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Tonotopic alterations in inhibitory input to the medial nucleus of the trapezoid body in a mouse model of Fragile X syndrome

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Abstract

Hyperexcitability and the imbalance of excitation/inhibition are one of the leading causes of abnormal sensory processing in Fragile X syndrome (FXS). The precise timing and distribution of excitation and inhibition is crucial for auditory processing at the level of the auditory brainstem, which is responsible for sound localization ability. Sound localization is one of the sensory abilities disrupted by loss of the Fragile X Mental Retardation 1 (*Fmr1*) gene. Using triple immunofluorescence staining we tested whether there were alterations in the number and size of presynaptic structures for the three primary neurotransmitters (glutamate, glycine and GABA) in the auditory brainstem of *Fmr1* knockout mice. We found decreases in either glycinergic or GABAergic inhibition to the medial nucleus of the trapezoid body (MNTB) specific to the tonotopic location within the nucleus. MNTB is one of the primary inhibitory nuclei in the auditory brainstem and participates in the sound localization process with fast and well-timed inhibition. Thus, a decrease in inhibitory afferents to MNTB neurons should lead to greater inhibitory output to the projections from this nucleus. In contrast, we did not see any other significant alterations in balance of excitation/inhibition in any of the other auditory brainstem nuclei measured, suggesting that the alterations observed in the MNTB are both nucleus and frequency specific. We furthermore show that glycinergic inhibition may be an important contributor to imbalances in excitation and inhibition in FXS and that the auditory brainstem is a useful circuit for testing these imbalances.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ROLE OF AUTHORS AK, EM, MH, ES

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: EM, MH, AK. Acquisition of data: EM. Analysis and interpretation of data: EM, ES. Drafting of the manuscript: EM, AK. Critical revision of the manuscript for important intellectual content: EM, AK, MH, ES. Statistical analysis: EM. Obtained funding: EM, AK, MH. Administrative, technical, and material support: EM, AK, MH, ES. Study supervision: EM, AK.

Keywords

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1. INTRODUCTION

Fragile X syndrome (FXS) is the most common monogenetic form of autism, affecting approximately 1 in 4000 males and 1 in 5000–8000 females (screening for FXS described in Lyons, Kerr, & Mueller, 2015). FXS is caused by the expansion of CGG repeats in the promoter of the *Fmr1* gene resulting in decreased protein translation of the gene product, Fragile X mental retardation protein (FMRP) (Kenneson, Zhang, Hagedorn, & Warren, 2001). FMRP is known to be a regulator of mRNA translation (E. Chen, Sharma, Shi, Agrawal, & Joseph, 2014), and its loss results in an increase in protein translation (Laggerbauer, Ostareck, Keidel, Ostareck-Lederer, & Fischer, 2001). In addition, FMRP regulates translation of mRNA in both dendrites and synapses of neurons (Antar, Afroz, Dictenberg, Carroll, & Bassell, 2004; Christie, Akins, Schwob, & Fallon, 2009). The clinical symptoms of FXS are impaired cognition, hyperactivity, seizures, attention deficits, long and immature dendritic spines, and hypersensitivity to sensory stimuli including auditory stimuli (Berry-Kravis, 2002; R. J. Hagerman & Hagerman, 2002; Irwin et al., 2001; Merenstein et al., 1996; Miller et al., 1999).

FMRP is highly expressed in auditory brainstem cells including the cochlear nucleus, MNTB, VNTB, and LSO (Beebe, Wang, & Kulesza, 2014). Notably, many of the sensory hypersensitivities seen in FXS are auditory in nature. Both patients with FXS and a mouse model of Fragile X show a higher rate of audiogenic seizures (Berry-Kravis, 2002; L. Chen & Toth, 2001), issues during sound localization, and trouble with communication—particularly in noisy environments (auditory processing issues in autism reviewed in Haesen, Boets, & Wagemans, 2011; O'Connor, 2012, FXS specifically in Rotschafer & Razak, 2014). Surprisingly, sound localization deficits have not been rigorously studied in either humans or in a mouse model of FXS. Similar auditory deficits have been seen in other disorders such as central auditory processing disorder (Shamma, 2008) and some forms of age-related hearing loss (Dubno et al., 2008; Goossens, Vercammen, Wouters, & Wieringen, 2016; He, Mills, Ahlstrom, & Dubno, 2008). For these latter conditions, the connection between the symptoms (trouble with sound localization and noisy environment cue disruption) and changes in excitation and inhibition in the sound localization pathway is better understood (Shamma, 2008). The hypothesis of the current study is that the auditory symptoms seen in FXS are due to an overall imbalance of excitatory and inhibitory synaptic inputs in the sound localization circuit located in the auditory brainstem. We will test this hypothesis by examining whether there are anatomical changes to glycinergic, GABAergic and glutamatergic synapses in the auditory brainstem that may underlie physiological changes to these synapses.

The auditory brainstem network is made up of discrete nuclei that are responsible for different aspects of sound stimulus encoding. Sound information that leaves the cochlea

travels down the auditory nerve and projects glutamatergic excitation onto the anteroventral cochlear nucleus (AVCN). The AVCN then transmits this information to several nuclei via strong glutamatergic synapses (Thompson & Schofield, 2000). One of these synapses is known as the calyx of Held and is a crossed projection from the AVCN to the medial nucleus of the trapezoid body (MNTB). Additionally, the AVCN also projects excitatory synapses contralaterally to the ventral nucleus of the trapezoid body (VNTB)(Thompson & Schofield, 2000; Robertson, 1996). The VNTB is known to project inhibitory input to the MNTB to fine-tune neural responses from the AVCN input (Albrecht, Dondzillo, Mayer, Thompson, & Klug, 2014; Kuwabara, DiCaprio, & Zook, 1991). Both the AVCN and MNTB project to the lateral superior olive (LSO); however, AVCN projects glutamatergic excitation from the ipsilateral ear, whereas MNTB contributes fast and well-timed glycinergic inhibition from the contralateral ear (Spangler, Warr, & Henkel, 1985), reviewed in Grothe, Pecka, & McAlpine, 2010; Tollin, 2003. The LSO is a specialized nucleus responsible primarily for processing of interaural level differences (ILDs). These differences are detected via excitation from the ear with higher level (sound pressure) through AVCN glutamatergic synapses and via inhibition from glycinergic synapses (MNTB) of the excitation from the contralateral ear further from the intense sound (Boudreau & Tsuchitani, 1968; Tsuchitani, 1977).

Several anatomical studies of autistic individuals have shown alterations in the size and orientation of brainstem nuclei, specifically a reduction in cell number within MNTB, MSO and LSO (Kulesza & Mangunay, 2008; Kulesza, Lukose, & Stevens, 2011). In an FXS knockout rat model, many brainstem neurons had a smaller soma and in the superior paraolivary nucleus (SPN) there was reduced expression of glutamic acid decarboxylase (GAD67) (Ruby, Falvey, & Kulesza, 2015). In the same study, the authors showed a reduction in number of calretinin positive terminals, which is a marker for calyx axons and terminals in the MNTB (Ruby et al., 2015). Another study in FXS mice showed increased vesicular GABA transporter protein (VGAT) staining in the MNTB compared to controls and smaller cell size in the MNTB and VCN of FXS mice (Rotschafer, Marshak, & Cramer, 2015). These results suggest that there are alterations in the balance of excitatory and inhibitory inputs to the auditory brainstem nuclei in FXS consistent with the hypothesis that hyperexcitability of sensory systems are one of the key components to altered sensory processing in autism and FXS (Rubenstein & Merzenich, 2003). However, none of these studies have investigated whether there are changes in glycinergic inhibition, which is the primary inhibitory neurotransmitter, in the auditory brainstem.

The current study aims to understand how loss of FMRP may change the presynaptic inputs onto auditory brainstem nuclei. We investigated whether there were changes in glutamatergic, GABAergic or glycinergic presynaptic structures in areas of the brainstem known to be important in the sound localization pathway. Specifically, we investigated the AVCN, VNTB, MNTB and the LSO of *Fmr1*^{-/-} mice. To achieve these aims, we performed triple immunofluorescence for vesicular glutamate transporter 2 (VGLUT2- a presynaptic glutamatergic marker), GAD67 (a presynaptic GABAergic marker), and glycine transporter 2 (GlyT2- a presynaptic glycinergic marker). The end goal of this study is to determine if there are anatomical changes to these auditory brainstem nuclei that may underlie and lead to functional changes in this circuit.

2. MATERIALS AND METHODS

All experiments complied with all applicable laws, NIH guidelines, and were approved by the University of Colorado IACUC.

Animals and tissue preparation

All experiments were conducted in C57BL/6J background (wildtype) with the hemizygous male and homozygous female *Fmr1*^{-/-} knockout strain maintained on this background (commercially available (Jackson Laboratory, Bar Harbor, ME), backcrossed for 5 generations, cryopreserved and then backcrossed for three generations). Animals were genotyped regularly using Transnetyx (Cordova, TN). Mice used in this experiment were adults (4 wildtype, 4 knockout) of both sexes (Ding, Sethna, & Wang, 2014) found that there were no differences in behavior, including audiogenic seizures (though in the C57BL/6J background these seizures are only observed around 22 days of age), between male and female *Fmr1*^{-/-} knockout mice on the C57BL/6J background), between 30 and 150 days old (Exact ages are C57BL/6J: 76, 71, 30 and 76 days old, *Fmr1*^{-/-}: 31, 149, 42, 59 days old). Mice were anesthetized with pentobarbital (120 mg/kg bodyweight) and transcardially perfused with ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.76 mM KH₂PO₄, 10 mM Na₂HPO₄ Sigma-Aldrich, St. Louis, MO), followed by perfusion with 4% paraformaldehyde (PFA). Following perfusion, animals were decapitated, brainstems removed and post-fixed in 4% PFA for one hour. Brainstems were then washed (3 × 10 min) in PBS and covered in 4% agarose. Fixed brains were then sliced into 100 µm coronal sections with a Vibratome (Leica VT1000s, Nussloch, Germany) that included, anteroventral cochlear nucleus (AVCN), medial nucleus of the trapezoid body (MNTB), lateral superior olive (LSO), and ventral nucleus of the trapezoid body (VNTB).

Immunofluorescence

Once the brainstem was sliced into roughly 10–20 sections/animal, free-floating slices were blocked in antibody media (AB media: 0.1 M phosphate buffer (PB: 50 mM KH₂PO₄, 150 mM Na₂HPO₄), 150 mM NaCl, 3 mM Triton-X, 1% bovine serum albumin (BSA)) and 5% NGS for one hour. In some animals Fab fragments (Jackson Immunoresearch, Westgrove, PA) were used in this step to increase the specificity of mouse on mouse primary antibody. However, there was no difference seen between sections within genotype containing Fab fragments versus those without and therefore Fab fragment use was discontinued. Following blocking, slices were incubated with primary antibody for GAD67, GlyT2, and VGLUT2 (Table 1, antibody characterization below) diluted in AB media and 1% NGS overnight at 4°C. Slices were then washed (3 × 10 min) in PBS followed by secondary antibody (Table 2, Thermo-Fisher, Waltham, MA) diluted in AB media and 1% NGS incubated for 1 hour at room temperature. Slices were then washed in PB and mounted on glass slides using Fluoromount-G (SouthernBiotech, Cat.-No.: 0100-01, Birmingham, AL) and coverslipped.

Antibody Characterization

Three primary antibodies were used for detection of GlyT2, GAD67 or VGLUT2, listed in Table 1. To visualize these primary antibodies, three complimentary fluorescent-conjugated secondary antibodies were used, listed in Table 2. The GlyT2 primary antibody, 272 003

(SySy, Göttingen, Germany; RRID: AB_2619997) is a rabbit polyclonal antibody to amino acids 1-229 of the rat GlyT2 protein. The staining pattern of this antibody is consistent with GlyT2 staining seen in other publications, of MNTB, AVCN and LSO ((Albrecht et al., 2014) (mouse), (Friauf, Aragon, Löhre, Westenfeller, & Zafra, 1999) (rat), (Mayer, Albrecht, Dondzillo, & Klug, 2014) (gerbil)). Additionally, specificity of this antibody has been characterized in Zafra et al., 1995 where immunoabsorption studies in rat immunocytochemistry were conducted and complete suppression of staining was seen. The primary antibody to GAD67, MAB5406 (Millipore, Temecula, CA; RRID: AB_2278725) is a mouse monoclonal antibody to amino acids 4-101 of the human GAD67 protein. This antibody has been well characterized in many studies ((Heusner, Beutler, Houser, & Palmiter, 2008); where they saw reduced immunostaining in a GAD67 knockout) and shows a similar staining pattern in these nuclei as seen previously (Albrecht et al., 2014). Additionally this antibody has been cited 12 times in the most recent version of the Journal of Comparative Neurology Database (V14). The VGLUT2 primary antibody, AB 2251 (Millipore, Temecula, CA; RRID: AB_1587626) is a guinea pig polyclonal antibody raised against a recombinant GST-tagged peptide (VQESAQDAYSYKDRDDYS), which corresponds to the rat C-terminus of the VGLUT2 protein. Staining seen with this antibody is consistent with VGLUT2 staining of these structures in other publications ((Rotschafer et al., 2015)(mouse), (Cooper & Gillespie, 2011)(rat)).

Imaging

Slides with brainstem slices were imaged using an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan). High-resolution (512×512 pixel), $60\times$ oil (numerical aperture 1.4), Z-stacks were taken through each section in 20 steps. Voxel volume varied between $0.09 \mu\text{m}^3$ (*Fmr1*^{-/-}) and $0.10 \mu\text{m}^3$ (B6) for the AVCN (n.s., $p = 0.27$), $0.07 \mu\text{m}^3$ (*Fmr1*^{-/-}) and $0.08 \mu\text{m}^3$ (B6) for the VNTB (n.s., $p = 0.41$), $0.22 \mu\text{m}^3$ (*Fmr1*^{-/-}) and $0.22 \mu\text{m}^3$ (B6) for the medial MNTB (n.s. $p = 0.87$), $0.21 \mu\text{m}^3$ (*Fmr1*^{-/-}) and $0.20 \mu\text{m}^3$ (B6) for the lateral MNTB (n.s., $p = 0.35$), $0.09 \mu\text{m}^3$ (*Fmr1*^{-/-}) and $0.10 \mu\text{m}^3$ (B6) for the lateral LSO (n.s. $p = 0.20$), and $0.10 \mu\text{m}^3$ (*Fmr1*^{-/-}) and $0.10 \mu\text{m}^3$ (B6) for the medial LSO (n.s. $p = 0.80$)(all values shown are medians, Mann-Whitney U statistical test). The AVCN was characterized by its morphology and was imaged by illuminating the most ventral portion of the cochlear nucleus on each side of the brainstem. There are several cell types within the AVCN, however we did not distinguish between these types. The LSO was characterized by the distinctive S-shape of the nucleus. In order to control for potential frequency specific changes in presynaptic staining in the LSO, images were taken of the most lateral and most medial portions of the LSO. The MNTB was localized based on the internal GlyT2 staining that commonly occurs in this nucleus due to presence of glycine internally to these cells. For medial MNTB, images were taken with the microscope justified to the most medial MNTB cells (with respect to the midline). For lateral MNTB, images were taken with the microscope justified to the most lateral MNTB cells. The VNTB was also characterized by its similar internalization of GlyT2 stain to the MNTB, however they are easily differentiated based on the VNTB's more ventral location. Only a few cells were seen in the VNTB per section and therefore the entirety of the VNTB was measured in each section for each animal. Image files were then saved as .oib files to be further analyzed by a custom MATLAB (The MathWorks, Inc., Natick, MA) toolbox (*imstack* goo.gl/0WhLZK)

developed to analyze these images. Total number of sections for each nucleus/genotype can be seen in Table 3. Representative images are taken at a higher resolution of 1024×1024 pixels with a 2× zoom magnification, and represent only one image in the 20 section Z-stack (Figures 1, 3, 5, 7, 9).

Quantification of images

Confocal image stacks were loaded into imstack using the bioformats toolbox (<http://downloads.openmicroscopy.org/bio-formats/5.2.4/>). Z-stacks for each channel were processed independently. To reduce background noise and eliminate uneven illumination, we performed a median and top-hat filter. We next used Otsu's method (Otsu, 1979) to calculate a threshold value for the entire stack. Due to the staining differences among the various nuclei type (e.g. Calyx of Held) that we examined, the threshold value was multiplied by a factor (eg. 0.5, 1) to ensure that the threshold intensity value fell above the background signal but below the intensity values for visually identified "presynaptic structures", thus maximizing the signal-to-noise ratio. For any given nucleus type, the same factor was used across genotypes. The image stack was then binarized using this adjusted threshold value. In this new, binarized stack, voxels with intensity values above the adjusted threshold value had a value of 1 and were considered to contain signal for GAD67, GlyT2, and VGLUT2 signal in the red, green, and blue channels, respectively. All other voxels in the binarized stack had a value of 0. For each channel, positive voxels throughout the z-stack were clustered into three-dimensional connected-components (contiguous groups of voxels) using a neighborhood connectivity of 26. A 26-connected voxel is a neighbor to other voxels that touches one its faces, edges, or corners. We then used the MATLAB regionprops function to calculate the volumes and centroid for each connected-component. We next calculated the mean volume + one standard deviation of all connected-components and used this value as our structure cut-off. In order to exclude any axons from our analysis, connected-components with volumes larger than the cut-off were eliminated while the remaining connected-components (with volumes falling below this cut-off) were considered to represent presynaptic structures. Thresholded images are included in each image figure (panels G–H) to illustrate the quantification of the images.

Statistical Analysis

Graphs were generated using Python (Python Software Foundation. Python Language Reference, version 2.7. Available at <http://www.python.org>, packages: Matplotlib (Hunter, 2007), Pandas (McKinney, 2010) and R for additional statistical analyses (R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>). A mixed effects model was used to account for repeat observations within one animal (lme4 package in R (Bates, Mächler, Bolker, & Walker, 2014)) with genotype as a fixed effect and animal as a random effect. Summary statistics for number and size of presynaptic structures is quantified in Tables 4 and 5. A Holm's correction was then applied to the p-values to account for multiple comparisons within each nucleus. An index of the ratio of excitation to inhibition (I_{sp} or Index of synaptic protein, see Rotschafer et al., 2015) was calculated using the value generated from the custom Matlab software that corresponded with number of pixels, in this case further called "presynaptic structures". The I_{sp} for GAD67 can be calculated as a ratio

of (VGLUT2-GAD67)/(VGLUT2+GAD67). The I_{sp} for GlyT2 can be calculated as a ratio: (VGLUT2-GlyT2)/(VGLUT2+GlyT2). For both GlyT2 and GAD67 I_{sp} , higher I_{sp} values can be considered greater excitation than inhibition (Rotschafer et al., 2015). I_{sp} results were also analyzed using a mixed effect model with a Holms p-value correction for multiple comparisons. Representative images were adjusted for brightness and contrast using Photoshop (Adobe Photoshop CS6, Mountainview, CA) purely for the purposes of illustration. All analyses were completed on unadjusted (by Photoshop) images only.

RESULTS

We used triple immunofluorescence staining in paraformaldehyde fixed tissue from *Fmr1*^{-/-} mice and B6 controls to examine the presynaptic inputs (glutamatergic, glycinergic and GABAergic) to different nuclei of the auditory brainstem to determine if there are imbalances in excitatory and inhibitory projections that might lead to sound localization issues in FXS.

Presynaptic structures of the AVCN

We first examined the inputs to the AVCN since it's the first nucleus along the ascending auditory pathway and receives excitatory input directly from the auditory nerve (Figures 1 and 2). Inhibitory inputs to this nucleus have been demonstrated (Adams & Mugnaini, 1987; Juiz, Helfert, Bonneau, Wenthold, & Altschuler, 1996; Mahendrasingam, Wallam, & Hackney, 2000; Saint Marie, Benson, Ostapoff, & Morest, 1991) and are thought to originate from the dorsal cochlear nucleus (DCN), VNTB, the SPN, or the cortex (Benson & Potashner, 1990; Kulesza & Berrebi, 2000; Lim, Alvarez, & Walmsley, 2000; Luo, Wang, Kashani, & Yan, 2008; Ostapoff, Benson, & Saint Marie, 1997; S. Zhang & Oertel, 1993). Representative images of the AVCN of *Fmr1*^{-/-} and B6 mice with triple staining of the three channels are shown in Figure 1. We found that in *Fmr1*^{-/-} mice, there was no change in the number of presynaptic structures for VGLUT2, GlyT2 or GAD67, however there was a trend towards increased number of VGLUT2 presynaptic structures in the *Fmr1*^{-/-} mice (not significant, $p = 0.0657$) (Figure 2a). In addition, there is no change in the mean size of any of the presynaptic structures (glutamatergic, glycinergic or GABAergic) in the AVCN (Figure 2b). The ratio of excitation to inhibition also (reflected as I_{sp} values) did not show any change between *Fmr1*^{-/-} mice and wildtype (Figure 2c). A diagram showing the projections to the AVCN and its downstream targets is shown in Figure 2D.

Presynaptic structures of the VNTB

Next, we examined if there are alterations in the inputs to the VNTB. The rationale for this set of experiments was that the VNTB receives excitatory input from the contralateral AVCN (Figures 3 and 4). VNTB also receives inhibitory inputs, but their origin is not well described (Robertson 1996). Importantly, the VNTB is the main extrinsic source of inhibitory inputs to the MNTB (Albrecht et al., 2014). Representative images of presynaptic inputs to VNTB cells in *Fmr1*^{-/-} and B6 animals are shown in Figure 3. We found that in *Fmr1*^{-/-} mice there was no change in either number or size of presynaptic structures compared to wild type controls (Figure 4a, 4b). Consistent with no change in number and size of GAD67 or GlyT2 presynaptic structures in VNTB, we see no difference in I_{sp} values

for VGLUT2/GlyT2 or VGLUT2/GAD67 ratios (Figure 4c). Connections between presynaptic nuclei innervating VNTB and its projections are shown in Figure 4e.

Presynaptic structures of the MNTB (medial and lateral)

We next examined the inputs to the MNTB, which is another target that receives neural excitation from the contralateral AVCN, although via a different group of neurons (Smith, Joris, Carney, & Yin, 1991). Additionally, the MNTB receives inhibitory inputs from the ipsilateral VNTB (Albrecht et al., 2014), as well as recurrent inhibition from MNTB axon collaterals (Dondzillo, Thompson, & Klug, 2016) (as shown in Figure 6d and 8d). The MNTB provides glycinergic inhibition to two independent sound localization processes, namely the analysis of interaural time differences at the MSO (Jeffress, 1948; Brand, Behrend, Marquardt, McAlpine, & Grothe, 2002) and the analysis of interaural intensity differences at the LSO (Caird & Klinke, 1983; Goldberg & Brown, 1969) reviewed by Grothe et al., 2010; Tollin, 2003), among other targets (in humans and rodents with lower frequency hearing). Because of this tonotopic organization of the MNTB, we decided to examine the low frequency contours and the high frequency contours of MNTB independently; the lateral region of the MNTB is involved in processing of low frequency sounds, while the medial region of MNTB is involved in the processing of high frequency sounds (Strumbos, Brown, Kronengold, Polley, & Kaczmarek, 2010; Tolnai, Hernandez, Englitz, Rübsamen, & Malmierca, 2008).

In the medial, high frequency region of MNTB, we observed a decrease in number of GlyT2 presynaptic structures, but not VGLUT2 or GAD67 structures in the *Fmr1*^{-/-} mouse compared to control (Figure 6a, Figure 5 for representative images). At the same time, the mean structure size for all three antibodies were not significantly different in *Fmr1*^{-/-} mouse compared to controls (Figure 6b). When the number of presynaptic structures was quantified as a ratio of VGLUT2/GlyT2 or VGLUT2/GAD67, we see no change in *I*_{sp} value (Figure 6c) between knockout and wildtype.

Interestingly, we did not see the same changes in the lateral MNTB as seen in the medial MNTB. The number of presynaptic structures labeled for GAD67, but not GlyT2 or VGLUT2, are significantly decreased in the *Fmr1*^{-/-} mouse compared to controls (Figure 8a, Figure 7 for representative images). Additionally, we see a trend towards an increase in mean size of VGLUT2 presynaptic structures, but no change in GAD67 or GlyT2 structure size (Figure 8b) in *Fmr1*^{-/-} mice compared to wildtype (not significant, *p* = 0.13992). When number of presynaptic structures was quantified as a ratio of VGLUT2/GAD67 staining, there is an increase in *I*_{sp} values for *Fmr1*^{-/-} mice suggesting increased glutamatergic excitation onto the MNTB that is in part due to a decrease in GABAergic inhibition (Figure 8c). As expected, based on the lack of change in number of GlyT2 labeled structures, we do not see a change in the ratio of VGLUT2/GlyT2 in *Fmr1*^{-/-} mice compared to controls (Figure 8c). The MNTB then projects glycinergic inhibition ipsilaterally to several other nuclei including the medial superior olive (MSO), lateral nucleus of the trapezoid body (LNTB), lateral lemniscus, and the LSO. Connections between presynaptic nuclei innervating MNTB and its projections are shown in Figures 6d and 8d.

Presynaptic structures of the LSO (medial and lateral)

Lastly, we examined the inputs to the LSO, which receives excitatory input from the ipsilateral AVCN and inhibitory input from the contralateral MNTB (as depicted in Figures 10d and 11d). The LSO is also organized tonotopically and receives tonotopically organized glycinergic inhibitory input from the medial MNTB to the medial LSO for high frequency sounds, and from the lateral MNTB to the lateral LSO for lower frequency sounds (Sommer, Lingenhöhl, & Friauf, 1993). Note that the MSO is very small in the mouse due their pronounced high-frequency hearing.

In the medial LSO, there was no change in the number or size of presynaptic structures for any of the three antibodies between *Fmr1*^{-/-} knockouts and controls (Figure 10a and 10b). Consistent with no change in number of presynaptic structures, there was no difference in the ratio of VGLUT2/GAD67 or VGLUT2/GlyT2 in the medial LSO between *Fmr1*^{-/-} mice compared to wildtype (Figure 10c). The same was true for the lateral LSO where there was no change in the number or size presynaptic structures for GlyT2, VGLUT2 or GAD67 in *Fmr1*^{-/-} mice compared to wildtype (Figure 11a and 11b, representative images in Figure 9). When we quantified the number of presynaptic structures as a ratio of VGLUT2/GAD67 or VGLUT2/GlyT2 (Figure 10f), we saw no difference in *I*_{sp} values between *Fmr1*^{-/-} mice and controls.

DISCUSSION

We used triple immunofluorescence staining to examine changes in presynaptic excitatory and inhibitory synapses in the auditory brainstem of *Fmr1*^{-/-} mice. The main findings of this study are: 1) No significant change in size of VGLUT2, GlyT2, and GAD67 for any of the nuclei examined 2) Tonotopic differences in contribution of GABA and glycine in the MNTB 3) there is a prominent role for glycinergic inhibition in FXS, while previous studies have focused much more on GABAergic inhibition.

AVCN

We see no change in overall number or size of presynaptic glutamatergic, glycinergic or GABAergic structures in the AVCN of *Fmr1*^{-/-} mice. However, we do see a trend towards increased number of glutamatergic synapses onto the AVCN. Inhibitory projections to the AVCN are thought to originate from the VNTB (Robertson 1996, Ostapoff et al., 1997) or from the DCN (Saint Marie et al., 1991). The role of this inhibition is not well understood, but is thought to play an important role in short-term response suppression otherwise known as “echo suppression” or “forward masking” (Backoff, Shadduck Palombi, & Caspary, 1997). It has been shown that individuals with Asperger syndrome (a form of autism) have a decreased wave III (which is thought to originate from the auditory nerve) in auditory brainstem responses (ABRs) when presented with a forward masking pattern, suggesting deficits in the auditory nerve processing, perhaps through increased inhibition (Källstrand, Olsson, Nehlstedt, Ling, & Nielzén, 2010). However, we did not see any changes in the inhibitory presynaptic terminals onto the AVCN and it is unclear if increased excitatory drive would contribute to deficits in forward masking in FXS. The AVCN is composed of several different glutamatergic cell types including spherical bushy cells, which project to

the LSO (Cant & Casseday, 1986) and globular bushy cells that project to the MNTB and VNTB (Bruce Warr, 1972; Helfert, Bonneau, Wenthold, & Altschuler, 1989; Kuwabara et al., 1991).

VNTB

In the VNTB of *Fmr1*^{-/-} mice we also observed no change in number of glutamatergic, glycinergic or GABAergic puncta. Anatomically, the VNTB receives excitatory input from the contralateral globular bushy cells of the AVCN (Bruce Warr, 1972; Helfert et al., 1989). Physiologically, it has been shown that excitatory synapses projecting from the AVCN are thought to be the primary regulator of VNTB synaptic firing (Robertson 1996). Where the inhibitory inputs (GABA and glycine) onto the VNTB originate from is not known. In guinea pigs, it has been shown that many of the cells in the VNTB have more GABAergic puncta than glycinergic suggesting that GABA plays a larger role in inhibition to this area (Helfert et al., 1989). However, Albrecht et al 2014 showed that, similar to the MNTB, the inhibitory projections to the VNTB show a developmental shift from more GABAergic inhibition in younger animals to primarily glycinergic inhibition in older animals. The function of auditory processing in the VNTB is not well understood, but one possible function is to provide fast and strong glycinergic inhibition onto the ipsilateral MNTB, where it may play a role in fine-tuning neural responses of MNTB cells (Albrecht et al., 2014; Awatramani, Turecek, & Trussell, 2004; Mayer et al., 2014). The VNTB also consists of other cell types that are GABAergic or cholinergic in nature and it is not known where these cells project (Helfert et al., 1989; Yao & Godfrey, 1998).

MNTB

We saw tonotopic differences in the MNTB of *Fmr1*^{-/-} mice, where in the medial, high frequency region of the MNTB, we see a decrease in glycinergic presynaptic structures, which could correlate with projections originating from the VNTB (though we did not see any changes in projections going to the VNTB). In contrast, in the lateral MNTB of *Fmr1*^{-/-} mice there was no change in glycinergic presynaptic structures, but a significant decrease in GABAergic puncta. The calyx of Held is a well-known and well-characterized strong glutamatergic synapse originating from the globular bushy cells in the AVCN and projecting to the contralateral MNTB (Guinan, Guinan, & Norris, 1972; Smith et al., 1991; Spangler et al., 1985) and we did not observe any alterations of this excitatory input in the knockout mice. Both GABAergic and glycinergic inhibition onto the MNTB have been shown to originate from the ipsilateral VNTB (Albrecht et al., 2014) and glycinergic axon collaterals from within the MNTB itself (Dondzillo et al., 2016). Similar to the VNTB, most of the inhibition onto the MNTB is GABAergic during development; however, this inhibition changes into faster glycinergic inhibition in adulthood (Kotak, Korada, Schwartz, & Sanes, 1998). Thus, one explanation for the differential alterations in high and low frequency contours of MNTB may be that the developmental shift from GABA to glycine is incomplete for afferents to the medial MNTB.

Traditionally, the MNTB has been thought to be merely a “relay station”, converting incoming excitation into outgoing inhibition that is then used for the computation of level difference information between the two ears in the LSO. However, recently it has been

shown that integration in the MNTB is likely more complex than previously thought (reviewed in Klug et al., 2012). Additionally, the MNTB receives prominent inhibitory inputs, which are inconsistent with the concept of a relay station (Albrecht et al., 2014; Awatramani et al., 2004; Dondzillo et al., 2016). In FXS, where it is thought that there is hyperexcitability of sensory systems due to imbalances in the excitation and inhibition (Rubenstein & Merzenich, 2003), impairments in MNTB processing may be particularly pronounced. However, recently it has been shown that despite changes in calyx morphology, there were no obvious changes in synaptic transmission *in vivo* in *Fmr1*^{-/-} mice (T. Wang, de Kok, Willemsen, Elgersma, & Borst, 2015). There was however a trend towards longer synaptic latencies in the *Fmr1*^{-/-} mice and this study did not specifically target the medial or lateral MNTB where we have seen (along with others, Strumbos et al., 2010) potential tonotopic differences in the MNTB of *Fmr1*^{-/-} mice. *In vitro* work in *Fmr1*^{-/-} mice has shown interactions between FMRP and Slack, resulting in reduced K_{NA} potassium conductance in *Fmr1*^{-/-} mice (M. R. Brown et al., 2010), and an increase in Kv3.1 currents in medial (high frequency) MNTB (Strumbos et al., 2010). These results would indicate that the MNTB of *Fmr1*^{-/-} mice would likely have reduced temporal fidelity during high-frequency firing and inaccurate coding of modulation rate of stimulus features. Any disruptions in MNTB firing would likely alter interaural level difference (ILD) coding in the LSO since timing and strength of the inhibitory input from the contralateral ear through the MNTB is crucial for coding of this information. However, the MNTB also projects glycinergic inhibition to several other brain areas including the lateral lemniscus, MSO, and LNTB, among others, which were not measured in this study (A. M. Thompson & Schofield, 2000).

LSO

In *Fmr1*^{-/-} mice, we saw no change number or size of glycinergic, GABAergic, or glutamatergic presynaptic structures in either the medial or lateral LSO. The LSO receives excitatory input from ipsilateral spherical bushy cells in the AVCN (Cant & Casseday, 1986) and glycinergic inhibition from the ipsilateral MNTB (Tsuchitani, 1977).

Electrophysiologically, alterations in LSO processing in *Fmr1*^{-/-} mice or humans have not been studied. However, several studies have examined ABRs in *Fmr1*^{-/-} mice and shown increased thresholds and decreased amplitudes of early ABR waveforms (which should correspond with brainstem level processing)(Rotschafer et al., 2015). In patients with FXS, ABR measurements show longer peak latencies and inter-peak intervals of wave I–III, which again corresponds to abnormal processing in the brainstem circuit level (Ferri et al 1987). Other studies however have shown dissimilar results or no differences in ABR measurements in FXS (Miezejeski et al., 1997; Roberts et al., 2005). However, with ABR measurements, there are many variables that can account for differences in findings such as electrode placement, stimulation, etc. (reviewed in (Ferber, Benichoux, & Tollin, 2016)). In autism, it has been shown that patients exhibit difficulties with sound localization on the vertical (monaural processing), but not horizontal plane (ILD processing)(Visser et al., 2013). However, this study was conducted in adults, and generically with people who have autism- not FXS. Whether there are in fact sound localization changes in patients with FXS has not been explored experimentally. One caveat to using the C57BL/6J strain (and many

mouse strains), is that these mice exhibit age-related hearing loss at fairly young ages, therefore we can not exclude the possibility that with our large age-range used in this study that one or more of the mice may have suffered from some age-related hearing loss (Zheng, Johnson, & Erway, 1999).

In summary, our study is the first to explore whether there are alterations in glycinergic inhibition in the auditory brainstem of *Fmr1*^{-/-} mice. We also found that there are nucleus specific and frequency specific alterations of excitation vs. inhibition in the auditory brainstem of the FXS knockout mouse. Additionally, our results suggest that due to the well-characterized functional roles of excitatory and inhibitory neurotransmitters in the auditory brainstem, the sound localization pathway is an ideal circuit by which to measure sensory alterations in FXS. A better understanding of alterations in lower auditory areas may be vital to understanding the complex sensory alterations that are occurring in patients with FXS, in particular how tonotopic alterations in inhibition to the MNTB might contribute to altered sound processing.

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Figure 1.

Triple immunofluorescent staining of presynaptic markers in the anteroventral cochlear nucleus (AVCN) in B6 (a–d) or *Fmr1*^{-/-} (e–h) mice respectively. Representative images for GAD67 (a, e), GlyT2 (b, f), VGLUT2 (c, g) and thresholded (d, h) staining of VGLUT2/GlyT2/GAD67 markers in B6 and *Fmr1*^{-/-} mice respectively. Scale bar is 20 microns.

Figure 2.

Fmr1^{-/-} mice have no change in size or number of VGLUT2, GlyT2 or GAD67 presynaptic structures in the AVCN. *Fmr1*^{-/-} mice have no change in number of VGLUT2 GlyT2, or GAD67 presynaptic structures (a). The mean structure size is unaltered in *Fmr1*^{-/-} mice compared to wildtype (b). The ratio (*I*_{sp}; see methods) of VGLUT2/GAD67 and VGLUT2/GlyT2 is unaltered in *Fmr1*^{-/-} mice (c). The AVCN receives excitation (magenta) from the cochlear nerve and inhibition (green (GlyT2)/blue (GAD67)) from other cochlear nuclei (d). AVCN projects excitation ipsilaterally to the LSO and contralaterally to VNTB and MNTB, box shows AVCN. D is dorsal, L is lateral, each box plot represents data from an individual animal within the box plot of the group, orange/red indicates a B6 animal, purple/blue are *Fmr1*^{-/-} animals

Figure 3.

Triple immunofluorescent staining of presynaptic markers in the ventral nucleus of the trapezoid body (VNTB) in B6 (a–d) or *Fmr1*^{−/−} (e–h) mice. Representative images for GAD67 (a, e), GlyT2 (b, f), VGLUT2 (c, g) and thresholded (d, h) staining of VGLUT2/GlyT2/GAD67 in B6 and *Fmr1*^{−/−} mice respectively. Scale bar is 20 microns.

Figure 4.

Fmr1^{-/-} mice have no change in size or number of presynaptic structures for VGLUT2, GlyT2 or GAD67 compared to wildtype in the VNTB. *Fmr1*^{-/-} mice have no change in number of GAD67, GlyT2, or VGLUT2 presynaptic structures compared to wildtype, (a). The mean structure size is unchanged in *Fmr1*^{-/-} mice compared to wildtype (b). The ratio (I_{sp} ; see methods) of VGLUT2/GAD67 or VGLUT2/GlyT2 is unchanged in *Fmr1*^{-/-} mice compared to wildtype (c). The VNTB receives excitation (pink) from the contralateral AVCN and projects inhibition to the ipsilateral MNTB (green (GlyT2)/blue (GAD67)), box shows VNTB (d). D is dorsal, L is lateral, each box plot represents data from an individual animal within the box plot of the group, orange/red indicates a B6 animal, purple/blue are *Fmr1*^{-/-} animals.

Figure 5.

Triple immunofluorescent staining of presynaptic markers in the medial portion of the MNTB in B6 (a–d) or *Fmr1*^{−/−} (e–h) mice. Representative images for GAD67 (a, e), GlyT2 (b, f), VGLUT2 (c, g) and thresholded (d, h) staining of VGLUT2/GlyT2/GAD67 in B6 mice and *Fmr1*^{−/−} mice respectively. Scale bar is 20 microns.

Figure 6.

Fmr1^{-/-} mice have fewer GlyT2 presynaptic structures compared to wildtype in the medial portion of the MNTB. *Fmr1*^{-/-} mice have fewer presynaptic GlyT2 structures compared to wildtype, but no change in VGLUT2 or GAD67 number (a). The mean structure size is unchanged in the medial MNTB of *Fmr1*^{-/-} mice compared to wildtype for VGLUT2, GlyT2 or GAD67 structure size (b). The ratio (I_{sp} ; see methods) of VGLUT2/GAD67 or VGLUT2/GlyT2 is unchanged in *Fmr1*^{-/-} mice compared to wildtype (c). The MNTB receives excitation (pink) from the contralateral AVCN, inhibition from the VNTB (green (GlyT2)/blue (GAD67)) and projects inhibition to the ipsilateral LSO, each box plot represents data from an individual animal within the box plot of the group, orange/red indicates a B6 animal, purple/blue are *Fmr1*^{-/-} animals ($p < 0.01 = **$).

Figure 7.

Triple immunofluorescent staining of presynaptic markers in the lateral portion of the MNTB in B6 (a–d) or *Fmr1*^{−/−} mice (e–h) mice. Representative images for GAD67 (a, e), GlyT2 (b, f), VGLUT2 (c, g) and thresholded (d, h) staining of VGLUT2/GlyT2/GAD67 in B6 and *Fmr1*^{−/−} mice respectively. Scale bar is 20 microns.

Figure 8.

Fmr1^{-/-} mice have fewer GAD67 presynaptic structures compared to wildtype in the lateral portion of the MNTB. *Fmr1*^{-/-} mice have fewer presynaptic GAD67 presynaptic structures compared to wildtype, but no change in VGLUT2 or GlyT2 (a). The mean structure size is unchanged for VGLUT2, GAD67 or GlyT2 structure size in *Fmr1*^{-/-} mice compared to wildtype (b). The ratio (*I_{sp}*; see methods) of VGLUT2/GAD67 or VGLUT2/GlyT2 is unchanged in *Fmr1*^{-/-} mice compared to wildtype (c). The MNTB receives excitation (pink) from the contralateral AVCN, inhibition from the VNTB (green (GlyT2)/blue (GAD67)) and projects inhibition to the ipsilateral LSO, box shows lateral MNTB (d). D is dorsal, L is lateral, each box plot represents data from an individual animal within the box plot of the group, orange/red indicates a B6 animal, purple/blue are *Fmr1*^{-/-} animals, (p < 0.05 = *).

Figure 9.

Triple immunofluorescent staining of presynaptic markers in the LSO in B6 (a–d) or *Fmr1*^{−/−} mice (e–h). Representative images for GAD67 (a, e), GlyT2 (b, f), VGLUT2 (c, g) and thresholded (d, g) staining of VGLUT2/GlyT2/GAD67 in B6 and *Fmr1*^{−/−} mice respectively. Scale bar is 20 microns.

Figure 10.

Fmr1^{-/-} mice have no change in size or number of presynaptic structures in the lateral LSO compared to wildtype. *Fmr1*^{-/-} mice have no change in number of presynaptic structures for VGLUT2, GlyT2 and GAD67 compared to wildtype in the lateral LSO (a). There is no change in size of presynaptic structures in *Fmr1*^{-/-} mice compared to wildtype (b). The ratio (I_{sp} ; see methods) of VGLUT2/GlyT2 or VGLUT2/GAD67 is unaltered in *Fmr1*^{-/-} mice compared to wildtype in the lateral LSO (c). The LSO receives excitation (pink) from the ipsilateral AVCN and inhibition from the ipsilateral MNTB (green (GlyT2)/blue (GAD67)), box shows lateral LSO (d). D is dorsal, L is lateral, each box plot represents data from an individual animal within the box plot of the group, orange/red indicates a B6 animal, purple/blue are *Fmr1*^{-/-} animals.

Figure 11.

Fmr1^{-/-} mice have no change in size or number of presynaptic structures in the medial LSO compared to wildtype. *Fmr1*^{-/-} mice have no change in number of presynaptic structures for VGLUT2, GlyT2 and GAD67 compared to wildtype in the medial LSO (a). There is no change in size of presynaptic structures in *Fmr1*^{-/-} mice compared to wildtype (b). The ratio (I_{sp} ; see methods) of VGLUT2/GlyT2 or VGLUT2/GAD67 is unaltered in *Fmr1*^{-/-} mice compared to wildtype in the medial LSO (c). The LSO receives excitation (pink) from the ipsilateral AVCN and inhibition from the ipsilateral MNTB (green (GlyT2)/blue (GAD67)), box shows medial LSO (d). D is dorsal, L is lateral, each box plot represents data from an individual animal within the box plot of the group, orange/red indicates a B6 animal, purple/blue are *Fmr1*^{-/-} animals.

Table 1

Primary antibodies used in immunofluorescence

Antibody	Immunogen	Manufacturer, species, mono or polyclonal, cat. or lot no., RRID	Concentration
Glycine-Transporter 2 (GlyT2, Slc6a5)	Recombinant protein (aa 1-229 of rat GlyT2)	SySy (Göttingen, Germany), rabbit polyclonal, 272 003, RRID: AB_2619997	1:2000
Glutamic acid decarboxylase 67 (GAD67)	Recombinant protein (aa 4-101)	Millipore (Temecula, CA), mouse monoclonal, MAB5406, 2726806, RRID: AB_2278725	1:1000
Vesicular glutamate transporter 2 (VGLUT2)	Recombinant GST tagged, VQESAQDAYSYKDRDDYS	Millipore (Temecula, CA), guinea pig polyclonal, AB2251, 2116703, RRID: AB_1587626	1:5000

Table 2

Secondary antibodies used in immunofluorescence

Catalog No., RRID	Manufacturer	Reactivity	Conjugate	Concentration
A-21105, AB_2535757	Thermo-Fisher	Goat anti-Guinea Pig IgG (H+L)	Alexa 633	1:1000
A-31553, AB_221604	Thermo-Fisher	Goat anti-Mouse IgG (H+L)	Alexa 405	1:1000
A-31572, AB_162543	Thermo-Fisher	Donkey anti-Rabbit IgG (H+L)	Alexa 555	1:1000

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Table 3

Number of sections per nucleus in wildtype or *Fmr1*^{-/-} animals

	AVCN	VNTB	Medial MNTB	Lateral MNTB	Medial LSO	Lateral LSO
<i>B6</i>	35	22	31	44	65	60
<i>Fmr1</i> ^{-/-}	22	13	36	35	53	57

Table 4

Descriptive statistics of presynaptic structures for each nucleus and antibody

Nucleus	<i>B6</i>			<i>Fmr1^{-/-}</i>			p-value
	Mean ± SEM	Median	IQR Q1–Q3	Mean ± SEM	Median	IQR Q1–Q3	
AVCN	VGLUT2	681.8 ± 40.6	643	420–720	883.0 ± 68.5	827	344–870
	GlyT2	503.4 ± 24.5	456	338–622	510.4 ± 22.5	503	276–578
	GAD67	581.8 ± 53.8	480	337–743	654.9 ± 78.3	553	366–792
VNTB	VGLUT2	613.5 ± 40.8	593	485–740	661.4 ± 91.5	680	487–750
	GlyT2	328.8 ± 16.3	330	267–384	293.8 ± 21.7	292	260–326
	GAD67	147.8 ± 41.1	68	39–96	355.3 ± 66.4	420	73–482
Medial MNTB	VGLUT2	409.8 ± 19.5	419	342–473	386.5 ± 23.7	344	267–450
	GlyT2	411.6 ± 32.0	394	256–520	286.5 ± 25.0	264	174–390
	GAD67	286.7 ± 19.6	269	202–351	243.0 ± 24.2	204	142–305
Lateral MNTB	VGLUT2	553.0 ± 21.4	547.5	462–623	488.8 ± 23.5	479	378–542
	GlyT2	370.0 ± 29.4	354	232–493	295.0 ± 21.6	278	206–364
	GAD67	383.9 ± 24.6	330.5	273–493	236.1 ± 17.9	203	162–332
Medial LSO	VGLUT2	368.6 ± 12.8	349	236–444	343.3 ± 17.9	364	316–427
	GlyT2	321.8 ± 9.1	314	273–362	306.6 ± 12.3	304	230–384
	GAD67	480.1 ± 18.1	511	379–587	442.6 ± 22.9	433	336–511
Lateral LSO	VGLUT2	331.0 ± 15.2	337.5	270–412	298.4 ± 11.9	301	229–358
	GlyT2	315.0 ± 7.7	311	279–353	291.9 ± 8.4	285	244–334
	GAD67	247.6 ± 19.4	204	153–319	250.8 ± 20.7	220	126–374

Table 5

Descriptive statistics of mean structure size for each nucleus and antibody

	Nucleus	B6			Fmr1 ^{-/-}			p-value
		Mean ± SEM	Median	IQR Q1-Q3	Mean ± SEM	Median	IQR Q1-Q3	
AVCN	VGLUT2	43.09 ± 1.5	43.6	36.2–47.5	49.7 ± 3.6	42.2	39.8–57.1	0.492
	GlyT2	40.1 ± 1.2	42.8	39.1–47.5	39.8 ± 1.9	39.0	37.2–50.2	0.502
	GAD67	40.8 ± 2.1	38.5	33.3–51.1	51.03 ± 5.6	37.3	36.5–63.8	0.239
VNTB	VGLUT2	36.3 ± 1.1	36.5	33.9–38.7	45.4 ± 3.0	49.1	38.5–53.0	0.414
	GlyT2	43.4 ± 1.5	44.5	36.8–47.7	44.9 ± 3.2	48.7	41.9–52.1	0.865
	GAD67	26.8 ± 1.5	25.5	21.3–30.5	37.8 ± 3.2	37.9	31.1–45.1	0.414
Medial MNTB	VGLUT2	40.5 ± 1.3	40.2	36.3–43.4	42.1 ± 1.1	40.6	37.4–43.7	1.0
	GlyT2	40.2 ± 1.8	36.7	33.6–43.9	35.3 ± 1.0	35.2	30.4–38.8	1.0
	GAD67	25.8 ± 0.77	25.5	23.5–27.3	27.9 ± 0.93	27.4	23.6–30.5	1.0
Lateral MNTB	VGLUT2	39.1 ± 1.0	39.2	33.8–42.9	44.6 ± 0.93	43.4	40.5–47.8	0.140
	GlyT2	40.7 ± 1.2	41.1	34.5–45.3	44.7 ± 1.3	44.3	38.8–48.7	0.420
	GAD67	26.5 ± 0.72	25.9	24.1–28.6	27.1 ± 0.85	25.8	24.8–29.6	0.691
Medial LSO	VGLUT2	21.8 ± 0.41	20.9	19.5–23.8	23.9 ± 0.46	23.3	21.5–25.9	0.294
	GlyT2	28.6 ± 0.50	27.8	25.1–30.7	30.6 ± 0.91	28.7	26.1–33.3	0.430
	GAD67	30.9 ± 0.84	29.6	27.5–35.2	33.9 ± 1.3	29.8	26.8–39.9	0.294
Lateral LSO	VGLUT2	22.0 ± 0.47	21.0	19.1–24.9	25.4 ± 0.84	24.1	20.6–28.5	0.647
	GlyT2	29.9 ± 0.72	29.4	25.3–33.6	32.8 ± 0.82	31.6	28.6–37.4	0.697
	GAD67	26.6 ± 0.84	24.4	21.8–30.1	30.6 ± 1.1	29.8	23.7–36.3	0.697