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Gene Expression Profiling in Trigeminal Ganglia from *Cntnap2*$^{-/-}$ and *Shank3b*$^{-/-}$ Mouse Models of Autism Spectrum Disorder

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Abstract—Sensory difficulties represent a crucial issue in the life of autistic individuals. The diagnostic and statistical manual of mental disorders describes both hyper- and hypo-responsiveness to sensory stimulation as a criterion for the diagnosis autism spectrum disorders (ASD). Among the sensory domain affected in ASD, altered responses to tactile stimulation represent the most commonly reported sensory deficits. Although tactile abnormalities have been reported in monogenic cohorts of patients and genetic mouse models of ASD, the underlying mechanisms are still unknown. Traditionally, autism research has focused on the central nervous system as the target to infer the neurobiological bases of such tactile abnormalities. Nonetheless, the peripheral nervous system represents the initial site of processing of sensory information and a potential site of dysfunction in the sensory cascade. Here we investigated the gene expression deregulation in the trigeminal ganglion (which directly receives tactile information from whiskers) in two genetic models of syndromic autism (*Shank3b* and *Cntnap2* mutant mice) at both adult and juvenile ages. We found several neuronal and non-neuronal markers involved in inhibitory, excitatory, neuroinflammatory and sensory neurotransmission to be differentially regulated within the trigeminal ganglia of both adult and juvenile Shank3b and Cntnap2 mutant mice. These results may help in disentangling the multifaced complexity of sensory abnormalities in autism and open avenues for the development of peripherally targeted treatments for tactile sensory deficits exhibited in ASD.

Key words: autism, sensory abnormalities, mouse models, trigeminal ganglion.

INTRODUCTION

Autism spectrum disorders (ASDs) form a multifaceted group of neurodevelopmental disorders with high heterogeneity of symptoms and severity. The diagnostic and statistical manual of mental disorders (DSM-V) describes autism as a pervasive neurological syndrome characterized by deficits in social interaction and repetitive/stereotyped behavior with several associated neurological symptoms. Despite being characterized by such a high degree of variability among individuals affected, it has been reported that 95% of autistic individuals show aberrant sensory experiences, suggesting sensory abnormalities as a typical feature in ASD. Indeed, sensory abnormalities are now described in the DSM-V as both hyper- and hypo-responsiveness to sensory stimulation demonstrating how sensory symptoms are fundamental for the description of the syndrome itself. Abnormal sensory reactivity represents a crucial issue in autism research since it likely contributes to other ASD symptoms such as anxiety, stereotyped behaviors, as well as cognitive and social dysfunctions (Ben-Sasson et al., 2007; Sinclair et al., 2017). Among the sensory domain affected in ASD, altered responses to tactile stimulation represent the most commonly reported sensory deficits in ASD (60.9%). Interestingly, hypo-responsiveness to tactile stimulation has been found to positively correlate with the severity of ASD core symptoms (Foss-Feig et al., 2012) and touch avoidance behavior in toddlers is predictive of ASD diagnosis later on in life (Mammen et al., 2015).

Although tactile abnormalities have been reported in monogenic cohorts of patients with ASD (Rett syndrome (Badr et al., 1987; Amir et al., 1999), fragile X syndrome (Rogers et al., 2003), Phelan-McDermid syndrome

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Abbreviations: ASD, autism spectrum disorders; CDFE, cortical dysplasia-focal epilepsy; CNS, central nervous system; Ct, cycle threshold; DRG, dorsal root ganglia; DSM-V, diagnostic and statiscical manual of mental disorders; FC, Fold change; KO, knockout; PMS, Phelan McDermid; PNS, peripheral nervous system; TG, trigeminal ganglion.

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(Tavassoli and Baron-Cohen, 2012), the underlying mechanisms are still under investigation. The first step in proper tactile perception starts in the periphery with the activation of the mechanosensory neurons which respond to innocuous tactile stimuli and mediate the perception of objects' shape and texture, vibrations, and skin strokes. These neurons in mammals are pseudo-unipolar and have their cell bodies in the dorsal root ganglia (DRG) and trigeminal ganglia (TG), as part of the peripheral nervous system (PNS), where they mediate tactile sensitivity from the body and head region respectively. DRG and TG neurons collect the somatosensory information from the periphery and send projections to the central nervous system (CNS), within the spinal cord for DRG neurons, and the brainstem for TG neurons.

Thus, the DRG and TG are initial sites of processing of tactile stimuli then conveyed to CNS. Ideally, these two structures represent potential sites of dysfunction underlying impairments in somatosensory perception in ASD individuals. While changes in the DRG have been reported in mouse models of ASD (Orefice et al., 2016; Orefice et al., 2019), to the best of our knowledge there is no evidence of TG neuron dysfunctions in ASD.

Mouse lines harboring ASD-relevant mutations have been recently used to assess the neurobiological underpinnings of abnormal sensory responses in ASD within the central and the peripheral nervous system (He et al., 2017; Balasco et al., 2019, 2020; Chelini et al., 2019; Orefice, 2020; Pizzo et al., 2020). Given the relevance of the whisker system in mice, the assessment of TG neurons (which mediate tactile sensitivity from the whiskers) represents a promising tool to study somatosensory processing defects in ASD. Mice use their whiskers for a variety of behavior such as object exploration (Brecht, 2007) and conspecific interaction (Ahl, 1986), and abnormalities in sensory perception through whiskers profoundly impact mouse behavior (Arakawa and Erzurumlu, 2015; Erzurumlu and Gaspar, 2020).

In this work, we used *Shank3b* and *Cntnap2* mutant mice as models for the Phelan McDermid (PMS) and cortical dysplasia-focal epilepsy (CDFE) respectively, two syndromic forms of autism. These two mouse models show hypo responsiveness to whisker dependent cues and altered activation of key regions involved in the somatosensory processing (Balasco et al., 2021; Balasco et al., 2022). Moreover, there are reports of DRG dysfunctions in *Shank3b* and other genetic mouse models of ASD (Orefice et al., 2019) which suggest that altered processing of sensory stimuli in the peripheral nervous system could be a common aspect of genetic mouse model of autism who shows hypo/hyper reactivity to sensory stimulation. PMS is caused by mutations to the *SHANK3* gene which codes for the SH3 and multiple ankyrin repeat domain protein 3 (Monteiro and Feng, 2017). This protein belongs to the family of Shank proteins and therefore acts as a major scaffolding protein within the postsynaptic density of excitatory neurons (Jiang and Ehlers, 2013). As a syndrome, PMS is described by intellectual disability, speech and developmental delay, and importantly ASD-related behaviors such as problems in communication and social interaction (Phelan and McDermid, 2012), as well as sensory hypo reactivity to tactile stimulation (Balasco et al., 2020). CDFE is caused by a recessive nonsense mutation in the *CNTNAP2* gene which codes for CASPR2. CASPR2 (CNTNAP2) is part of the neurexin family of transmembrane proteins and is involved in neuron-glia interactions, potassium channel clustering on myelinated axons, dendritic arborization, and spine development (Poliak et al., 1999; Poliak et al., 2003; Anderson et al., 2012). CDFE syndrome is a rare disorder characterized by intellectual disability, ASD-like behaviors, language regression, and focal epileptic seizures from childhood. *Shank3b* and *Cntnap2* knockout mice display autistic-like characteristics as repetitive grooming and impaired social interaction among others thus are considered reliable models for ASDs (Peça et al., 2011; Peñagarikano et al., 2011; Vogt et al., 2018).

Given the fundamental role of early-life tactile experiences in shaping the acquisition of normal social behavior and communication skills in humans as well as in rodents, we hypothesized that peripheral tactile processing defects might contribute to ASD symptomatology. We recently reported aberrant somatosensory processing in the central nervous system of adult *Shank3b* and *Cntnap2* mutant mice following whisker stimulation (Balasco et al., 2020; Balasco et al., 2021). Nonetheless, to infer a possible peripheral contribution to such defects, here we performed a gene expression profiling of TG in *Cntnap2*$^{-/-}$ and *Shank3b*$^{-/-}$ mice, in both adult and juvenile stages.

EXPERIMENTAL PROCEDURES

Animals. All experiments were carried out following the Italian and European directives (DL 26/2014, EU 63/2010) and were approved by the Italian Ministry of Health and the University of Trento animal care committee. All surgical procedures were performed under anesthesia to minimize animal suffering. Animals were housed in a 12 h light/dark cycle with food and water available ad libitum. Shank3b and Cntnap2 mutant mice were crossed at least five times into a C57BL/6 background before mating. Heterozygous mating (*Shank3b*$^{+/-}$ x *Shank3b*$^{+/-}$ and *Cntnap2*$^{+/-}$ x *Cntnap2*$^{+/-}$) was used to generate the wild-type (WT) and knockout (KO) homozygous littermates used in this study. PCR genotyping was performed following the Jackson Laboratory protocol (https://www.jax.org). 40 sex-matched adult (6 months old) littermates (10 *Shank3b*$^{+/+}$ and 10 *Shank3b*$^{-/-}$ [5M and 5F for both genotypes]; 10 *Cntnap2*$^{+/+}$ and 10 *Cntnap2*$^{-/-}$ [5M and 5Ffor both genotypes]) and 16 sex-matched juvenile (P30) littermates (3 *Shank3b*$^{+/+}$ and 3 *Shank3b*$^{-/-}$ [2M and 1F for both genotypes]; 5 *Cntnap2*$^{+/+}$ and 5 *Cntnap2*$^{-/-}$ [3M and 2F for both genotypes]) were used for qRT-PCR. 16 sex-matched adult littermates (4 *Shank3b*$^{+/+}$ and 4 *Shank3b*$^{-/-}$; 4 *Cntnap2*$^{+/+}$ and 4 *Cntnap2*$^{-/-}$) and 16 sex-matched juvenile littermates (4 *Shank3b*$^{+/+}$ and 4 *Shank3b*$^{-/-}$; 4 *Cntnap2*$^{+/+}$ and 4 *Cntnap2*$^{-/-}$) were used for western blot experiments. P30 animals were used to get insight about the

neurodevelopmental effect of the mutations on trigeminal ganglia.

Trigeminal Ganglia Dissection. Animal were anesthetized by a mix of 5 mg/kg Xilazine and 20 mg/kg Zoletil, and then subjected to decapitation. Trigeminal ganglia (TG) were rapidly removed with surgical tweezers immediately after the skull's opening and the brain's removal. The trigeminal ganglia are easily visible at the base of the skull following the removal of the brain where they appear as two white bands running parallel in a rostro-caudal direction. Trigeminal ganglia were washed in cold PB buffer and stored at −80 °C.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Total RNAs were extracted from the trigeminal ganglia of adult and young mutant (*Shank3b*$^{-/-}$ and *Cntnap2*$^{-/-}$) and control mice (*Shank3b*$^{+/+}$ and *Cntnap2*$^{+/+}$) with RNeasy Mini Kit (QUIAGEN) and retro-transcribed to cDNA with a SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. qRT-PCR was performed in a CFX96TM Real-Time System (Bio-Rad, USA), using SYBR Green master mix (Bio-Rad). Primers (Sigma) were designed on different exons to avoid the amplification of genomic DNA (Table 1). The CFX3 Manager 3.0 (Bio-Rad) software was used to perform expression analyses (Sgadò et al., 2013). Mean cycle threshold (Ct) values from replicate experiments were calculated for each marker and β-actin (used as a standard for quantification), and then corrected for PCR efficiency and inter-run calibration. The expression level of each mRNA of interest was then normalized to that of β-actin for both genotypes. No differences were found among Ct of actin for wild-type and mutant mice of both genotype in each condition (*Shank3b* WT vs KO P30: 18.73 vs 18.38; *Cntnap2* WT vs KO P30: 20.27 vs 20.24; *Shank3b* WT vs KO Adults: 16.46 vs 16.61; *Cntnap2* WT vs KO Adults: 16.6, $p > 0.05$). For RT-qPCR experiments, the expression levels of each marker (normalized to that of β-actin) were compared from triplicate experiments performed on RNA pools. Specifically, ten animals for genotype (10 *Shank3b* WT and 10 *Shank3b* KO; 10 *Cntnap2* WT and 10 *Cntnap2* KO) were pooled for adults' trigeminal ganglia experiments, three animals per genotypes (3 *Shank3b* WT and 3 *Shank3b* KO) and 5 animals per genotype (5 *Cntnap2* WT and 5 *Cntnap2* KO) were pooled for juvenile trigeminal ganglia experiments. Data can be found in Supplementary Tables S2-5.

Primers. Fourty eight genes were selected for testing via RT-qPCR on adult TG tissue, whereas a subset of 19 genes was selected for testing in juvenile TG tissue. Genes were selected to identify specific markers of sensory, inhibitory, and excitatory neurons, as well as neuroinflammation and neuroprotection. Primer specificity was verified using the In-Silico PCR (UCSC Genome Browser) and Primer Blast (NCBI) resources. Primer sequences are listed in Supplementary Table 1 (S1).

Gene expression Analysis. All raw Ct values resulting from qRT-PCR experiments were normalised and calculated into Fold change values via the Livak method. Fold change (FC) represents the expression ratio of KO groups (*Shank3b*$^{-/-}$ or *Cntnap2*$^{-/-}$) relative to their respective WT controls (*Shank3b*$^{+/+}$ or *Cntnap2*$^{+/+}$). Therefore, fold changes lower than 1 represent a downregulation in the expression of a target gene while those greater than 1 report an upregulation of the target gene relative to the control sample. Statistical analysis was performed by unpaired t-test using the GraphPad Prism 8 software with a significance level set at $p < 0.05$.

Protein extraction and western blots

Tissue samples were lysed in lysis buffer (Tris 10 mM, pH 7.4, 0.5% NP-40, 0.5% TX-100, 150 mM NaCl plus Protease Inhibitor Cocktail Tablets, Roche) via mechanical homogenization. Diluted samples (2:1 in 4× Laemmli sample buffer containing 100 mM DTT) were boiled 8 min at 95 °C and loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using precasted gels (Any kD Bio- Rad) and then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked for 20 min in 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.01% Tween-20 (TBS-T). Blots were probed with anti-GFAP (DAKO GA524, kindly provided by Dr. Luciano Conti, University of Trento, Italy; 1:1000) or anti-GAD67 (Sigma MAB5406; 1:5000) in 5% (w/v) non-fat dry milk. After incubation with primary antibodies, membranes were washed three times with TBS-T (10 min each), then probed with a 1:10,000 dilution of anti-mouse HRP conjugated (Jackson ImmunoResearch 115-035-003) or anti-rabbit HRP conjugated (Jackson ImmunoResearch 111-035-045) accordingly for 1 h at RT. After two washes with TBS-T and one with Milli-Q water, signals were revealed using the ECL Prime western blotting Detection Kit (GE Healthcare) and visualized with a ChemiDoc Imaging System (Bio-Rad). The final quantification of proteins detected by primary antibodies were obtained by densitometric analysis of the western blots, normalizing each signal on the corresponding total protein lane (obtained by the enhanced tryptophan fluorescence technology of stain-free gels, BioRad). Statistical analysis of WB data was performed by unpaired t-test using the GraphPad Prism 8 software with a significance level set at $p < 0.05$.

RESULTS

Fig. 1 describes the experimental workflow used to profile gene expression in trigeminal ganglia from *Shank3b* and *Cntnap2* mutant mice. A total of 48 genes were tested in the trigeminal ganglia of WT and KO *Shank3b* and *Cntnap2* adult mice. A subset of 19 genes were selected post-hoc and chosen for testing in trigeminal ganglia from juvenile mutant mice and controls (P30 WT and KO).

Adult mice. Numerous markers for sensory neurons were significantly upregulated in the trigeminal ganglia of either *Shank3b*$^{-/-}$ or *Cntnap2*$^{-/-}$ adult mice (Fig. 2(**A**)). *Cck* (FC = 2.164, $p = 0.0001$), *Sst* (FC = 1.387, $p = 0.0333$), *P2X7* (FC = 1.201, $p = 0.0378$), and

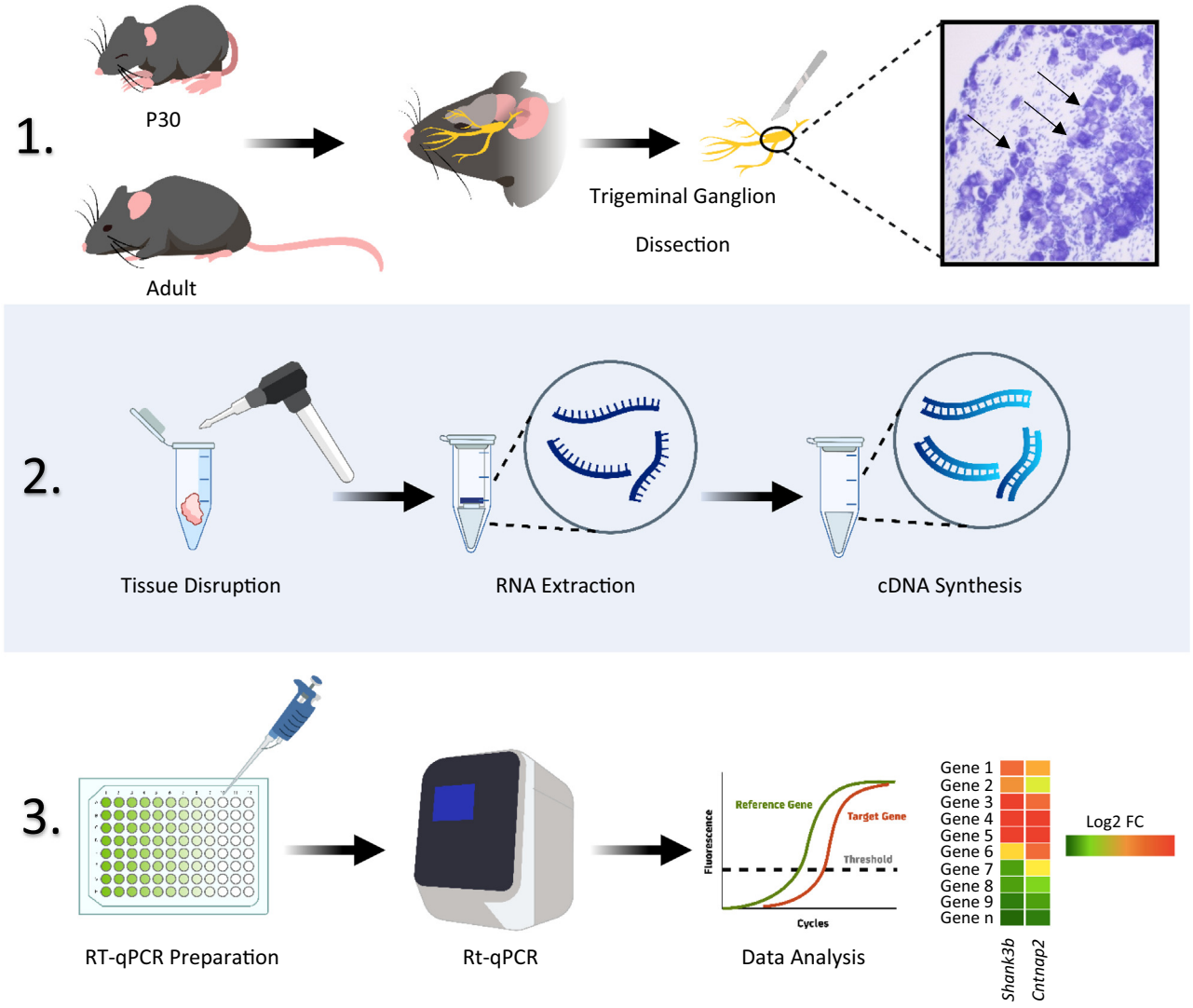

Fig. 1. Schematic of the experimental workflow. Adult and P30 mice of both *Shank3b* and *Cntnap2* mice were sacrificed and trigeminal ganglia (TG) collected. A Nissl staining of the TG is presented. Arrows indicates pseudo-unipolar neurons (in light blue). TG is then mechanically disrupted and the RNA extracted and converted in cDNA. RT-qPCR is performed and run on a Real-time PCR thermal cycler. Data are extracted and analyzed as described.

Calca (FC = 1.222, p = 0.0019) show significant increases in expression values in *Cntnap2*$^{-/-}$ trigeminal ganglia, similarly as *Tacr1* (FC = 1.435, p = 0.0006) and *Trpa1* (FC = 1.154, p = 0.0185) in *Shank3b*$^{-/-}$ samples. As in *Cntnap2* KO TG, *Calca* mRNA was also upregulated in *Shank3b*$^{-/-}$ TG (FC = 1.114, p = 0.0144).

Shank3b$^{-/-}$ and *Cntnap2*$^{-/-}$ adult mice also showed significant alteration in the expression of inhibitory markers (Fig. 2(**B**)) such as a downregulation in *Gabra1* (for SHK FC = 1.435, p < 0.0001; for CNT FC = 1.072, p = 0.0038) in the trigeminal ganglia. There was a significant decrease in the genetic expression of *Gad2* (FC = 0.2379, p < 0.0001) in the TG of Shank3b$^{-/-}$ adults. On the other hand, genes such as *Pvalb* (FC = 2.313, p < 0.0001), *Gabra2* (FC = 1.549, p < 0.0001), *Gabrb2* (FC = 1.399, p = 0.0021), and *Gabrb3* (FC = 1.450, p = 0.0434) were upregulated in the TG of these same mice. Markers for excitatory neuronal subtypes were also significantly differentially expressed in the trigeminal ganglia of KO adult animals (Fig. 2(**A**)). In *Shank3b* KO mice, *Kcna1* was downregulated (FC = 0.8985, p = 0.0447). Additionally, an upregulation of *mGluR2* (FC = 132.0, p = 0.0103) was found in Cntnap2 KO animals.

Several markers for neuroinflammation (Fig. 2(**C**)) were downregulated in the adult *Shank3b*$^{-/-}$ TG, namely *CCL-5* (FC = 0.5115, p = 0.0012), *Tnf* (FC = 0.8403, p = 0.0226), *Iba1* (FC = 0.7728, p = 0.0476), and *Tmem-119* (FC = 0.7799, p = 0.0103). qRT-PCR showed an increased expression of *S100B* mRNA in *Shank3b* KO TG (FC = 1.519, p = 0.0321). In *Cntnap2*$^{-/-}$ mice there was also an upregulation of *iNOS* expression (FC = 1.864, p = 0.0003). Conversely, *Il-1b* expression showed to be significantly decreased in *Cntnap2*$^{-/-}$ samples (FC = 0.7344, p = 0.0023).

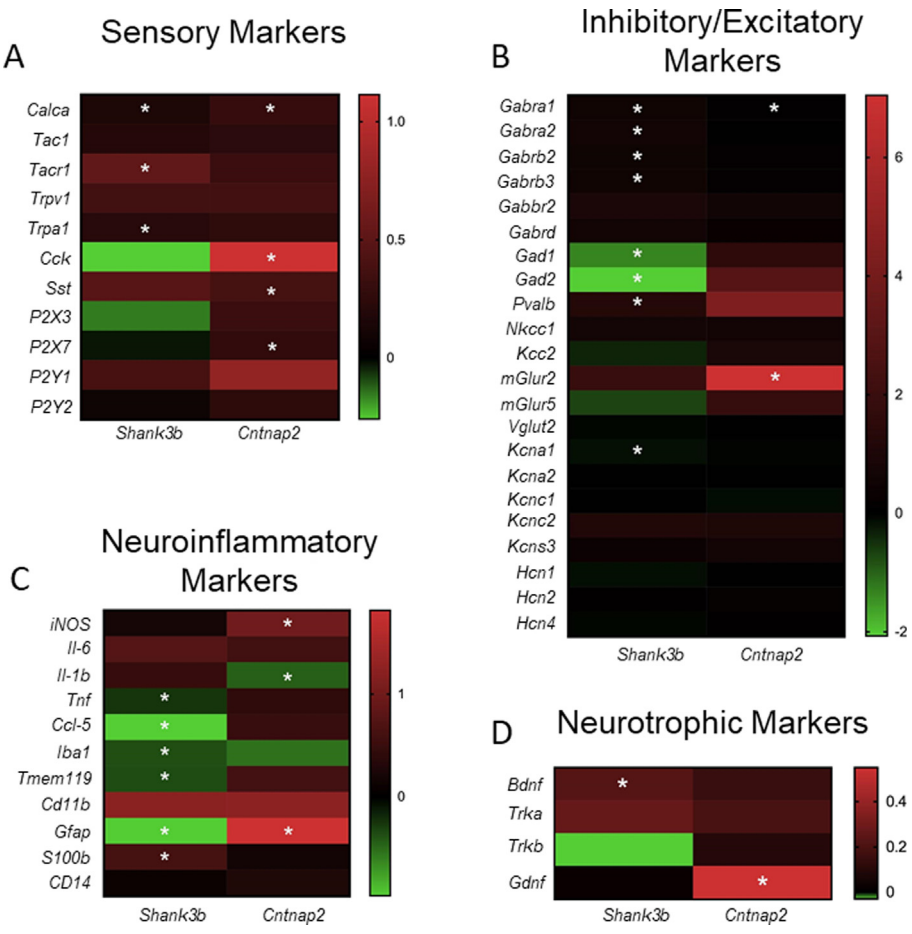

Fig. 2. Heatmap of Log2 fold change values for adult *Shank3b* and *Cntnap2* TG. Values above 0 indicate an upregulation of the respective gene in KO mice relative to WT controls (in red). Conversely values below 0 indicate a downregulation of a gene in KO animals relative to WT controls (in green). Genes annotated with an asterisk (*) show a significant difference in expression values ($p < 0.05$).

Finally, a significant increase in the gene expression was found among the neurotrophic markers tested in adult samples (Fig. 2(**D**)), with *TrkA* (FC = 1.205, $p = 0.0332$) showing an upregulation in *Shank3b*$^{-/-}$ samples and *Gdnf* (FC = 1.464, $p = 0.0404$) in *Cntnap2*$^{-/-}$ samples.

Juvenile mice. qRT-PCR experiments on juvenile mice (Fig. 3) showed that only *Shank3b*$^{-/-}$ TGs display a significant difference in the expression of sensory markers, namely a decrease in *P2X3* mRNA expression (FC = 0.6916, $p = 0.0031$) and an increased expression of *Cck* (FC = 4.087, $p = 0.0220$).

Within the inhibitory markers tested, *Gabrd* was upregulated in *Shank3b*$^{-/-}$ trigeminal ganglia (FC = 1.241, p 0.0016), while *mGlur5* is upregulated in *Shank3b*$^{-/-}$ (FC = 2.277, $p = 0.0494$) among the excitatory markers tested.

Of the neuroinflammatory markers tested in P30 mice, *Il-1b* was the only one resulting in a significant difference in fold change showing an upregulation (FC = 1.817, $p = 0.0107$) in *Cntnap2*$^{-/-}$ juvenile mice relative to controls.

Lastly, in the category of neurotrophic markers, both *TrkA* and *TrkB* were downregulated (FC = 0.7446, $p = 0.0472$ for *TrkA*; FC = 0.8958, $p = 0.0175$ for *TrkB*), while *Gdnf* was upregulated (FC = 2.086, $p = 0.0053$) in the Shank3b$^{-/-}$ TG.

Gad1* and *Gfap. The expression profile of *Gad1* and *Gfap* was of particular interest as these genes showed differential expression in both adult and juvenile *Shank3b*$^{-/-}$ and *Cntnap2*$^{-/-}$ mice. At P30, *Gad1* expression in both *Shank3b*$^{-/-}$ and *Cntnap2*$^{-/-}$ TG was significantly higher relative to WT controls (FC = 2.193, $p = 0.0103$ for *Shank3b*$^{-/-}$; FC = 1.750, $p = 0.0397$ for *Cntnap2*$^{-/-}$). In adulthood, a significant downregulation of these gene was found only in the case of *Shank3b*$^{-/-}$ TG (FC = 0.3933, $p < 0.0001$) (Fig. 4(**A,B**)).

Gfap expression showed opposed trends throughout development in the two mouse strains. *Gfap* mRNA was upregulated at P30 (FC = 4.823, $p = 0.0403$) and downregulated in adulthood (FC = 0.5118, $p = 0.0030$) in *Shank3b*$^{-/-}$ mice. In contrast, juvenile *Cntnap2*$^{-/-}$ mice showed *Gfap* downregulation (FC = 0.7848, $p = 0.0173$) followed by its upregulation in adulthood (FC = 2.821, $p = 0.0117$) (Fig. 4(**C,D**)).

GAD1 and GFAP protein expression. To assess whether transcript alterations in *Gad1* and *Gfap* were accompanied by protein deregulation, we performed western blot experiments focusing with GAD67 and GFAP proteins in trigeminal ganglia form *Shank3b* and *Cntnap2* mice during adult (Fig. 5) and juvenile stages (Fig. 6). In adult animals, GAD67 expression was unaltered in both *Cntnap2*$^{-/-}$ and *Shank3b*$^{-/-}$ mice (Fig. 5 (**A,C,D**); $p > 0.05$). This was in line with our RT-qPCR data showing no alteration of *Gad1* mRNA in *Cntnap2* mice (Fig. 4(**B**)) but not in *Shank3b* mice that showed upregulation instead (Fig. 4(**A**)). In addition, in adult animals, while GFAP protein expression was reduced in *Cntnap2*$^{-/-}$ mice, no difference were found in *Shank3b*$^{-/-}$ mice (Fig. 5(**B,E,F**) $p > 0.05$). Our RT-qPCR instead reported increased *Gfap* mRNA levels in *Cntnap2*$^{-/-}$ mice (Fig. 4(**D**)) while reduced mRNA levels in *Shank3b*$^{-/-}$ mice (Fig. 4(**C**)). In juvenile animals, GAD67 protein expression was unaltered in both *Cntnap2*$^{-/-}$ and *Shank3b*$^{-/-}$ mice (Fig. 6(**A,C,D**); $p > 0.05$) while increased *Gad1* mRNA levels were found in both mutant lines (Fig. 4(**A,B**)). Moreover, GFAP protein expression was reduced in *Cntnap2*$^{-/-}$ mice (Fig. 6(**B,E**); $p < 0.05$) while unaltered in *Shank3b*$^{-/-}$ mice (Fig. 6(**B,F**);

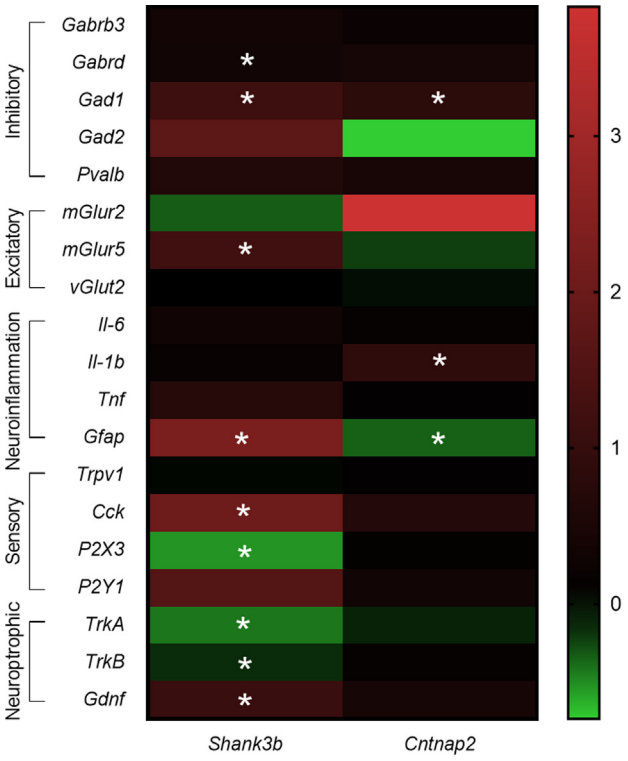

Fig. 3. Heatmap of Log2 fold change values for juvenile (P30) *Shank3b* and *Cntnap2* TG. Values above 0 indicate an upregulation of the respective gene in KO mice relative to WT controls (in red). Conversely values below 0 indicate a downregulation of a gene in KO animals relative to WT controls (in green). Genes annotated with an asterisk (*) show a significant difference in expression values ($p < 0.05$).

$p > 0.05$). This was in line with our RT-qPCR data showing decreased *Gfap* mRNA levels in *Cntnap2*$^{-/-}$ mice (Fig. 4(**D**)) but not in *Shank3b*$^{-/-}$ mice that showed increased *Gfap* mRNA levels instead (Fig. 4(**C**)). A summary of these findings can be found in Fig. 7.

DISCUSSION

The TG is among the first stations in tactile sensory processing in the mouse somatosensory system. Alteration of this crucial structure may hence have a role in sensory processing abnormalities. The present investigation sought to test several neuronal and non-neuronal markers involved in cellular mechanisms (inhibition, excitation, neuroinflammation) within the trigeminal ganglia of both adult and juvenile *Shank3b* and *Cntnap2* mouse models of ASD. Untangling the role of such markers based solely on their gene expression throughout development carries an inherent level of difficulty as several cellular processes may interfere with and influence the resulting behavioral phenotypes. Nonetheless, the present work is the first to describe gene expression alteration in trigeminal ganglia of genetic mouse models of ASD and give possible support for the involvement of particular markers within the TG in ASD.

The present study revealed significant alterations in markers for sensory neurons in the TG of *Shank3b*$^{-/-}$ and *Cntnap2*$^{-/-}$ mice, even at juvenile ages. Specifically, we showed an increased mRNA expression in several sensory markers involved in nociception, such as *Trap1*, *Calca*, and *Tacr1* in *Shank3b*$^{-/-}$ and *Calca* and *Sst* in *Cntnap2*$^{-/-}$ TG. Furthermore, *P2X7*, a purinergic receptor expressed by satellite glial cell and involved in pain transmission (Ren and Illes, 2022) is upregulated in *Cntnap2*$^{-/-}$ adults. Such findings converge with previous studies reporting instances of increased pain sensitivity in ASD patients and animal models (Failla et al., 2020; Zhang et al., 2021). Other researchers however find a hyposensitivity in response to stimuli among ASD individuals and models (Allely, 2013; Dhamne et al., 2017). Altered expression of sensory markers *P2X3* and *Cck* in juvenile *Shank3b*$^{-/-}$ mice (downregulated and upregulated respectively) attest to early transcriptomic changes in the trigeminal ganglion.

Dysregulation in GABA receptor expression and interneuron functioning is commonly seen among humans with ASD as well as within animal models of this disorder (Cellot and Cherubini, 2014; Vogt et al., 2018; Zhao et al., 2022). In line with this, the present study found that inhibitory markers, the majority of which GABAergic, were among the most altered in *Shank3b*$^{-/-}$ and *Cntnap2*$^{-/-}$ TG. Specifically, we found an upregulation of *Gabra1*, *Gabra2*, *Gabrb2*, and *Gabrb3* mRNA in *Shank3b*$^{-/-}$ and *Cntnap2*$^{-/-}$ trigeminal ganglia. Previous studies in the cerebral cortex of ASD mouse models found instead the reduction of GABA receptor units such as GABAA receptors (Cellot and Cherubini, 2014; Fatemi et al., 2014; Orefice et al., 2019; Zhao et al., 2022). Upregulation found in GABAergic markers in the trigeminal ganglia could be interpreted as underlining increased inhibition or alternatively be a compensatory reaction to a decrease in GABA availability at the synapse. Further research is needed to properly address these points. In juvenile mice, on the other hand, an upregulation was found among *Shank3b*$^{-/-}$ TG for *Gabrd*, a gene whose dysfunction promotes psychomotor delay and epilepsy (Windpassinger et al., 2002).

Our qRT-PCR results also unveiled an upregulation of the metabotropic glutamate receptor 5 (*mGluR5*) in the trigeminal ganglia of *Shank3b*$^{-/-}$ juvenile mice. The role of mGluR5 in ASD is supported by studies that associate its increased activity with the manifestation of symptoms in Fragile X Syndrome (Yan et al., 2005). We also found downregulation of *Kcna1* in *Shank3b*$^{-/-}$ adult TG which encodes for the Kv1.1 voltage-gated potassium channel subunits that regulate axonal excitability and whose loss of function missense mutations are often associated with ataxia and epilepsy (Thouta et al., 2021). These results, suggesting an increased excitability in both *Shank3b* and *Cntnap2* models, seem to conflict with our findings on inhibitory markers. It is imperative to underline, however, that contrasting evidence on the role of excitatory markers in ASD is available (Oka and Takashima, 1999; Lohith et al., 2013; Chana et al., 2015; Cai et al., 2019) and that the present results are of smaller magnitude than that of other marker types.

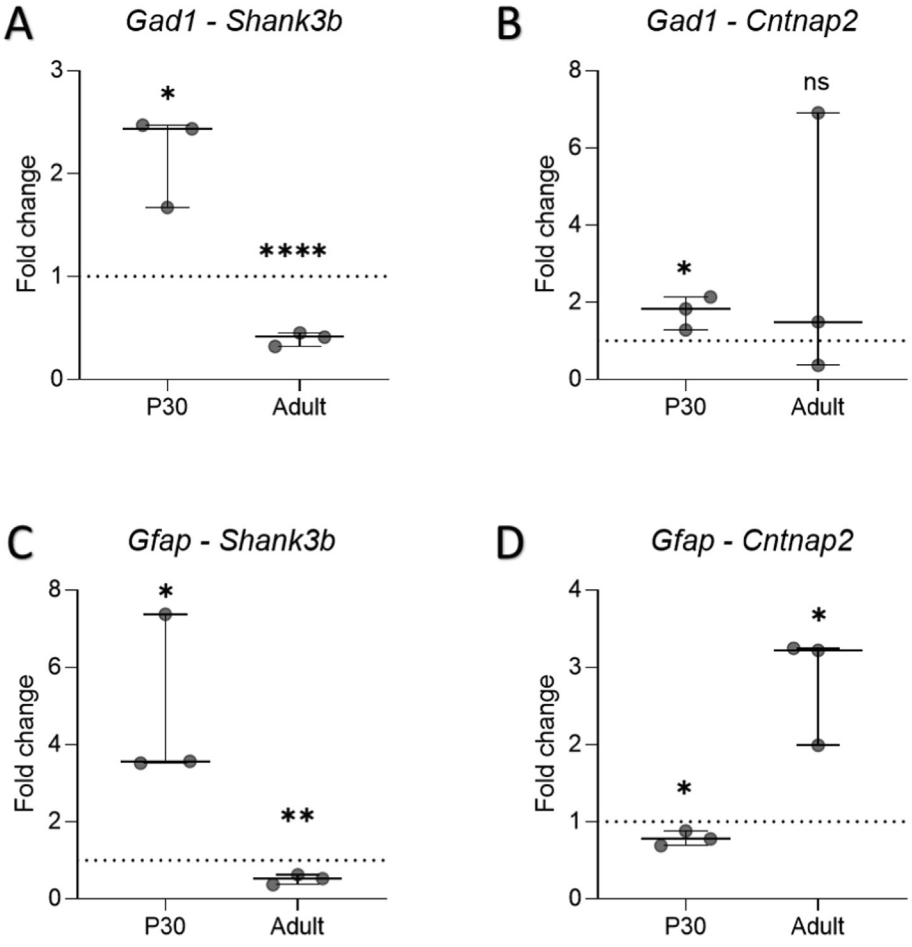

Fig. 4. Expression of *Gad1* and *Gfap* mRNAs in Shank3b (**A,C**) and Cntnap2 (**B,D**) adult and juvenile (P30) TG. Values are reported as fold changes respect to WT controls.

Hence, further investigation is crucial for untangling the influence of excitatory gene expression in ASD.

Neuroinflammation has been proposed to contribute the development of ASDs by prompting neuronal dysfunction that characterize these conditions (Eissa et al., 2020). Several lines of research document the elevated expression of inflammatory markers such as pro-inflammatory cytokines in the brain and blood of ASD subjects (Varga et al., 2005; Molloy et al., 2006; Li et al., 2009; Rodriguez and Kern, 2011; Zhao et al., 2021). Our experiments showed an upregulation of inflammatory markers such as *S100b*, *iNOS*, and *Il-1b* in the TG of *Shank3b*$^{-/-}$ and *Cntnap2*$^{-/-}$ mice. However, other inflammatory markers (*Tnf*, *Iba1*, *Ccl-5*, and *Tmem-119*) were instead downregulated in the TG of *Shank3b*$^{-/-}$ and *Cntnap2*$^{-/-}$ mice. While no evidence of immune dysfunction in the trigeminal ganglia from ASD individuals or animal models have been reported so far, the implications of our data based solely on transcript changes require further investigation.

Finally, we also tested neurotrophic factors and their receptors. Neurotrophic factors are essential regulators of neuronal maturation including synaptic synthesis and have been implicated in the origins of neurodevelopmental disorders such as ASD and ADHD (Nickl-Jockschat and Michel, 2011). While no differences in mRNA expression were found for *Bdnf* (brain derived neurotrophic factor) in both mutant mouse lines, mRNA for *Trkb* receptor was found downregulated in the TG of juvenile *Shank3b*$^{-/-}$mice. NGF (nerve growth factor) is another neurotrophic factor crucial to typical development which has been found to be altered in ASD (Dinçel et al., 2013; Mostafa et al., 2021). Again, while *Ngf* itself was not altered in both models, our results do show a downregulation of its receptor *TrkA* in *Shank3b*$^{-/-}$ juvenile TG. The glial-derived nerve growth factor *Gdnf* was likewise found to be upregulated in *Shank3b*$^{-/-}$ juvenile and *Cntnap2*$^{-/-}$ adult TG. *Gdnf*, whose upregulation is associated with ADHD (Galvez-Contreras et al., 2017), supports cell survival (specifically of dopaminergic cells) (Lin et al., 1993).

Among the markers tested in the present study, the expression profiles of *Gad1* and *Gfap* mRNA are particularly relevant. While *Gad1* is downregulated in *Shank3b*$^{-/-}$ adults, its expression is upregulated in KO juveniles of both lines. *Gad1* encodes for the GAD67 enzyme crucial to GABA synthesis, particularly during early neurodevelopment (Feldblum et al., 1993) but also in the glutamate cycle, and its dysregulation may be involved in creating an excitatory/inhibitory imbalance (Rubenstein and Merzenich, 2003). Interestingly, increased levels of GABA, glutamate and glutamine were also found in *Cntnap2* rats (Möhrle et al., 2021). Lower levels of both *GAD1* mRNA and protein have been found in the postmortem brains of autistic adults (Fatemi et al., 2002; Yip et al., 2007; Chao et al., 2010; Zhubi et al., 2017) as well as in animal models (Peñagarikano et al., 2011) in several areas throughout the cerebral cortex. On the other hand, increased GAD1 expression has been documented in the cerebellum of postmortem autistic brains (Yip et al., 2008) and the prefrontal cortex of ASD animal models (El Idrissi et al., 2005; Hou et al., 2018). *Gad1* overexpression correlates with increased GABA synthesis and therefore promotes inhibition (Dicken et al., 2015). Despite reports of GABA expression in the trigeminal ganglia its effect seems to be neuromodulatory and of signaling with satellite glial cells (Hayasaki et al., 2006). Chemogenetic depolarization of GABAergic DRG reduces peripherally-induced nociception, while decreasing inhibition by introducing GABA receptor antagonists on sensory ganglia trigger nociception (Du et al., 2017). Moreover, recent work on

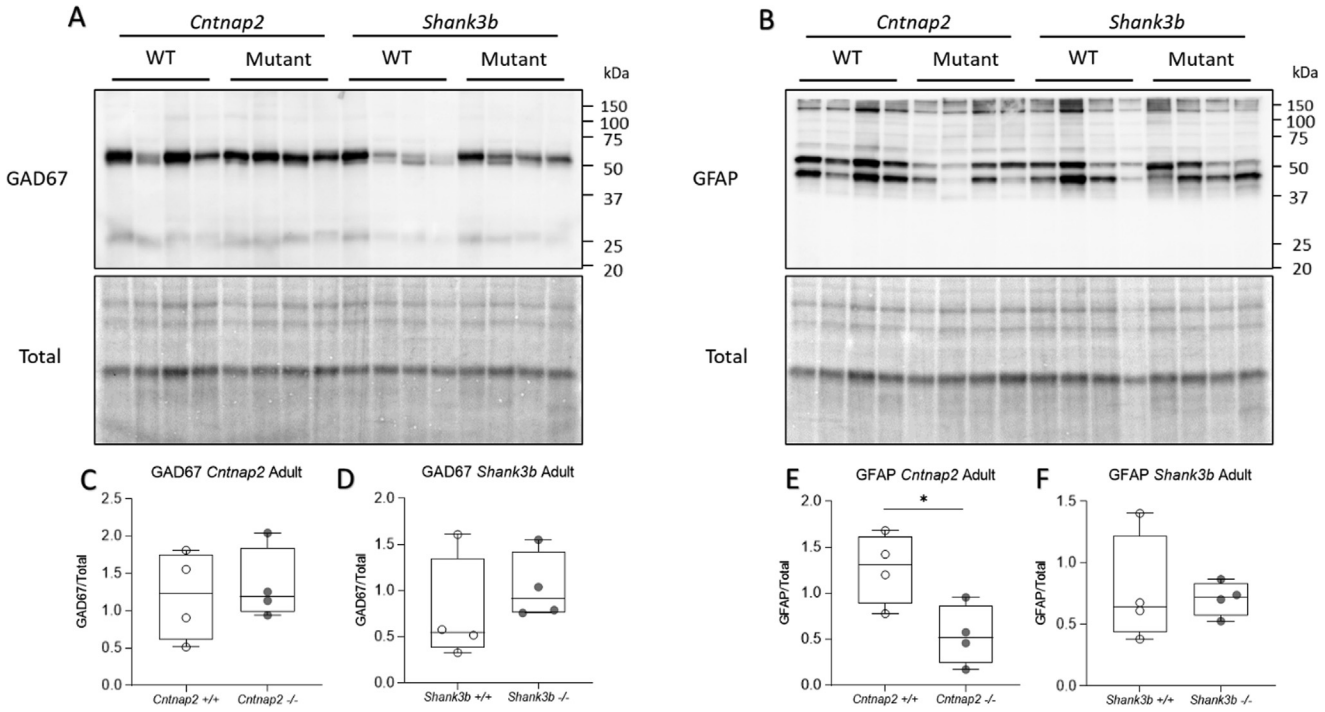

Fig. 5. Representative western blots (**A,B**) and their quantification (**C-F**) on trigeminal ganglia from *Shank3b* and *Cntnap2* adult animals.

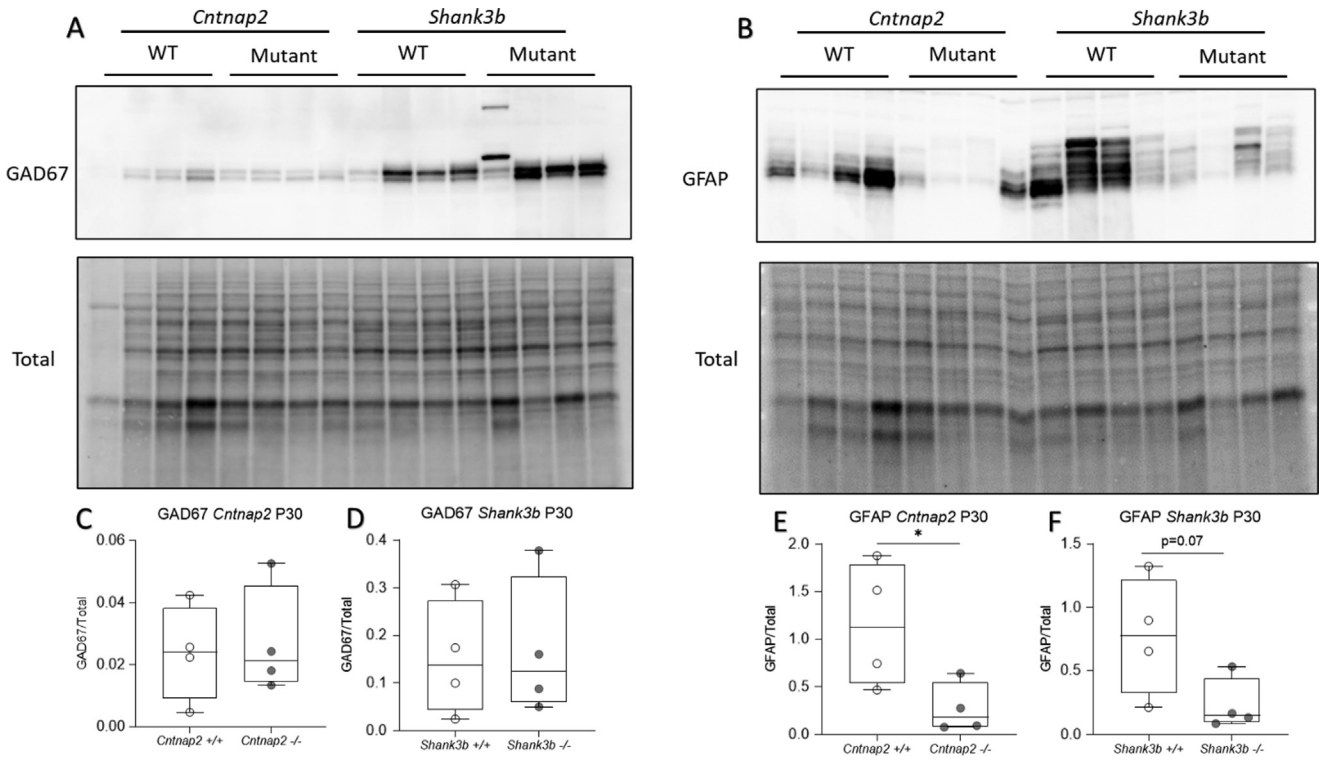

Fig. 6. Representative western blots (**A,B**) and their quantification (**C-F**) on trigeminal ganglia from *Shank3b* and *Cntnap2* P30 animals.

cortical networks (Haroush and Marom, 2019) suggests that inhibition may reduce discrimination between stimuli. However, western blot experiments on GAD67 protein expression did not show differences in *Shank3b* and *Cntnap2* TG in both developmental stages. Numerous studies indicate that many ways for regulating the expression of the *GAD* gene and its protein levels exist, including transcriptional and posttranscriptional mechanisms (Pinal et al., 1997; Yanagawa et al., 1997; Soghomonian and Martin, 1998). Interestingly, a discrepancy between *Gad1* mRNA and GAD67 protein expression was also found in the cerebral cortex of patient with

	Shank3b				*Cntnap2*			
	Adult		P30		Adult		P30	
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
GAD67	Down	No diff.	Up	No diff.	No diff.	No diff.	Up	No diff.
GFAP	Down	No diff.	Up	No diff.	Up	Down	Down	Down

Fig. 7. Summary of mRNA data and protein data from RT-qPCR and western blot experiments respectively.

schizophrenia (Dracheva et al., 2004) suggesting a fine regulatory activity between transcript production and protein expression.

The present study also found *Gfap* mRNA upregulation in the juvenile *Shank3b*$^{-/-}$ and in the adult *Cntnap2*$^{-/-}$ TG. Conversely, *Gfap* mRNA was downregulated in the TG of *Shank3b*$^{-/-}$ adults and *Cntnap2*$^{-/-}$ juveniles. The *Gfap* gene codes for GFAP expressed in glial cells, in the TG specifically in satellite glial cells (Stephenson and Byers, 1995). GFAP expression has been reported to be deregulated in the brain of autistic subjects (Laurence and Fatemi, 2005; Edmonson et al., 2014; Crawford et al., 2015). In contrast to our RT-qPCR data, we did not find differences in GFAP protein expression in both adults and juvenile Shank3b mutants. On the other hand, while *Gfap* mRNA was found upregulated in *Cntnap2* $^{-/-}$ adult mice, GFAP protein showed reduced expression instead. GFAP protein expression was similarly decreased in juvenile *Cntnap2*$^{-/-}$ mice, consistent with reduced *Gfap* mRNA expression. These result support the existence of a difference in the post-transcriptional control of *Gfap* gene expression in the trigeminal ganglion. Alternatively, the stability of *Gfap*-mRNA may differ in the TG respect to other regions. It is still difficult to say whether the alterations in *Gfap* mRNA levels is caused directly by *Shank3b* or *Cntnap2* deficiency or is mediated by other factors within the trigeminal ganglion. Indeed, it has been shown that GFAP synthesis is also regulated by growth factors and cytokines (Laping et al., 1994), which we also found altered at mRNA level in TG of both mutant mice. Further investigation is needed to understand the functional regulation of GFAP expression in the trigeminal ganglion of mouse models of ASD.

In this study, we uncovered for the first time significant differences in gene expression of important markers of neuronal and glial function in the TG of *Shank3b*$^{-/-}$ and *Cntnap2*$^{-/-}$ mice at young and adult age with some of them confirmed at protein level via western blot. A true understanding of the functional significance of these alterations in gene expression found in the TG of *Shank3b*$^{-/-}$ and *Cntnap2*$^{-/-}$ will only be achieved prior further investigations. Such results open avenues for the development of peripherally targeted treatments for tactile sensory deficits observed in ASD.

AUTHOR CONTRIBUTIONS

AGCC performed rt-qpcr experiments, analysed data and drafted the manuscript; VB performed western blot experiments and analyzed data; EB provided funding; YB provided funding and edited the manuscript; LB designed and supervised the study, analyzed data, and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2023.08.028.

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