-His) plates (Panel A, C, E) and High stringency plates (- Ade/ -Leu/-His/-Trp) plates (Panel B, D, F). Positive Control (+) was the fusion protein of the bait and prey vectors pGBKT753 and GADT7T co- transformed in Yeast strain AH109. Negative Control (-) is the empty bait vector. *clones 25 and 26 were not plated due to contamination.

2.2 Results for Specific aim 2:

2.2.1 Identifying the expression of GlyT2 in the CNS using a transgenic mouse model.

As mentioned previously, GlyT2 is exclusively glycinergic and is an excellent marker to identify glycinergic synapses in the CNS. For almost all major neurotransmitters, there exists abundant information about the cell bodies that produce these neurotransmitters as well as the areas to which they send their projections. For example, dopaminergic neurons are found in the substantia niagra whereas serotonergic cell bodies are found in the nucles of Raphe. However, there isn't much information in the literature about the existence of exclusively glycinergic cell bodies or their projections to different areas of the brain. Hence, I decided to address this question as part of my second specific aim that by trying to identify glycinergic cell bodies in the glycine-rich areas of the brain such as the brainstem, cerebellum and the spinal cord using a transgenic mouse model. The information gleaned from this project would be useful in identifying dedicated glycinergic circuit, which could be manipulated and studied in detail using optogenetics and electrophysiological techniques.

2.2.2 Experimental design: A transgenic mouse expressing green fluorescent protein under the control of the GlyT2 promoter was obtained from Dr. H. U. Zeilhofer, University of Erlangen-Nurnberg, Germany (Zeilhofer et al., 2005) and used for the studies described in this section. In addition, a highly specific polyclonal antibody against the N-terminus tail of GlyT2, synthesized and tested for specificity in the Miranda lab was used for all immunostaining experiments.

Immunohistochemistry: For immunohistochemistry, 1 adult transgenic mouse was deeply anesthetized with nembutal (50 mg/kg; i.p.) and perfused through the ascending aorta with 4% paraformaldehyde (pH 7.4). The brain was post-fixed, cryo-protected in sucrose and cut parasaggitally at 30-micrometer section thickness with a sliding microtome (Leica Inc.). Sections were collected in PBS and stored in an antifreeze solution (15% glucose and 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) prior to use. Series of adjacent sections were mounted to observe the GFP expression by observing the tissue under fluorescent microscope (Zeiss Inc.) Also, sections next in series were used for performing Nissls staining, that provides information of the cytoarchitecture of the region and helps locate anatomical landmarks.

2.2.3 Results: Figure 5 represents the first experiment to identify the glycine-rich areas of the CNS. Sagittal sections from different levels show that the majority of GFP signal is localized to the areas in the mid brain and hindbrain: the colliculi- both superior and inferior, pons, suprachiasmatic nucleus, cerebellum and the medulla. Several nuclei containing cell bodies of GFP positive cells are restricted to the colliculi and several areas of the pons.

In the next experiment, sections were stained with DAPI; a nuclear stain to identify the glycinergic cellbodies in these glycine rich areas and Figure 6 shows the presence of glycinergic cell bodies in the brainstem and cerebellum areas.

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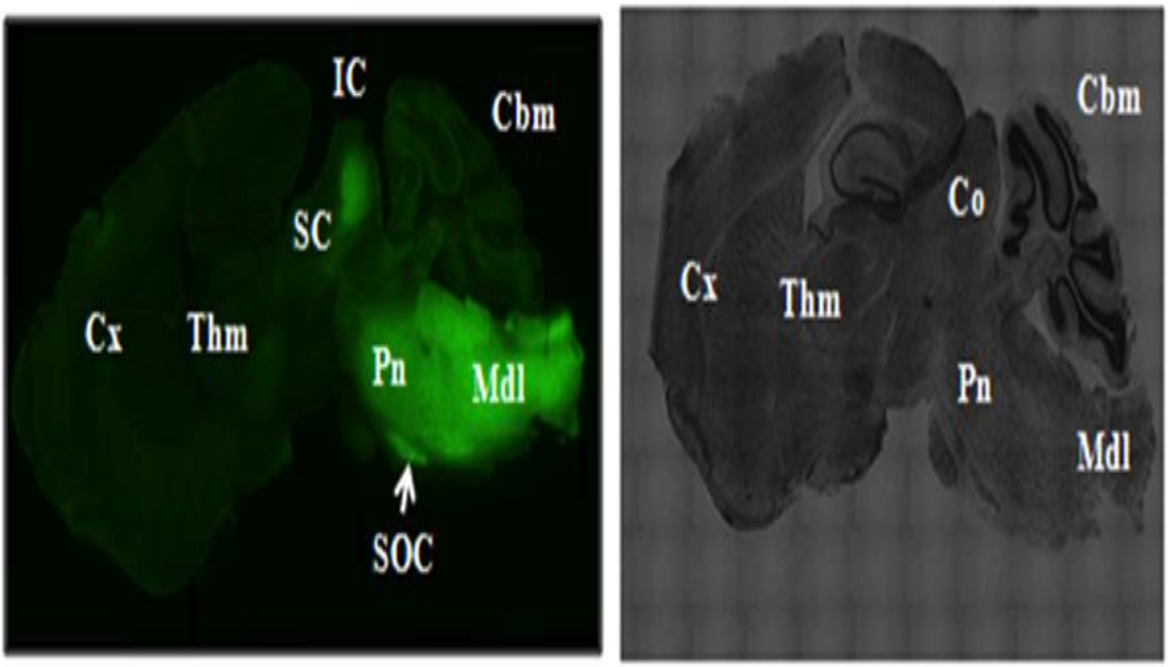

Figure 5: 10X saggital Section 109 (30 µm, paxinos mouse brain atlas 5th Ed.) of eGFP- GlyT2 mouse brain. B: Nissl stain
Abbreviations: Cx: Cortex, Thm: Thalamus, CO: Colliculi, SC: Superior Colliculus,IC: Inferior colliculus, Pn: Pons, SOC: Superior Olivary nucleus complex, Mdl: Medulla, Cbm: Cerebellum.

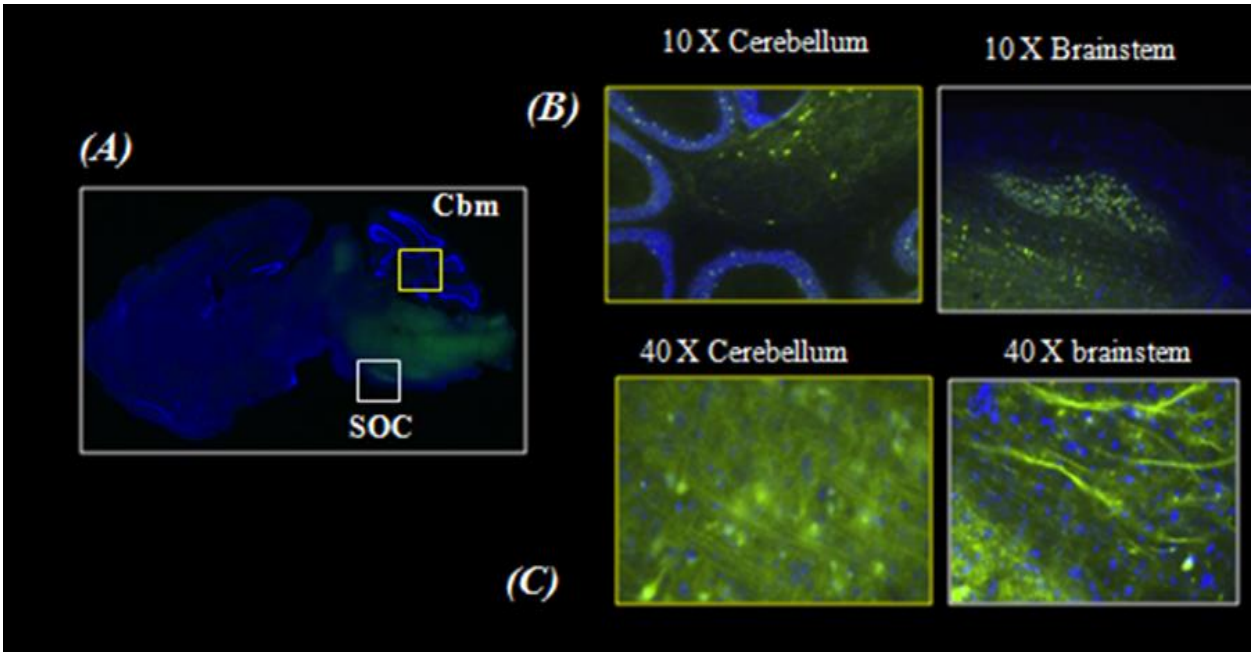

Figure 6. A) 10X saggital section 109 (30 µm) of e-GFP-GlyT2 mouse brain stained with DAPI(nuclear stain).
B) 10X image of cerebellum and Brainstem and Superior Olivary complex.
C) 40X image of Cerebellum Brainstem and Superior Olivary nucleus indicating the presence of glycine- rich (green) areas and cell bodies (blue).
Glycine- rich fibers are visible in the brainstem.
Abbv: Cx: Cortex, SOC: Superior Olivary Complex

From the above experiment, it is pretty clear that there appears to be a rich network of glycinergic fibers that appear to criss-cross the brainstem area along with a large number of cell bodies, which seem to be positive for glycine. While these findings not only confirm the already known truth about abundant glycine in the caudal areas of the nervous system but also open avenues for further research about the presence of glycine in new areas of the brain such as the colliculi as well as numerous cell bodies (GFP-positive) in the pons and the superior olivary complex area.

Following these preliminary studies, I decided to focus on identifying expression of the GlyT2 in these areas. The first step was to optimize the GlyT2 antibody dilution in order to visualize its distribution in areas such as the pons and the inferior colliculus. Optimization experiments, vigorous testing and comparisons with stringent controls such as no primary controls and pre-adsorption studies led to the successful visualizing of GlyT2 staining using the following dilution of antibodies: 1:2000 primary GlyT2 Ab (rabbit) and secondary Ab anti-rabbit 1:10K Alexa 488. In order to visualize neuronal cell bodies, NeuN antibody (mouse) was used, at a dilution of 1:2000 and secondary antibody, Alexa 647 (anti-mouse; dilution?).

Figure 7 shows the presence of cell bodies that test positive for GlyT2 (stained yellow in the merge column). The above experiment was repeated using the same section but incorporating NeuN in order to identify neurons in the inferior colliculus. Figure 8 shows the presence of some cells that test positive for NeuN as well as for GlyT2 indicating that these are glycinergic neurons found in the inferior colliculus area.

The expression of GlyT2 in the inferior colliculus is a novel result and holds significance with exciting possibilities for further studies, which will be elaborated upon in the Discussion section of this thesis.

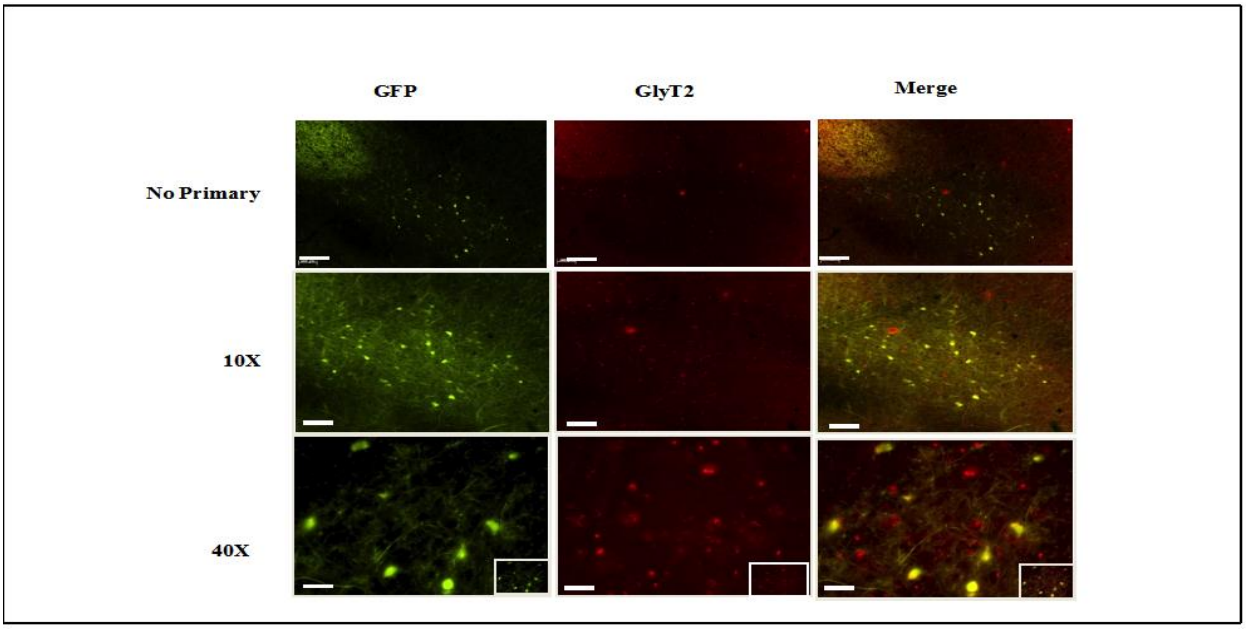

Figure 7: Immunostaining of GlyT2 in the IC region of saggital section 109 (30 µm) e- GFP- GlyT2 transgenic mouse brain.
Top panel: No primary control (Tissue incubated with blocking solution for Primary incubation, secondary antibody: Cy3 1:10,000). Middle panel: Lower magnification, GlyT2 (red) Lower panel: Higher magnification, GlyT2 (red).

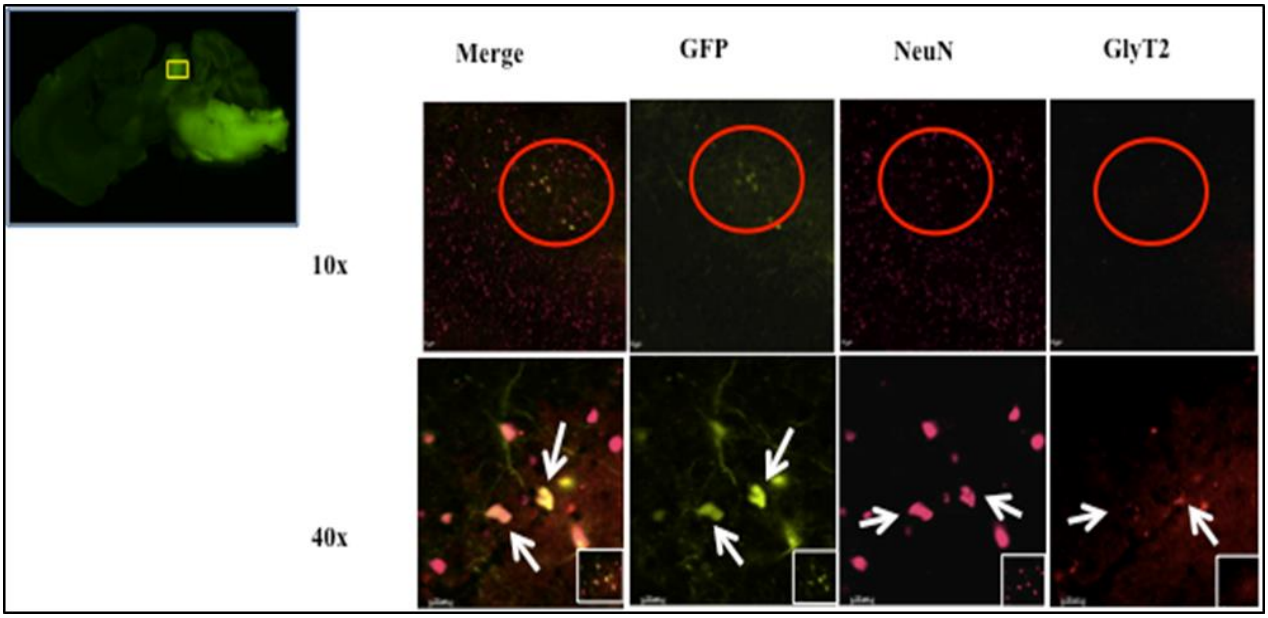

Figure 8: Immunostaining of GlyT2 neurons in the IC region of saggital section 109 (30 µm) e- GFP- GlyT2 transgenic mouse brain.
Panel A & B show lower (10X) and higher (40X) magnification images of the IC neurons (stained with NeuN, pink) staining positive for GlyT2 (Red),
Some of these GlyT2 positive neurons co- localize with glycine- rich cell bodies (yellow).

2.2.4 GlyT2 expression in the brainstem:

Studies performed in rats, guinea pigs, bats and cats have indicated the presence of glycine in the lower auditory areas of the brainstem region such as the superior olivary complex (Aoki E, 1988) (Adam, 1979) (M. Merchána, 2005). It is understood that glycine plays an important role in modulating inhibitory neurotransmission in conjunction with GABA (Coleman JL, 2011). With this knowledge it is reasonable to presume that there is a probability of finding expression of glycine transporters in these auditory nuclei areas as well since almost all of them project to the inferior colliculus where the expression of GlyT2 has already been shown previously in the results. Figure 9 depicts the expression of GlyT2 in the brainstem in a saggital section of the transgenic mouse brain whereas Figure 10 depicts the expression of GlyT2 in the lower auditory nuclei of the brainstem such as the MVPO, LVPO, VCA, and other regions.

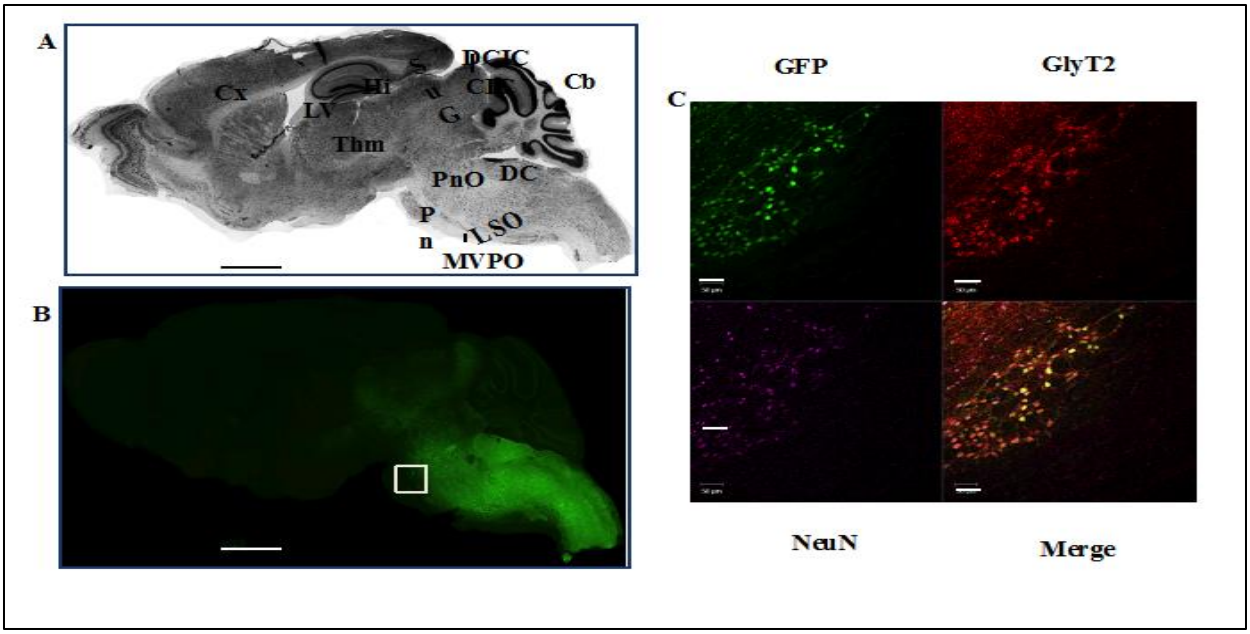

Figure 9: Immunostaining of GlyT2 neurons in the BRAINSTEM region of saggital section 109 (30 µm) e- GFP- GlyT2 transgenic mouse brain.
A) Nissl staining B) GFP fluroscence (green)
C) GlyT2 positive neurons observed in the Pons region of the brainstem. (GlyT2- red, NeuN- Pink, Merge- yellow)

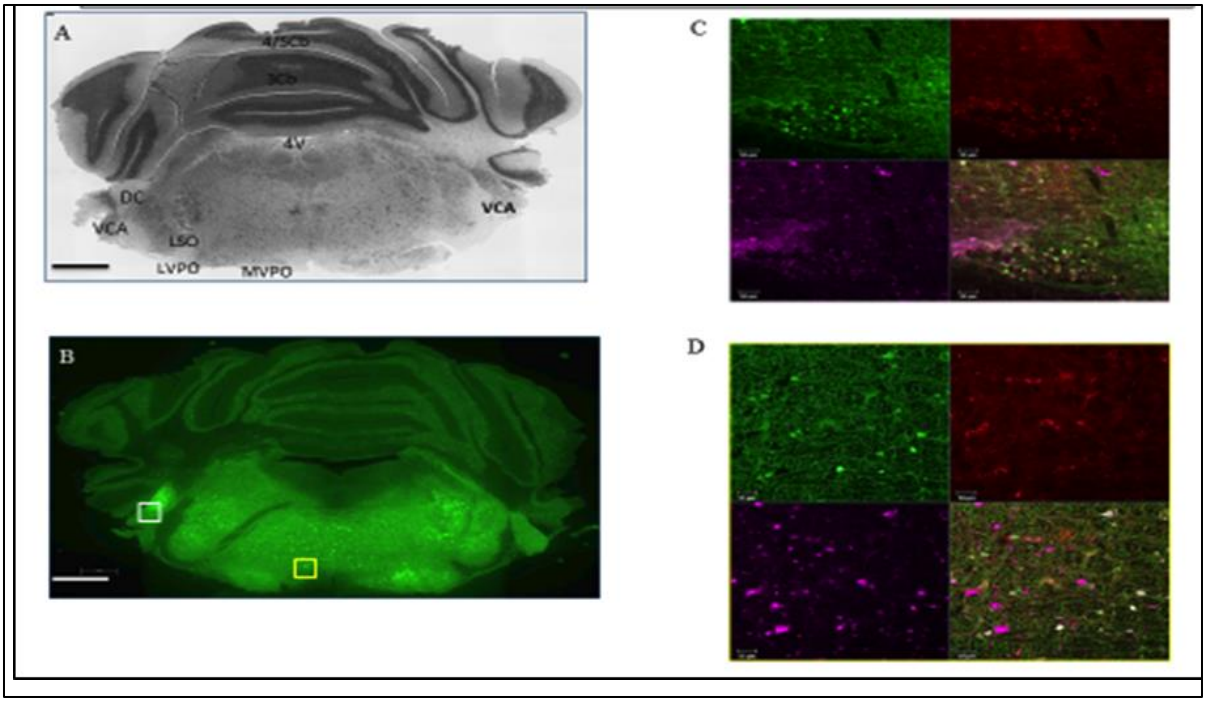

Figure 10: Immunostaining of GlyT2 neurons in the BRAINSTEM region, coronal section 77 (paxinos mouse brain atlas 5th Ed., 30 µm) e- GFP- GlyT2 transgenic mouse brain. A) Nissl staining of section 77. B) GFP (green) signal, C and D) GlyT2 staining (red) visualized in the VCA (white square), MVPO (yellow square). Neurons are stained with NeuN (pink).

Abbv: 4/5 Cb: Cerebellar lobe, DC: Dorsal coclear nucleus, VCA: Ventral Cochlear nucleus, MVPO, LVPO: Medial and lateral periolivary nuclei, LSO: Lateral superior olive, 4V: Fourth ventricle.

As observed in figure 5, there seem to a rich network of putative glycinergic cell bodies and fibers in the brainstem area especially in the pons area as well areas comprising the superior olivary complex such as the lateral periolivary nucleus, medial periolivary nucleus and the ventral periolivary nucleus (LVPO, MVPO, VLPO respectively). Hence, there was a high probability of identifying GlyT2 expression in these areas as well. Figure 9 represents the expression of GlyT2 in the pons as visualized in a saggital section while Figure 10 shows the expression of GlyT2 positive neurons in the cochlear nucleus (Ventral area, VCA) as well as the lateral periolivary nucleus in the coronal section 78.

CHAPTER 3: DISCUSSION

There has been a steady increase in studying glycine as a neurotransmitter and the various components involved in mediating glycinergic neurotransmission. The duality of glycine's role, as an inhibitory neurotransmitter at glycinergic synapses and as a modulator of excitatory glutamatergic neurotransmission mediated by NMDA (N-methyl-D-aspartate) receptors, makes it an interesting target for research and especially for designing therapeutic interventions against a host of ailments such as schizophrenia and alcohol and drug addiction to name a few (Johnson and Ascher, 1987).

The glycine transporters GlyT1and GlyT2 play an important role in regulating the glycinergic neurotransmission by controlling the amount of glycine available at the synapse and by promoting its re-uptake to ensure its repackaging into the synaptic vessels to sustain a new round of inhibition (Eulenberg V, 2005) (Eulenburg V, 2010). There is no surprise, therefore, that both the transporter proteins are linked to a number of disorders that are on the rise today such as schizophrenia, alcohol and drug addiction, hyperekplexia, epilepsy, breathing and muscle disorders. (Harvey R, 2008) (Sager JJ, 2011)

While GlyT1 is definitely better studied of the two transporters, the focus of this research is GlyT2, the lesser known but highly interesting glycinergic transporter that is solely devoted to the task of clearance of glycine from the synapse back into the pre-synaptic neuron to make it available for repackaging into synaptic vesicles. GlyT2 is exclusively neuronal, found abundantly in the caudal areas of the CNS such as brainstem, cerebellum and spinal cord. Its importance can be gauged by the fact GlyT2$^{-/-}$ mice do not survive more than 2 weeks after birth and show poor growth and reflexes, acute respiratory distress, muscle spasticity etc. (Gomeza. J, 2003). Mutations in the GlyT2 gene also give rise to hyperkplexia aka Startle's disease. GlyT2 is

not just implicated in disorders but is an attractive therapeutic target for therapies against chronic pain as well as the development of effective analgesics and anesthetics. (Dohi, 2009) (Masson J, 1999; Coleman JL, 2011) (Davies, Chung et al. 2010).

Because of an ever-increasing interest in GlyT2, it is a highly exciting area of research and presents some unique aspects to study. As highlighted in figures 1 & 2, the presence of a long 201 amino acid tail at the N-terminus, a feature not shared with any other member of the SLC6 family of neurotransmitter transporter (including GlyT1), is the focus of this research. Previous studies done using dopamine, serotonin transporters as well as receptors like AMPA has shown that the N-terminus of these proteins often acts as a binding site for a number of proteins that are implicated in assisting the anchorage of these receptors and transporters at the plasma membrane or proteins that are involved in and are a part of signaling cascade that regulate the assembly, recycling and functions of the transporters. (Eriksen J, 2010) (Lee K, 2004).

Based on these studies, it is reasonable to hypothesize that this long N-terminal tail of GlyT2 could serve as binding site for proteins that can have an impact on the anchorage, distribution and/or recycling of the transporter. In order to test this hypothesis, I performed a yeast two-hybrid assay that is a fast, convenient and fairly reliable technique to identify potential interacting proteins. To accomplish this, a fusion protein of the N-terminal domain of GlyT2 N-terminal expressed in yeast plasmid pGBKT7-53 was constructed and rigorously tested by expressing this fusion protein in the yeast strain AH109 and by performing a western blot to conform its expression in the yeast strain as shown in Figure 3. Once it was ascertained that the fusion protein is abundantly expressed and isn't toxic to the yeast strain, the N-terminus of GlyT2 was used as 'bait' to screen a normalized human brain cDNA library (Clontech Inc.) to

identify potential interactors. The transformed yeast strain was plated on low stringency (-Leu/-Trp) plates and high stringency (-Ade/-leu/-Trp/-His) plates to identify true positive interactions. By this screen, 74 clones were obtained on the high stringency plates and were utilized for identifying the interacting proteins. Only the clones growing on high stringency media were identified (indicating higher degree of true interactions) and studied further. Further, as shown in Figure 4, drop tests were performed for clones 1 to 26 to test the validity of our results and to identify clones that grow better (hence indicating better chances of positive interactions). Once it was confirmed that all the 26 clones are growing on the high stringency SD media, the plasmids from clones 1 to 26 were recovered from these transformed yeasts by the Yeast plasmid recovery kit protocol (Clontech). These recovered plasmids were then transformed in competent E.coli bacterial cells (Agilent technologies, US) and the purified DNA was sequenced to reveal their identity as listed in Table 1.

Out of the 26 clones that were sequenced, most of the potential interactor proteins are cytoskeletal proteins (MAP2, CKAP5, MAP Tau etc), some involved in signaling (PTPN2, Cam Kinase II) to name just a few. This validates the hypothesis that the N-terminus of GlyT2 is an important binding site for a number of proteins. Moreover, pull down assays done in the lab by Dr. Luciana Gentil, a former Research Associate in theMiranda Lab also led to the identification of cytoskeletal proteins such as actin, beta tubulin and NSF (N-ethyly amide sensitive factor, a protein involved in fusion of synaptic vesicle with the plasma membrane), which provides independent corroboration of the yeast two hybrid results. Currently, studies are underway that would confirm these interactions in vitro and in vivo thus providing more insights into the nature of these interactions and their impact on the activity of the transporter. Some of these proteins

could serve as potential therapeutic targets for alleviating some of the GlyT2 associated disorders listed previously.

The second important aspect of this research has been the identification of GlyT2 in glycine rich areas of the brain. As mentioned previously, GlyT2 is exclusively neuronal and hence is a reliable marker for glycinergic neurons. Even though it can be inferred that the expression of GlyT2 would coincide with the presence of glycine, there hasn't been much research done in identifying exclusively glycinergic areas in the CNS. For instance, it is well know that dopaminergic cells are found in substantia nigra and project to areas such as the ventral tegmental area, nucleus accumbens, and striatum. Not much is known about the presence of glycinergic cell bodies or their projections into different regions of the brain. This knowledge could potentially lead to an excellent understanding of disorders associated with glycinergic neurotransmission and pave the way for therapeutic intervention against these complications. The identification of these glycinergic cell bodies could lead to identification of glycinergic circuits that can be amenable to optogenetic manipulations and provide better insights into understanding glycinergic inhibitory neurotransmission.

The use of the transgenic mouse model, expressing GFP under the influence of GlyT2 promoter (eGFP- GlyT2), kindly provided by Dr. H. U. Zeilhofer, University of Erlangen-Nurnberg, Germany (Zeilhofer et al., 2005), gives an excellent understanding of the distribution of GlyT2 in the CNS. The animals were perfused and their brains were sectioned in a sagittal plain, mounted in glycerol to visualize the fluorescence, and alternate sections were used to perform Nissl in order to identify anatomical landmarks and cytoarchitecture of the brain tissue. Figure 5 confirms the expression of glycine in the caudal areas of the nervous system like the brainstem, cerebellum and the spinal cord and interestingly, reveals presence of glycine in the

mid brain areas specifically the colliculi. Subsequently, some of these sections were stained with DAPI (1:1000) in order to localize the glycinergic cell bodies in areas rich in GFP signal (Figure 6) and it is clear that there are abundant glycinergic cell bodies distributed all over the areas of the hind brain such as the cerebellum, pons, various nuclei of the superior olivary complex like the LSO, MVPO, LVPO to name a few. But the presence of a rich network glycinergic fibers and interspersed among them, a few cell bodies in the colliculi is an important discovery that could highlight previously neglected role of glycine in this area.

The inferior colliculus is an important component of the auditory pathway as it receives ascending projections from nearly all brainstem auditory nuclei, and also from the contralateral inferior colliculus. It acts as a relay station and projects to the median geniculate nucleus, which in turn carried information to the auditory cortex (Coleman JL, 2011) (Adam, 1979) (M. Merchána, 2005). The inferior colliculus is the responsible for integrating multi-modal sensory perception and plays an important role in detection of pitch, mediating startle response and vestibulo-ocular reflex etc. Most of the studies about teasing out the role of the inferior colliculus and its projections have been done using animals such as bats, cats and gerbils etc. and there are virtually no studies using mouse models. Presently, there is a renewed interest in the circuitry of the inferior colliculus and studies are being undertaken to decipher the source of auditory hallucinations (hearing noises), which are a vivid feature of Schizophrenia (Shergill SS, 2000). From the previously published work, it is clear that the inferior colliculus is rich in fibers that are mostly GABAergic and that the inhibitory inputs received from the brainstem nuclei to the inferior colliculus are also predominantly GABAergic and glycinergic (M. Merchána, 2005). Further, results shown in figures 5 and 6 highlight the need to study the presence of glycinergic

cell bodies in this area, which could provide some clues about the projections of the glycinergic fibers that are so abundant in this region of the brain.

The immediate goal was to identify whether these IC cell bodies are neuronal or glial and this was accomplished by identifying expression of GlyT2 in this area of the mouse brain. The e-GFP- GlyT2 transgenic animal is an excellent model for these studies but there has been a concern about the leaky expression of GFP in some areas. Hence, in order to overcome the leaky expression of the GFP and to accurately identify GlyT2 positive neurons, a highly specific GlyT2 polyclonal antibody was produced in the lab, which was exhaustively optimized in order to visualize GlyT2 in the brain. Figure 7 shows the presence of a small number of cell bodies that test positive for GlyT2 in the CIC as compared to a no primary control. Some of the populations of cells that don't stain with GlyT2 do stain positively for GAD67 (a marker for GABA, unpublished data from the Miranda Laboratory) thereby highlighting the presence of a mixed population of cells in this region, which is in confirmation with previously published studies.

The above experiment was repeated using the same section but incorporating NeuN, a neuronal marker to confirm the presence of glycinergic neurons in the inferior colliculus. As seen in Figure 8, there exists a distinct population of cell bodies that test positive for NeuN as well as for GlyT2 confirming that these are glycinergic neurons found in the inferior colliculus. This is a novel result and, coupled with the knowledge that there is an abundant network of glycine- rich fibers in this area, it is reasonable to speculate that some of these fibers must be ascending projections from the lower areas of the brainstem auditory nuclei just as there could be a population of fibers that must be projecting from the inferior colliculus to the median geniculate nuclei and to the Auditory cortex. While tracing these projections is beyond the scope of this research, it was our intention to identify the expression of GlyT2- positive in the

brainstem auditory nuclei, which, as mentioned previously, project to the IC. Figure 9 shows the presence of a distinct population of neurons in the pons area, which test positive for GlyT2. Studies done using GAD67 have also indicated the presence of GABAergic neurons in this area (data not shown) thereby confirming what is already known about the presence of GABA in the pons. Figure 10 shows the expression of GlyT2 in the lower auditory nuclei of the brainstem such as the MVPO, LVPO, and VCA etc. as seen in a coronal section of the transgenic mouse brain. Moreover, as observed in the inferior colliculus area, it seems that there is a rich network of glycinergic cell fibers in the brainstem especially in the pons area as well. The possibility of a distinct population of glycinergic neurons amongst these mixed population of glycine/ GABAergic neurons is a novel result and points to the importance of assisting GABA in fine-tuning various aspects of auditory information.

It is interesting to note that this research provides evidence for the existence of GlyT2 positive neurons in not just the inferior colliculus but also in some brainstem nuclei like the cochlear nuclei and the periolivary complex. These findings can pave the way for future neuronal tracing projects that will help identify the target areas of the glycinergic neurons of the inferior colliculus, which can be useful in identifying the important functions associated with such pathways. As mentioned previously, mutations in the GlyT2 can give rise to hyperekplexia characterized by exaggerated startle response to acoustic or visual stimuli and it would be interesting to know if there is any possibility of isolating the glycinergic circuit that contributes to this condition. Preliminary tracing experiments done in this study using the transgenic mouse model (eGFP- GlyT2), using FluroGold as a retrograde tracer, have confirmed the existence of projections between the brainstem auditory nuclei and the IC and also identified the expression of GlyT2 in both the areas (unpublished data, Miranda Lab). Therefore, it would be interesting to

study the role of the transporter in mediating these important auditory functions and to identify it as a potential target for designing therapeutic interventions for a host of complications previously mentioned. Currently, there are a number of fMRI studies being done in human subjects that are trying to understand the role of various auditory nuclei including the IC in contributing to the auditory hallucinations that are a distinct hallmark of schizophrenia and other psychotic conditions.

This research brings to light certain important aspects of GlyT2 and its important role in inhibitory neurotransmission.

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