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Expression of Cntnap2 (Caspr2) in multiple levels of sensory systems

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ABSTRACT

Genome-wide association studies and copy number variation analyses have linked contactin associated protein 2 (Caspr2, gene name *Cntnap2*) with autism spectrum disorder (ASD). In line with these findings, mice lacking Caspr2 (*Cntnap2*$^{-/-}$) were shown to have core autism-like deficits including abnormal social behavior and communication, and behavior inflexibility. However the role of Caspr2 in ASD pathogenicity remains unclear. Here we have generated a new Caspr2:tau-LacZ knock-in reporter line (*Cntnap2*$^{tlacz/tlacz}$), which enabled us to monitor the neuronal circuits in the brain expressing Caspr2. We show that Caspr2 is expressed in many brain regions and produced a comprehensive report of Caspr2 expression. Moreover, we found that Caspr2 marks all sensory modalities: it is expressed in distinct brain regions involved in different sensory processings and is present in all primary sensory organs. Olfaction-based behavioral tests revealed that mice lacking Caspr2 exhibit abnormal response to sensory stimuli and lack preference for novel odors. These results suggest that loss of Caspr2 throughout the sensory system may contribute to the sensory manifestations frequently observed in ASD.

1. Introduction

Caspr2 (human gene name CNTNAP2) is a neuronal cell adhesion protein, of the neurexin superfamily, initially described in myelinating nerves. It serves to cluster voltage-gated potassium (Kv) channels at the juxtaparanode, a membrane domain adjacent to the node of Ranvier (Poliak et al., 1999, 2003) and can also form a barrier holding nodal components in place (Gordon et al., 2014). In the CNS, in mature pyramidal neurons, Caspr2 is found in axons, dendrites, dendritic spines and the soma. It is present in a subset of excitatory synapses where it colocalizes with GluA1 (Varea et al., 2015). Supporting this, Caspr2 (together with its binding partner TAG1/Contactin 2) was found in the synaptic plasma membranes fraction of the forebrain (Bakkaloglu et al., 2008). Elimination or reduction of Caspr2 resulted in decreased spine density and altered spine morphology as well as in lower levels of AMPA receptor subunit on the spines (Anderson et al., 2012; Varea et al., 2015). In the absence of Caspr2 new synaptic spines are unable to stabilize (Gdalyahu et al., 2015) which may explain the decreased spine density seen in the null mice.

Over the past few years many studies have shown association between Caspr2 and several mental disorders including: autism spectrum disorder (ASD) (Alarcon et al., 2008), schizophrenia, bipolar disorder (Wang et al., 2010), epilepsy (Mefford et al., 2010), Alzheimer's disease (van Abel et al., 2012) and language disorders (Newbury et al., 2011). Many of these studies have focused on ASD, and using linkage and genome-wide association studies (GWAS) as well as copy number variation (CNV) analyses have clearly associated between Caspr2 and ASD (Alarcon et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008; Li et al., 2010; O'Roak et al., 2011). Additionally, common variants in Caspr2 have also been associated with ASD (Anney et al., 2012; Arking et al., 2008; Stein et al., 2011). Numerous mutations in Caspr2 have been identified, in many different regions of the protein, both intra- and extra-cellularly, and have been associated with ASD (Bakkaloglu et al., 2008). Furthermore, variants in the 5′ promoter of Caspr2 were shown to be risk factors for ASD. Some of these variants were shown to have reduced transcriptional efficiency leading to lower levels of expression of Caspr2 (Chiocchetti et al., 2014).

Caspr2 null mice can serve as a model for ASD as they exhibit many of its characteristics. They display epileptic seizures as well as some core autism related deficits. These include stereotypic motor movements, behavioral inflexibility, and communication and social abnormalities. Morphologically, these mice show astrogliosis in the hippocampus, neuronal migration abnormalities and a reduced number of GABAergic interneurons. These mice also exhibit reduced neuronal synchrony in the cortex. Additional evidence that these phenotypes are in fact autism related is demonstrated by the reduction in the repetitive behavior when treating the mice with Risperidone, an approved drug for symptomatic treatment of ASD (Penagarikano et al., 2011). Furthermore, treating these mice with oxytocin rescued the social deficits found in the null mice (Penagarikano et al., 2015).

However, the cellular and molecular mechanisms that implicate Caspr2 in ASD pathogenicity are still unknown. To address this question it is crucial to establish which networks of the brain express Caspr2 and

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at which developmental stage. In this study we generated a reporter mouse line in which tau-LacZ replaces the first exon of Caspr2 (*Cntnap2*$^{tlacz/tlacZ}$), which allowed us to study the brain areas and networks expressing Caspr2. We describe a comprehensive atlas of Caspr2 expression including expression in the cortex hippocampus and many areas of the thalamus as well as in many components of the limbic system. We found expression of Caspr2 in many sensory processing areas as well as in the primary sensory organs. Moreover, we demonstrated that Caspr2 null mice have impaired sensory processing.

2. Methods and materials

2.1. Derivation of mutant mice

The targeting construct was designed to replace the first exon encoding the ATG and the signal sequence of Caspr2 with a tau-LacZ gene and an oppositely directed *neo* gene (Fig. 1A). The construct was cloned from a 129SvJ genomic phage library. Genomic fragments of 3.3 and 4.0 kb located upstream and downstream of exon 1, respectively, were cloned into the pKO-SelectNeo vector (Stratagene, La Jolla, USA). This strategy resulted in a deletion of 828 bp, including the first exon. R1 ES cells were transfected with the linearized targeting construct, and recombinant ES clones were selected with G418. Clones exhibiting correctly targeted integrations were identified by Southern hybridization. Chimeric mice were generated by aggregation of the targeted ES cells. They were mated with ICR females, and germ-line transmission was detected by coat color and Southern analysis of tail DNA. Genotyping of progenies was performed by PCR of genomic DNA using primer sets derived from the deletion in Caspr2-targeted allele (5′-TTGGGTGGAGAGGCTATTCGGCTATG-3′ to 5′-TCAGAGTTGATACC CGAGCGCC-3′), as well as by using primers for the LacZ gene (5′-CTGGATAACGACATTGGCGTAAG-3′ to 5′-AGATCCCAGCGGTCAAAA CAG-3′). Mice carrying the mutant allele were backcrossed for 10 generations onto C57B6/J (Jackson) background. All experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by the Weizmann's Institutional Animal Care and Use Committee.

2.2. Antibodies

Rabbit anti Caspr2 antibody was raised by immunizing rabbits with an Fc-fusion protein containing the extracellular domain of human Caspr2 until the fibrinogen like domain. Other antibodies used were mouse anti beta-galactosidase (G8021, Sigma Aldrich, Rehovot, Israel), mouse anti tubulin (SAP.4G5, Sigma), chicken anti pan Neurofascin (AF3235, R&D systems, Minneapolis, USA) and goat antibody anti ChAT (AB144P, Merck-Millipore, USA). 488-, and Cy3- coupled antibodies were obtained from Jackson ImmunoResearch.

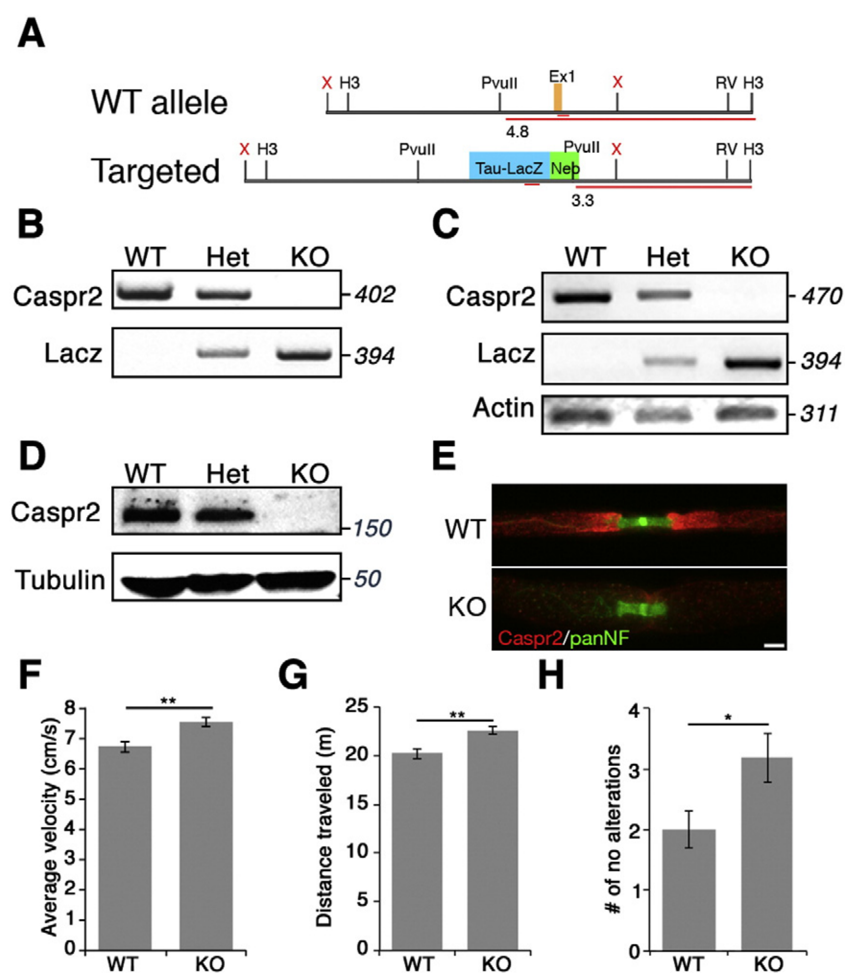

Fig. 1. Replacement of the first exon of Caspr2 with a tau-LacZ results in a complete knockout with a phenotype comparable to the Caspr2 knockout mice. A. Schematic representation of WT genomic Caspr2, targeting vector (construct) and targeted mutant allele. B. PCR analysis of genomic tail DNA of the indicated genotypes detecting the WT and targeted Caspr2-LacZ alleles. C. RT-PCR analysis of brain mRNA revealed the absence of a complete Caspr2 transcript coupled to the presence of the tau-LacZ gene in null mice. Actin levels were used as controls. D. Western blot analysis of brain using antibodies that recognize the Caspr2, or tubulin as control. No signal is detected in Caspr2-lacZ null mice. E. Immunolabeling of teased sciatic nerves from Caspr2-LacZ mice using antibodies to Caspr2 (red), and a nodal and paranodal marker Neurofascin (panNF; green). F-G. Average velocity (F) and total distance traveled (G) over a five minute period. n = 13 for each genotype. H. Number of no alterations in ten trials of a spontaneous T-maze alteration test. n = 11 for each genotype. $^*p < 0.05$. Scale bar 5 μm.

2.3. Immunoblotting

Freshly dissected tissues were homogenized in RIPA buffer (50 mM Tris–HCl pH = 7.4, 1% NP-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, proteinase inhibitor (Sigma Aldrich, Rehovot, Israel)), incubated on ice for 30–60 min, centrifuged at 10,000 *g* for 30 min, and the supernatant was collected for further use. SDS-PAGE sample buffer was added and protein lysate and resolved in Tris-Acetate acrylamide gels. Immunoblotting was done as previously described (Gollan et al., 2002).

2.4. RT-PCR

Total RNA was isolated from freshly dissected rat brains of the indicated age using TRI-reagent (Sigma Aldrich, Rehovot, Israel). cDNA were obtained with Super Script reverse transcriptase II (Invitrogen, Carlsbad, USA) and were normalized among different samples with actin-specific primers. Primers against Caspr2 (GGAATGGAGAAGGTCACATCG and ACCAGAGAAAGGTATGCCTCC-3′), LacZ (CTGGATAACGACATTGGCGT AAG and AGATCCCAGCGGTCAAAACAG) and actin (GAGCACCCTGTGCT GCTCACCGAGG and GTGGTGGTGAAGCTGTAGCCACGCT) were used.

2.5. X-gal staining

Adult brain, spinal cord, and optic nerves were obtained from 1% PFA perfused mice. The tissues were then left overnight in 1% PFA, 30% sucrose in PBS followed by 2 h in 0.5% glutaraldehyde, 30% sucrose in PBS. Developing mice brains were fixed in 1% PFA for 0.5–2.5 h followed by 2 h in 0.5% glutaraldehyde and were then left overnight in 30% sucrose. For sectioning tissues were embedded in OCT (Tissue-Tek) and frozen on dry ice. 10–50 μm thick sections were prepared using a sliding microtome. Adult cochlea were fixed using 4% PFA and 0.2% glutaraldehyde in PBS for 30 min on ice. Retinas were fixed with 4% PFA for 30 min on ice. For olfactory system staining the head was cut sagittally down the midline and was fixed in 4% PFA on ice for 30 min. Staining was done with 1 mg/ml X-GAL (Sigma Aldrich, Rehovot, Israel) in 20 mM Tris ph = 7.3, 5 mM ferricyanide, 5 mM ferrocyanide, 0.01% sodium-deoxycholate, 0.02% NP-40 and 2 mM MgCl2 in PBS overnight at 37 °C. Images were obtained using a Nikon E800 microscope or Panoramic MIDI scanner (3DHistech, Budapest, Hungary).

2.6. Immunofluorescence

Tissues were removed and fixed in 4% paraformaldehyde for 30 min on ice. For sectioning tissues were cryo-protected in 30% sucrose (in PBS) over-night at 4 °C, embedded in OCT (Tissue-Tek), and frozen on dry ice. Sciatic nerves were desheathed and teased on SuperFrost Plus slides (Menzel-Gläser, Thermo Scientific, Braunschweig, Germany). Slides were air-dried over-night, and then kept frozen at −20 °C. Blocking and permabilaztion were done by incubation for 45–60 min in 5% normal goat serum and 0.5% Triton X-100, in PBS at room temperature. Primary antibodies were diluted in 5% normal goat serum and 0.2% Triton X-100, in PBS and incubated over-night at 4 °C. Secondary antibodies were incubated for 45 min at room temperature in 5%

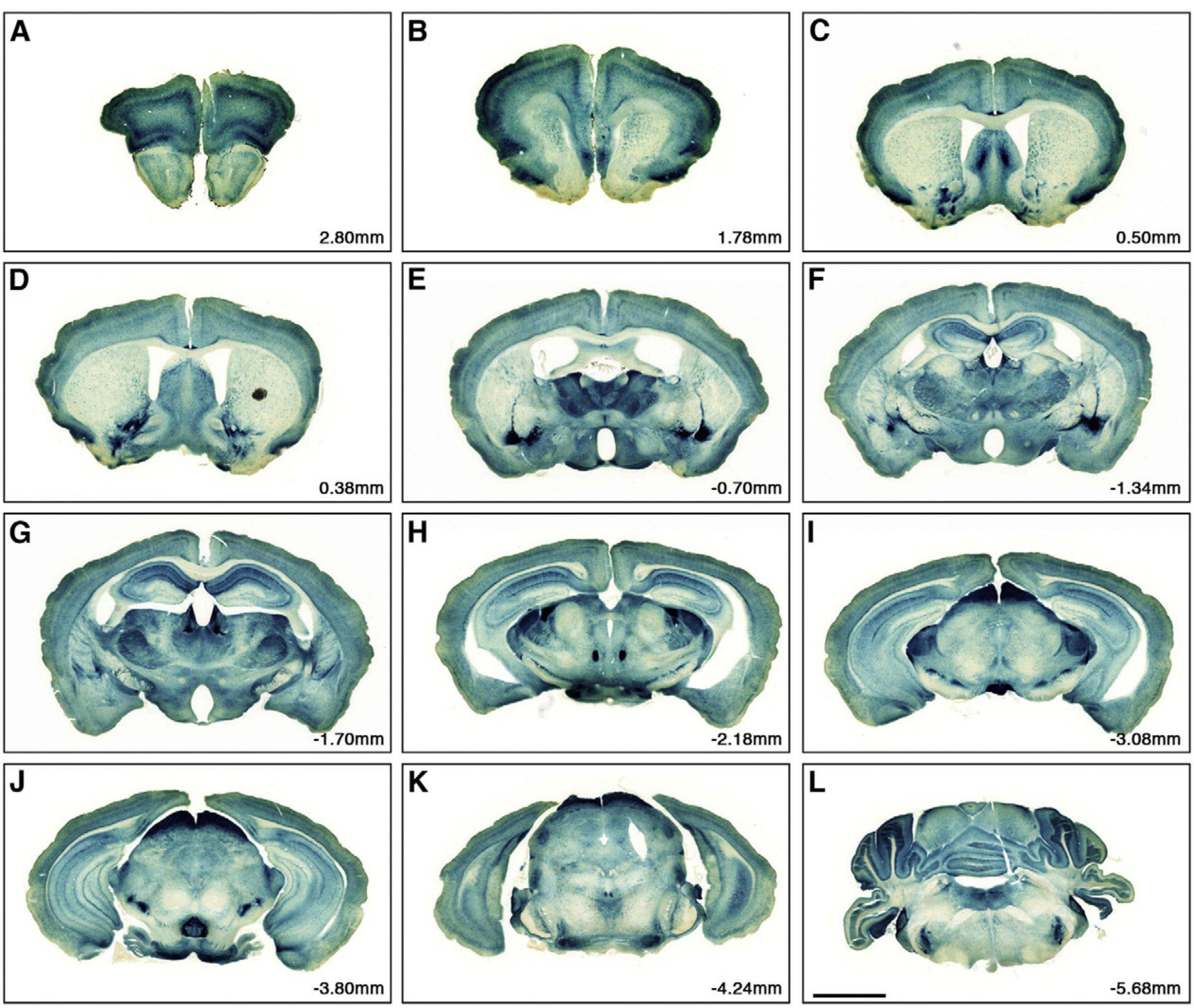

Fig. 2. Caspr2 expression in the adult mouse brain. Serial sections through the brain show Caspr2 expression in many areas detailed in Table 1. Scale bar 2 mm.

Table 1
Brain areas expressing Caspr2.

Abbrev.	Name	Sensory system	Ref
12	hypoglossal nucleus		
ACo	anterior cortical amygdaloid nucleus	Olfactory	(1)
AD	anterodorsal thalamic nucleus		
AHiPM	amygdalohippocampal area posterior medial		
AI	agranular insular cortex		
AL	nucleus ansa lenticularis		
AM	anteromedial thalamic nucleus		
APTD	anterior pretectal nucleus dorsal	Somatosensory/visual	(2, 3)
Arc	arcuate hypothalamic nucleus		
ATg	anterior tegmental nucleus		
AVDM	anteroventral thalamic nucleus dorsomedial		
AVVL	anteroventral thalamic nucleus ventrolateral		
bic	brachium inferior colliculus	Auditory	(4)
BSTLP	bed nucleus of the stria terminalis lateral division posterior	Olfactory	(5)
BSTLV	bed nucleus of the stria terminalis lateral division ventral	Olfactory	(5)
BSTMA	bed nucleus of the stria terminalis medial division medial	Olfactory	(5)
CA1	CA1 field hippocampus		
CA2	CA2 field hippocampus		
Ce	central amygdala nucleus	Somatosensory	(6)
CGPn	central gray pons	Somatosensory	(7)
CM	central medial thalamic nucleus		
CPu	caudate putamen		
Cu	cuneate nucleus	Somatosensory	(8)
DC	dorsal cochlear nucleus	Auditory	(4)
df	dorsal fornix		
DLG	dorsal lateral geniculate nucleus	Visual	(9)
DLO	dorsolateral orbital cortex		
DLPAG	dorsal lateral periaqueductal gray	Somatosensory	(7)
DM	dorsomedial hypothalamic nucleus		
DMPAG	dorsomedial periaqueductal gray	Somatosensory	(7)
DMPn	dorsomedial pontine nucleus		
DMSP5	dorsomedial spinal trigeminal nucleus	Somatosensory	(10)
DT	dorsal terminal nucleus of the accessory optic tract	Visual	(11)
ECIC	external cortex inferior colliculus	Auditory	(12)
ECu	external cuneate nucleus	Somatosensory	(13)
fi	fimbria hippocampus		
fr	fasciculus retroflexus		
GRC	granular layer cochlear nucleus	Auditory	(12)
HDB	nucleus horizontal limb digonal band	Olfactory	(14)
IAD	interanterior dorsal thalamic nucleus		
IAM	interanteromedial thalamic nucleus		
ic	internal capsule		
IG	indusium griseum	Olfactory	(15)
IMD	intermediodorsal thalamic nucleus		
IP	interpeduncle nucleus		
IPACL	interstriatal nu. posterior limb–anterior commissure, lateral		
IPACM	interstriatal nu. posterior limb–anterior commissure, medial		
LA	lateral amygdaloid nucleus	Auditory	(16)
Lat	lateral (dentate) cerebellar nucleus		
LDDM	laterodorsal thalamic nucleus dorsomedial	Somatosensory	(17)
LDTg	laterodorsal tegmental nucleus ventral	Visual/auditory/somatosensory	(18)
LDVL	laterodorsal thalamic nucleus ventrolateral	Somatosensory	(17)
LHB	lateral habenula		
LM	lateral mammillary nucleus		
LPBC	lateral parabrachial nucleus central		
LPBD	lateral parabrachial nucleus dorsal		
LPMR	lateral posterior thalamic nucleus mediocaudal		
LSD	lateral septal nucleus dorsal		
LSI	lateral septal nucleus intermediate		
LSO	lateral superior olive	Auditory	(4)
LT	lateral terminal nucleus accessory optic tract	Visual	(19)
MCPO	medial preoptic nucleus central	Olfactory	(20)
MDC	mediodorsal thalamic nucleus central	Olfactory	(21)
MDL	mediodorsal thalamic nucleus lateral	Olfactory	(21)
MDM	mediodorsal thalamic nucleus medial	Olfactory	(21)
ME	median eminence		
MEA	medial amygdaloid nucleus anterior	Olfactory	(5)
MGD	medial geniculate nucleus dorsal	Auditory	(12)
MGM	medial geniculate nucleus medial	Auditory	(12)
MGV	medial geniculate nucleus ventral	Auditory	(12)
MHB	medial habenular nucleus		
ML	medial mammillary nucleus lateral		
MM	medial mammillary nucleus		

(continued on next page)

Table 1 (*continued*)

Abbrev.	Name	Sensory system	Ref
MO	medial orbital cortex		
MPA	medial preoptic area	Olfactory	(5)
MPB	medial parabrachial nucleus	Gustatory	(22)
MPOM	medial preoptic nucleus medial	Olfactory	(5)
MVeMC	medial vestibular nucleus mediocaudal	Vestibular	(23)
MVePC	medial vestibular nucleus paravicel	Vestibular	(23)
MZMG	marginal zone of the medial geniculate	Somatosensory/auditory	(24)
PaAP	paraventricular hypothalamic anterior parvicellular		
PAG	periaqueductal gray	Olfactory	(5)
PaV	paraventricular hypothalamic nucleus ventral		
PC	paracentral thalamic nucleus		
Pir	piriform cortex	Olfactory	(25)
PL	paralemniscal nucleus	Auditory	(26)
PLi	posterior limitans thalamic nucleus	Visual	(11)
Pn	pontine nucleus		
Po	posterior thalamic nuclear group	Visual	(27)
Pr	prepositus hypoglossal nucleus		
Pr5DM	principal sensory trigeminal nucleus dorsomedial	Somatosensory	(13)
Pr5VL	principal sensory trigeminal nucleus ventrolateral	Somatosensory	(13)
PrC	precommissural nucleus		
PV	paraventricular thalamic nucleus		
PVA	paraventricular thalamic nucleus anterior		
PVN	paraventricular hypothalamic nucleus		
py	pyramidal tract		
RC	raphe capsule		
RPO	rostral periolivary region	Auditory	(28)
RRF	retrorubral field		
S1	somatosensory 1	Somatosensory	(29)
SC	superior colliculus	Visual	(30)
SChDM/VL	suprachiasmatic nucleus dorsomadial/ventrolateral		
SG	suprageniculate thalamic nucleus	Visual/auditory/somatosensory	(31)
SGL	superficial glial layer, cochlear nucleus	Auditory	(4)
SI	substantia innominata	Gustatory	(22)
SNC	substantia nigra compacta		
Sol	solitary tract nucleus	Gustatory	(22)
SP5I	spinal trigeminal nucleus interpolar	Somatosensory	(13)
SP5O	spinal trigeminal nucleus oral	Somatosensory	(13)
SP5OVL	spinal 5 nucleus oral ventrolateral	Somatosensory	(13)
SPF	subparafasciular thalamic nucleus		
SPO	superior paraolivary nucleus	Auditory	(12)
STh	subthalamic nucleus		
Sub	submedius thalamic nucleus	Somatosensory	(32)
SubB	subbrachial nucleus		
SuM	supramammillary nucleus		
SuML	supramammillary nucleus lateral		
TC	tuber cinereum area		
Te	terete hypothalamus		
tfp	transverse fibers pons		
Tz	nucleus trapezoid body	Auditory	(12)
VCP	ventral cochlear nucleus posterior	Auditory	(12)
VL	ventrolateral thalamic nucleus		
VLG	ventrolateral geniculate nucleus	Visual	(9)
VLGMC	ventrolateral geniculate nucleus magnocellular	Visual	(9)
VM	ventromedial thalamic nucleus		
VMH	ventromedial hypothalamic nucleus	Olfactory	(5)
VP	ventral pallidum		
VPL	ventral posterolateral thalamic nucleus	Somatosensory	(33)
VPM	ventral posteromedial thalamic nucleus	Gustatory/somatosensory	(33)
VTRZ	visual tegmental relay zone	Visual	(34)
Xi	xiphoid thalamic nucleus		

normal goat serum and 0.1% Triton X-100, in PBS. Samples were mounted with elvanol. Images were taken using Nikon eclipse 90i microscope or Zeiss LSM710 confocal microscope.

2.7. Behavioral analysis

3 chamber olfactory test — non-social odors used were: distilled water, banana extract and almond extract (Bakto-flavors, USA). Both Odors were diluted 1:100 in distilled water. 100 μl of the odor was soaked into a 2 × 2 cm piece of Whatman paper. For social olfactory stimuli bedding was taken from cages with at least 4 animals which had not been changed for 4 days. All odor stimuli were placed in a small plastic weighing boat to prevent cross contamination. Odors were placed in cages in opposing corners of the side chamber of the 3-chamber setup. Each trial lasted 5 min after which the mouse was removed to a separate room while the odors were changed (about one minute). The cage was cleaned with 70% ethanol between trials. Interaction time was measured as time in which the nose of the mouse was within 10 cm of the cage. Video capture, tracking and analysis were performed using Ethovision 9 (Noldus, Wageningen, The Netherlands). Preference was determined as the time spent interacting with a stimulus as a portion of the time interacting with both stimuli. Statistical significance was determined by single sampled student t-test ($\mu = 0.5$).

No alteration T-maze — mice were placed at the base of a T shaped gated Plexiglas maze and were allowed to choose between the two arms. A decision was considered to be when the whole body of the mouse passed the gate at which point the gate was closed and the mouse was allowed 10 s to explore the arm of the cage. After this time the mouse was returned to the base of the maze for 5 s. This was repeated ten consecutive times. Student t-test was used to determine significance.

Hyperactivity — speed and distance traveled were measured over three five-minute trials. The average for each animal was used as a data point. Student t-test was used to determine significance.

3. Results

3.1. Generation and characterization of *Cntnap2*$^{tlacz/tlacZ}$ mice

In order to study the spatial and temporal expression pattern of Caspr2 a knock-in strategy was used, in which the first exon of Caspr2 was replaced with a tau-LacZ cassette (*Cntnap2*$^{tlacz/tlacZ}$; Fig. 1A). The tau localizes the LacZ to the soma and axon of neurons allowing the study of the cells and networks expressing Caspr2. The correct homologous recombination was confirmed by genomic PCR (Fig. 1B) and by southern blot (data not shown). The insertion of the LacZ cassette concomitantly with the knockout of Caspr2 were also verified using cDNA PCR to test for the presence of mRNA (Fig. 1C) and Western blot to test the protein expression (Fig. 1D). We also verified the absence of Caspr2 from the juxtaparanode of myelinating axons (Fig. 1E). Examining the behavior of these mice showed that similar to previously described *Cntnap2*$^{-/-}$ mice (Penagarikano et al., 2011), these mice were also hyperactive as measured by both speed and distance traveled over a 5-min period (Fig. 1F–G). They also displayed the same behavioral inflexibility seen in the *Cntnap2*$^{-/-}$ mice as measured by the no alterations T-maze (Fig. 1H).

3.2. Comprehensive analysis of Caspr2 in the CNS

To examine the networks and brain areas that express Caspr2, serial coronal sections from the *Cntnap2*$^{tlacz/tlacZ}$ brain were stained using X-gal (Fig. 2). A detailed list of brain areas positive for this staining can be found in Table 1. Comparing the staining pattern from the homozygous *Cntnap2*$^{tlacz/tlacZ}$ and from the heterozygous *Cntnap2*$^{+/tlacZ}$ brains showed differences in levels of expression but no gross differences in brain regions expressing Caspr2 (Fig. S1). Particularly strong expression was seen in the cortex, hippocampus, substantia nigra, interpeduncle nucleus, pontine nucleus and the medial mammillary nucleus (Fig. 3). β-gal labeling partially co-localizes with paravalbumin positive cells in the piriform cortex, indicating that Caspr2 is expressed by interneurons in the neocortex (data not shown).

3.3. Dynamic expression of Caspr2 during development

As ASD is a neurodevelopmental disorder the temporal expression of Caspr2 was examined. Caspr2 has previously been shown to be expressed starting prenatally and gradually increasing through to adulthood (Poliak et al., 1999). Examining parasagittal sections for Caspr2 expression at different time points during development and in the adult (Fig. 4A, B) revealed that Caspr2 expression starts prenatally and is greatly increased postnatally. At embryonic day 18 (E18) Caspr2 expression could mainly

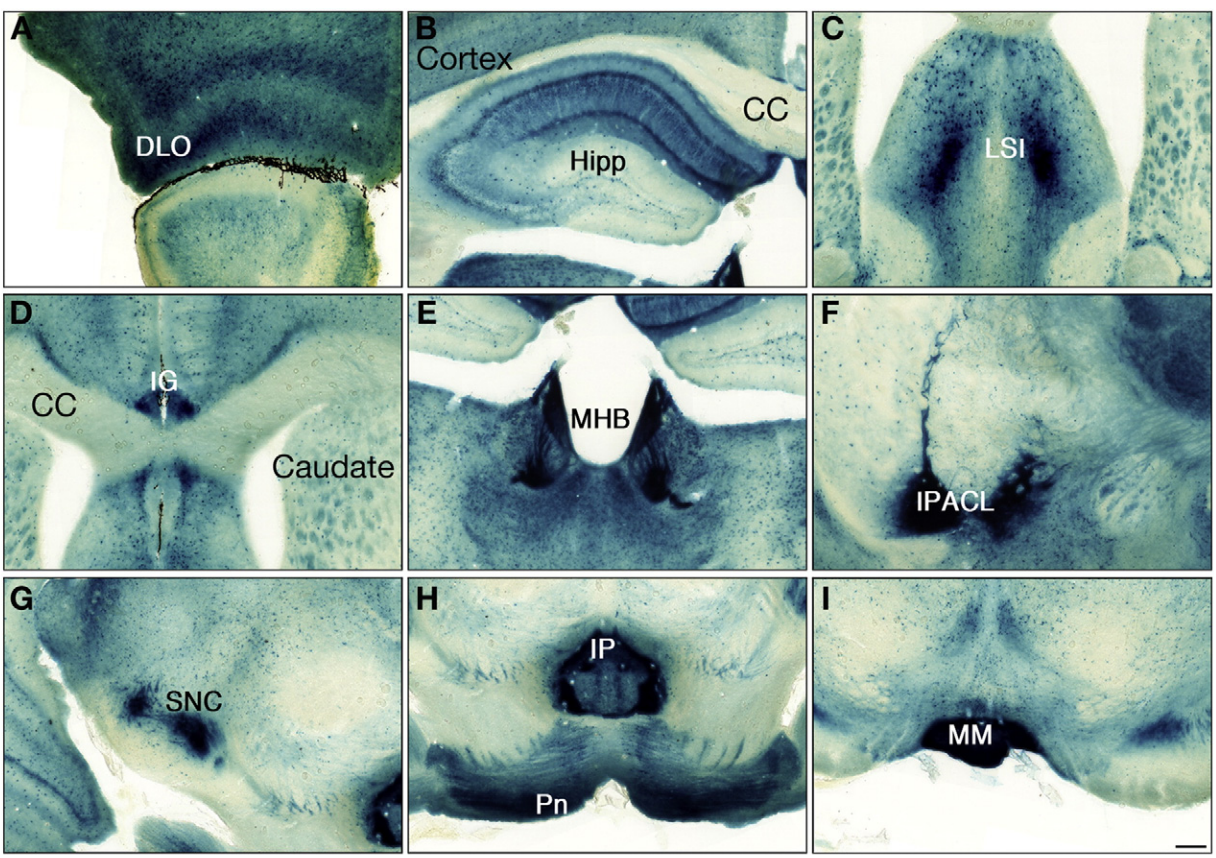

Fig. 3. Areas of the brain expressing high levels of Caspr2. High magnification of coronal section areas expressing high levels of Caspr2. DLO — dorsolateral orbital cortex (A). Hipp — hippocampus (B). LSI — lateral septal nucleus, intermediate (C). IG — indusium griseum (D). MHB — medial habenula (E). IPACL — interstriatal nucleus of the posterior limb of the anterior commissure, lateral part (F). SNC — substantia nigra compacta (G). IP —interpeduncle nucleus, Pn — pontine nucleus (H). MM — medial mammillary nucleus (I). CC — corpus callosum. Scale bar 200 μm.

be detected in the developing brain stem, thalamus and hypothalamus. At postnatal day 0 (P0) the strong expression could also be seen in the midbrain (superior and inferior coliculli). By postnatal day 7 (P7) the expression of Caspr2 could be seen in many layers of the cortex. At postnatal day 14, Caspr2 was detected in all layers of the cortex. At this age we also detected an increase in its expression in the olfactory bulb, as well as in the white matter tracts of the developing cerebellum. The overall pattern of expression shows a posterior to anterior progression pattern during development. Higher magnification of the hippocampus showed that expression of Caspr2 starts in the dentate gyrus (DG) at E18 and that during development this expression in the DG becomes much weaker and instead CA1 and CA2 expression becomes much more pronounced (Fig. 4C). Higher magnification of the cortex showed Caspr2 expression in the marginal zone at E18 and P0, which gradually migrated into deeper layers of the cortex (Fig. 4D). This expression pattern in the cortex and hippocampus is very similar to that of Reelin.

3.4. Caspr2 expression in sensory modalities

Examining the list of brain areas expressing Caspr2 revealed that many of these areas are involved in sensory processing as can bee seen by the marked areas in Table 1. These areas were not restricted to any single sense but rather were found in all sensory pathways. These areas were found at all processing levels from brain stem nuclei through to the thalamus and into the cortex. In the brain stem Caspr2 expression was seen in the solitary tract nucleus and the dorsal cochlear nucleus (Fig. 5D′), which are the first brain areas to receive input from the gustatory and auditory sensory organs, respectively. Caspr2 expression could also be found in other brain stem nuclei including the medial vestibular nucleus (MVeMC) and the dorsomedial spinal trigeminal nucleus (DMSP5) (Fig. 5D′). In the thalamus many sensory processing brain areas also expressed Caspr2 including the ventral posterolateral thalamic nucleus (VPL), the ventral posteromedial thalamic nucleus (VPM; Fig. 5C′)

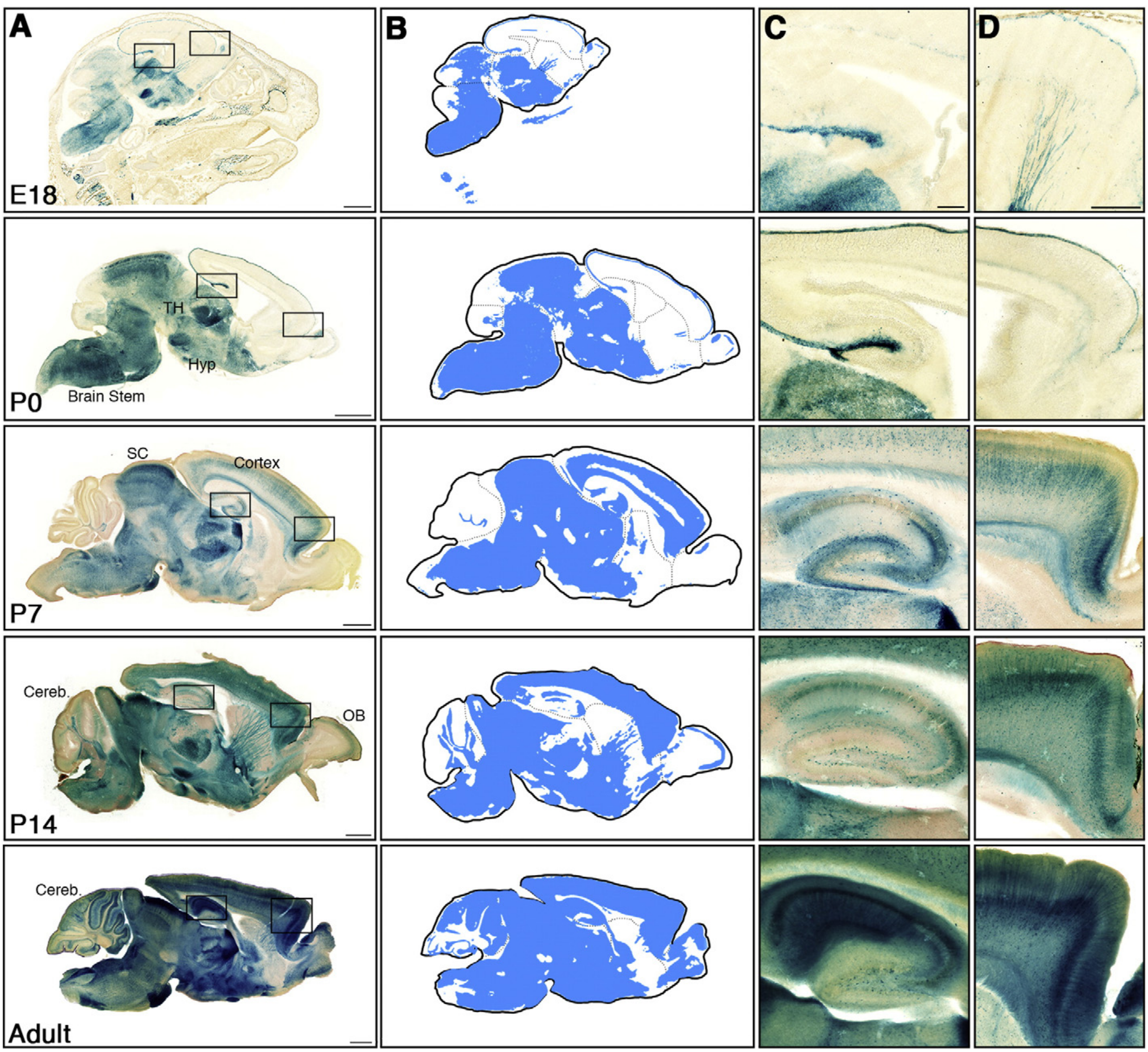

Fig. 4. Caspr2 is dynamically expressed in the cortex and hippocampus during brain development. A-B. Staining of null mice brains from embryonic day 18 (E18) to adult (A) and a schematic representation of these stainings (B). C. High magnification of the hippocampus shows expression of Caspr2 starting in the dentate gyrus at E18 and gradually moving towards the CA1 region in the adult. **D.** High magnification of the cortex reveals expression starting at the surface and later moving in to deeper levels of the cortex. TH — thalamus. Hyp — hypothalamus. SC — superior colliculus. Cereb — cerebellum. OB — olfactory bulb. Scale bar: 1 mm(A), 200 μm(C) and 500 μm (D).

and the medial geniculate nucleus dorsal (MGD/MGV; Fig. 5B′). In the cortex high levels of expression were seen in the piriform cortex (Fig. 5A′) an area of the cortex involved in olfactory processing.

As Caspr2 is expressed in many brain areas involved in sensory processing, the expression of Caspr2 was examined along the whole sensory pathway starting from the primary sensory organ. In the visual system Caspr2 expression could be detected at the retina (Fig. 6A and enlarged in the inset). Examining the layers of the retina for LacZ expression using immunofluorescence showed that Caspr2 was expressed in retinal ganglion cells and in ChAT positive amacrine cells (Fig. S2). The expression continued through the optic nerve (Fig. 6B) and into the lateral geniculate nucleus (LGN; Fig. 6C) and the superior colliculus (SC; Fig. 6D). In the auditory system Caspr2 expression was first detected in the spiral ganglion cells (Fig. 6E) and in the inner spiral plexus of the cochlea (Fig. 6F). In the brain Caspr2 expression was seen in the dorsal cochlear nucleus (DC; Fig. 6G), which is the first area to receive auditory from the periphery. In higher brain areas expression was seen in the medial geniculate nucleus (MGN; Fig. 6H). In the somatosensory system expression was found in the footpad during development (Fig. 6I) and in the dorsal root ganglion (Fig. 6J). In the spinal cord expression was seen in the dorsal horns, the area conveying sensory information (Fig. 6K). Further downstream Caspr2 expression was also found in the ventral posterolateral thalamic nucleus (VPL; Fig. 6L). In addition to this pathway Caspr2 expression was also found in the innervation of the whiskers (Fig. 6M) as well as in the trigeminal ganglion (data not shown). The gustatory system also showed Caspr2 expression at all levels of processing starting in the innervation of the tongue (Fig. 6N) and in nerve endings in the tongue (Fig. 6O). Expression was also found in solitary tract nucleus (Sol; Fig. 6P) the first area of the brain to receive gustatory information. In the thalamus Caspr2 expression was seen in the ventral posteromedial thalamic nucleus (VPM) a thalamic area involved in gustatory processing. These findings are summarized in Table 2.

3.5. Expression and role of Caspr2 in the olfactory system

The mouse olfactory system is divided into main and accessory systems (Fig. 7A). In the main olfactory system Caspr2 expression was found in single olfactory sensory neurons (OSN; Fig. 7B) in the main olfactory epithelium with the axons of these neurons converging towards the olfactory bulb (Fig. 7C). In the olfactory bulb (OB) Caspr2 expression was found in the glomeruli level (GL) as well as at the external plexiform layer (EPL) and mitral cell layer (Mi; Fig. 7D). In the accessory olfactory system expression was found predominantly in basal vomeronasal sensory neurons (VSN; Fig. 7E). Whole mount staining showed Caspr2 expression in the vomeronasal organ (VNO) with VSN axons converging on the accessory olfactory bulb (AOB; Fig. 7F). Examining the AOB in both whole mount (Fig. 7G) and sections (Fig. 7H) showed Caspr2 expression in the posterior part of the AOB, which is the area that receives input from the basal VSNs. In higher brain regions Caspr2 expression was found in both primary and accessory olfactory processing areas including the bed nucleus of the stria terminalis (BST), the medial preoptic area (MPA), the medial thalamus and the piriform cortex (Fig. 7I–K). A summary of these results is found in Table 2.

As Caspr2 is expressed throughout the sensory pathways, from sensory organ to cortical processing, we set to test whether specific sensory modalities are affected in the null mice. To this end we chose the olfactory system, as it is one of the major senses controlling mice behavior. A Previous report has shown that Caspr2 null mice are not anosmic as they were able to find food hidden bellow the cage bedding (Penagarikano et al., 2011). A modified 3-chamber test was performed to test the role of Caspr2 in the olfactory system. In this test novel odors were used as stimuli either compared to no odor or compared to a familiar odor (Fig. 7L). The mice were tested for time interacting with the odor, measured by time that the nose of the mouse was found in close proximity to the wire cup containing the odor. *Wt* and

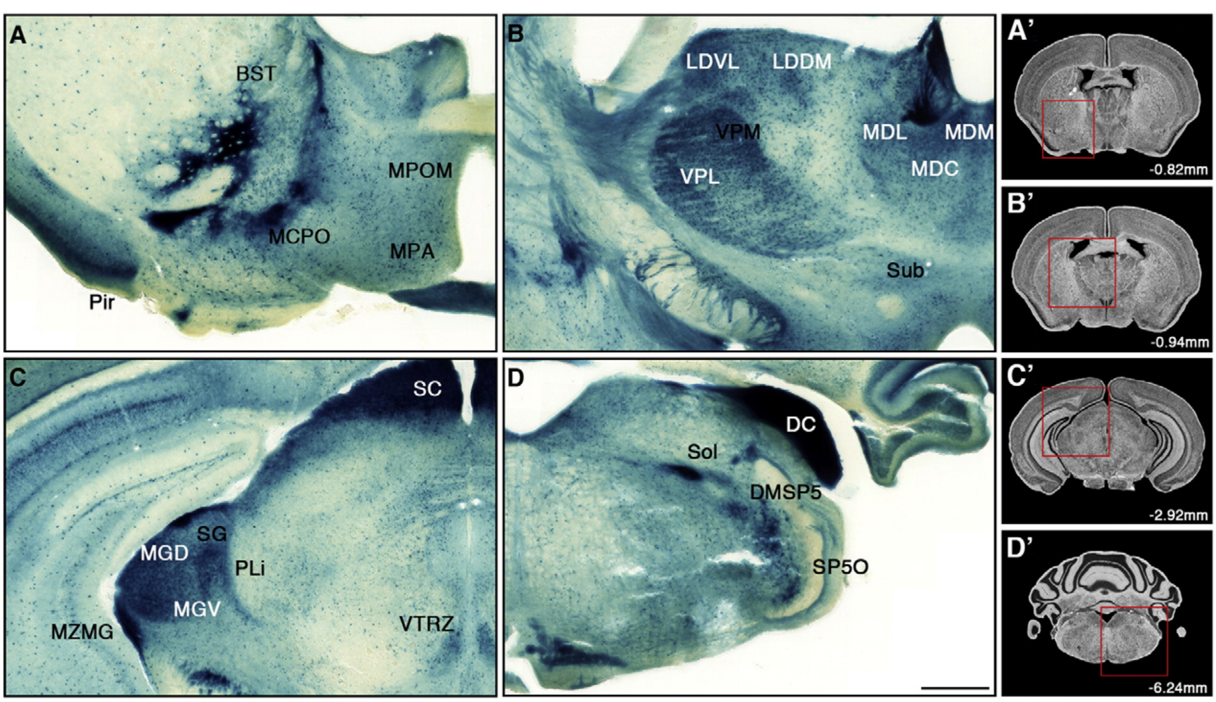

Fig. 5. Caspr2 is expressed in many brain areas involved in sensory processing. Different brain bregmas (A′–D′; taken from www.mbl.com; (Rosen et al., 2000) show Caspr2 in many brain areas involved in sensory processing (A–D; marked in gray in Table 1). BST — bed nucleus of the stria terminalis. DC — dorsal cochlear nucleus. DMSP5 — dorsomedial spinal trigeminal nucleus. LDDM — laterodorsal thalamic nucleus dorsomedial. LDVL — laterodorsal thalamic nucleus ventrolateral. MCPO — medial preoptic nucleus central. MD (L/C/M) — mediodorsal thalamic nucleus (lateral/central/medial). MGD — medial geniculate nucleus dorsal. MGV — medial geniculate nucleus ventral. MPA — medial preoptic area. MPOM — medial preoptic nucleus medial. MZMG — marginal zone of the medial geniculate. Pir — Piriform cortex. PLi — posterior limitans thalamic nucleus. SC — superior colliculus. Sol — solitary tract nucleus. SP5O — spinal trigeminal nucleus, oral. Sub — submedius thalamic nucleus. VPL — ventral posterolateral thalamic nucleus. VTRZ — visual tegmental relay zone. Images of bregmas are taken from the mouse brain library (Rosen et al., 2000). Scale bar: 500 μm.

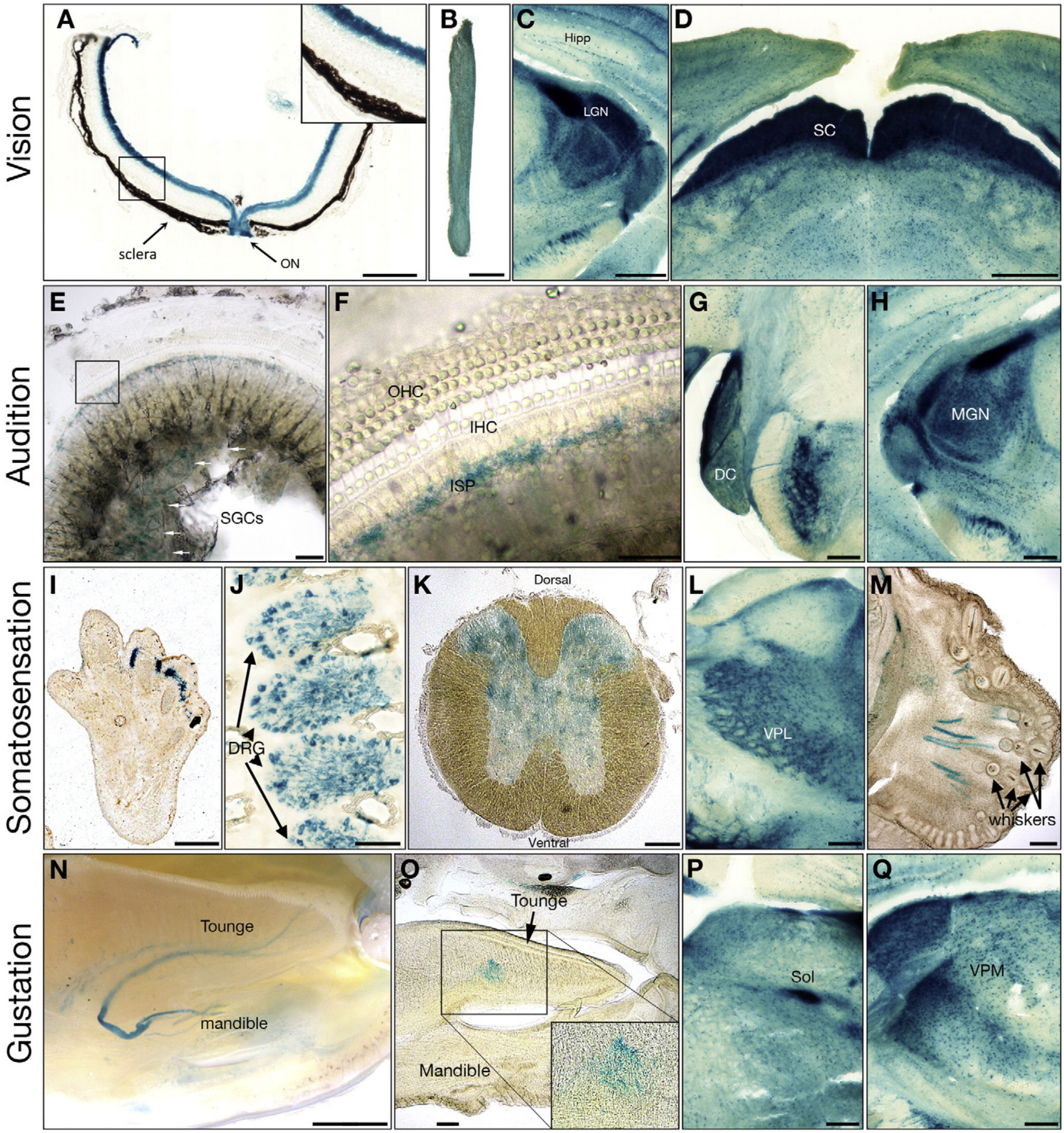

Fig. 6. Caspr2 is expressed in all sensory modalities. A–C. Caspr2 expression in the visual system. A. Caspr2 is expressed in the retina of adult mice (enlarged in inset) and in the optic nerve (ON). B. The optic nerve, leading from the retina to the brain, also expresses Caspr2. C. Caspr2 is expressed in the lateral geniculate nucleus (LGN), the first brain area to process visual information. D. Caspr2 is also expressed in the other visual pathway leading from the retina to the superior colliculus (SC). D–H. Caspr2 expression in the auditory system. D. Whole mount staining of the cochlea shows expression in the spiral ganglion cells (SGC) and in the inner spiral plexus (ISP). E. Higher magnification of the cochlea shows staining in the ISP but not in the inner or outer hair cells (IHC/OHC). E. Caspr2 expression can also be seen in dorsal cochlear nucleus (DC), the first brain area to process caspr2. H. In the thalamus Caspr2 expression can be detected in the medial geniculate nucleus (MGN), the next stage of the auditory pathway. I–L. Caspr2 expression in the somatosensory system. I. Caspr2 is expressed in nerves innervating the footpad of E15 mice. J. Dorsal root ganglia (DRG) of E15 mice also express Caspr2. K. Expression is also seen in the dorsal (somatosensory) part of the spinal cord. L. In the brain Caspr2 is expressed in the ventral posterolateral thalamic nucleus (VPL) an area of the brain involved in somatosensory processing. M. Expression in the somatosensory system can be seen in the innervation of the maxillary whiskers of E18 mice. N–Q. Caspr2 expression in the gustatory system. N. Whole mount staining reveals expression in the innervation of the tongue. O. Sectioning of the tongue shows staining in axon bundles within the tongue (enlarged in the inset). P. Caspr2 is expressed in the solitary tract nucleus (Sol), the first brain area to receive gustatory input. Q. In the thalamus, Caspr2 is expressed in the ventral posteromedial thalamic nucleus (VPM) an area involved in gustatory processing. Scale bar: A–D, I — 500 μm, E, N — 100 μm, F — 80 μm, G, L, M, P, Q — 250 μm, J, K, O — 200 μm.

Cntnap2$^{tlacz/tlacZ}$ mice showed no preference for either side when presented with no odor (water vs. water). However, when presented with a novel odor compared to no odor (banana vs. water) or a novel odor compared to familiar odor (almond vs. banana) *wt* but not null mice showed a preference for the novel odor (Fig. 7M). This effect was also found when performing the test using social odors. *Wt* but not null mice showed a preference for novel male odors over familiar male odors (novel vs. familiar) as well as for female odors over male odors (female vs. male; Fig. 7N).

4. Discussion

Over the past few years many independent genetic screens have shown a link between Capsr2 and ASD (Alarcon et al., 2008; Anney et al., 2012; Arking et al., 2008; Bakkaloglu et al., 2008; Li et al., 2010; O'Roak et al., 2011; Stein et al., 2011). Furthermore, Caspr2 null mice (*Cntnap2*$^{-/-}$) were shown to have core autism related deficits (Penagarikano et al., 2011) making these mice an excellent model for studying ASD and for resolving the role that Caspr2 plays in these disorders. To understand this role it is essential to know which brain areas and neuronal networks express Caspr2. To this end we generated a Caspr2:tau-LacZ reporter line (*Cntnap2*$^{tLacZ/tLacZ}$) in which the first exon of Caspr2 was replaced by a tau-LacZ reporter leading to the expression of the tau-LacZ reporter under the Caspr2 promoter. The tau localizes this reporter to the soma, allowing us to investigate the spatial and temporal expression pattern of Caspr2, as well as to the axons, which allows us to elucidate the networks in which these cells are found (Callahan and Thomas, 1994).

Here we show that Caspr2 is expressed in various brain areas and is dynamically expressed in the cortex and hippocampus during development. When examining brain areas which have been linked to ASD we could often detect Caspr2 expression. For example, in the limbic system (Chen et al., 2015) we saw Caspr2 expression in the hippocampus, lateral habenula, amygdala, septal nuclei and mammillary bodies. In addition, in the cortex, in which many abnormalities have been linked to ASD (Chen et al., 2015), we could detect Caspr2 in all areas examined. During development the dynamic expression of Caspr2 in the cortex and hippocampus is very similar to that of Reelin, a protein that is involved in the regulation of neuronal migration and modulation of synaptic plasticity (Lakatosova and Ostatnikova, 2012). Both Reelin and Caspr2 have previously been linked to ASD (Poot, 2015; Wang et al., 2014). However, it should be noted that these two proteins likely affect cell migration differently, as Caspr2 null mice exhibit migration abnormalities of upper layer but not of deeper layer neurons (Penagarikano et al., 2011), while in *reeler* mice all layers of the cortex are disrupted (Lakatosova and Ostatnikova, 2012).

Comparing the expression pattern of Caspr2 described here to that previously described in humans using in-situ hybridization, shows that the Caspr2 cortical expression resembles that found in humans (Bakkaloglu et al., 2008). This resemblance was also found when comparing Caspr2 expression in developing mice brains to that found in developing human fetal brains (Abrahams et al., 2007). In both mouse and human developing brains Caspr2 expression was found in the cortical subplate and marginal zone while in the adult expression was found in all cortical layers. Caspr2 expression was also found in all other developing brain areas which were shown to have high levels of expression in humans, including the amygdala, caudate putamen and thalamus (Abrahams et al., 2007). However, the localization of the tau-LacZ to the axon in addition to the soma, allowed us to elucidate not only the cells expressing Caspr2, but also the network in which these cells are found.

When analyzing the areas and networks expressing Caspr2 in the brain we saw that many of them are involved in sensory processing. These areas were found in all sensory modalities and were not restricted to any specific one. Moreover, Caspr2 was found to be expressed in the brain areas containing the first sensory synapse in the different sensory modalities: the LGN (visual), DC (auditory), Sol (gustatory), SP5O (somatosensory) and the glomerular layer of the olfactory bulb (olfactory). The areas of Caspr2 expression were often found within a particular sensory processing path. For example, in the olfactory system the following parasympathetic pathway, involved in many social behaviors including defensive responses against predators and reproductive related behaviors (Pardo-Bellver et al., 2012), was Caspr2 positive: vomeronasal organ (VNO)–accessory olfactory bulb (AOB)–medial amygdaloid nucleus (MEA)–bed nucleus of the stria terminalis (BST)–medial preoptic area (MPA)–central gray pons (CGPn). Another example is in the auditory system in which Caspr2 is expressed in the following pathway: cochlear nucleus (DC)–superior olivary nucleus (LSO)–Inferior colliculus (ECIC)–medial geniculate nucleus (MGD)–cortex (Carr and Edds-Walton, 2008).

When examining these pathways we set out to determine how early on in the pathway is Caspr2 expressed. We discovered that all the primary sensory organs express Caspr2. For example, in the olfactory system Caspr2 expression was detected in both the main olfactory epithelium as well as in the vomeronasal organ. In both these systems Caspr2 was only found in a subset of sensory neurons (OSNs and VSNs) and was not found ubiquitously in all these cells. In the accessory olfactory system cells expressing Caspr2 were found primarily in a subpopulation of VSNs in the basal part of the VNO which sends processes to the posterior area of the AOB. In a similar manner, in the main olfactory system, only a subpopulation of OSN expresses Caspr2. Similarly, in the visual system, Caspr2 is expressed only by a subset of retinal ganglion and amacrine cells. Further research is needed to determine the exact identity of cells expressing Caspr2, as it appears that it is not limited to a single cell type (data not shown).

As Olfaction is a central sensory system for mice we tested whether the null mice showed any abnormal olfactory processing. To this end we performed behavioral tests measuring the response of Capr2 null mice to novel olfactory stimuli. This approach revealed that the Caspr2 null have abnormal olfactory preferences demonstrating that Caspr2 is involved in olfactory sensory processing in both the primary and accessory pathways. It is not needed for mice to smell as Caspr2 null mice are not anosmic, but rather our results suggest that Caspr2 plays a more regulatory role in sensory processing.

This expression of Caspr2 in the sensory systems is of importance as it is estimated that over 90% of individuals with ASD have sensory abnormalities (Leekam et al., 2007), with abnormalities found in all sensory modalities (Marco et al., 2011) and across all ages and spectrum of severity (Ben-Sasson et al., 2009). In fact, even in Kanner's first description of autism, patients were described as having sensory abnormalities (Kanner, 1943). These sensory symptoms are so widely prevalent among patients with autism that the American Psychiatric Association has added sensory abnormalities as a criteria for diagnosing ASD

Table 2
Caspr2 positive areas in sensory pathways.

Sense	Caspr2^{+} area within primary sensory organs	Caspr2^{+} brain regions in sensory pathways
Auditory	Spiral ganglion cells (SGCs)/inner spiral plexus (ISP)	Dorsal cochlea nucleus (DC)–medial geniculate nucleus (MGN)
Vision	Retinal ganglion cells (RGCs)/amacrine cells	Lateral geniculate nucleus (LGN)/superior colliculus
Gustatory	Nerve endings	Solitary tract nucleus (Sol)–ventral posteromedial thalamic nucleus (VPM)
Somatosensory	Foot pad — DRG/whiskers — trigeminal ganglion	Ventral posterolateral thalamic nucleus (VPL)
Olfactory (main)	Olfactory sensory neurons (OSNs)	Olfactory bulb (OB)–medial preoptic area (MPA)–medial thalamus
Olfactory (accessory)	Vomeronasal sensory neurons (VSNs)	Accessory olfactory bulb (AOB)–bed nucleus stria terminalis (BST)

in the latest version of the DSM (DSM V) (American Psychiatric Association, 2013). These symptoms can be extremely debilitating and have great implications to the everyday activities of these patients and their families (Behrmann and Minshew, 2015). Moreover, the severity of these sensory deficits has been linked to the severity of affective symptoms such as negative emotionality, anxiety, and depression (Ben-Sasson et al., 2008). Another study by Lane and colleagues showed that 50% of the variance in maladaptive behavior could be explained by sensory function (Lane et al., 2010). However, to date, the underlying neurobiology of the sensory symptoms of ASD is still unclear (Hazen et al., 2014). There are currently a few theories regarding the neurological mechanism involved in these sensory processing abnormalities. As multisensory integration and higher level processing were shown to be abnormal in patients with ASD, abnormal functioning in cortical layers has been proposed to play a role (Marco et al., 2011). Another hypothesis suggested by these authors is a disruption in connectivity between cortical and subcortical regions (Marco et al., 2011). Others have suggested that disruption in the hypothalamic-pituitary-adrenal (HPA) axis and amygdala may explain these abnormalities (Mazurek et al., 2013). Based on our findings we cannot rule out any of the above hypotheses as we see Caspr2 expression in the cortex, subcortical regions and in the amygdala. However, the expression of Caspr2

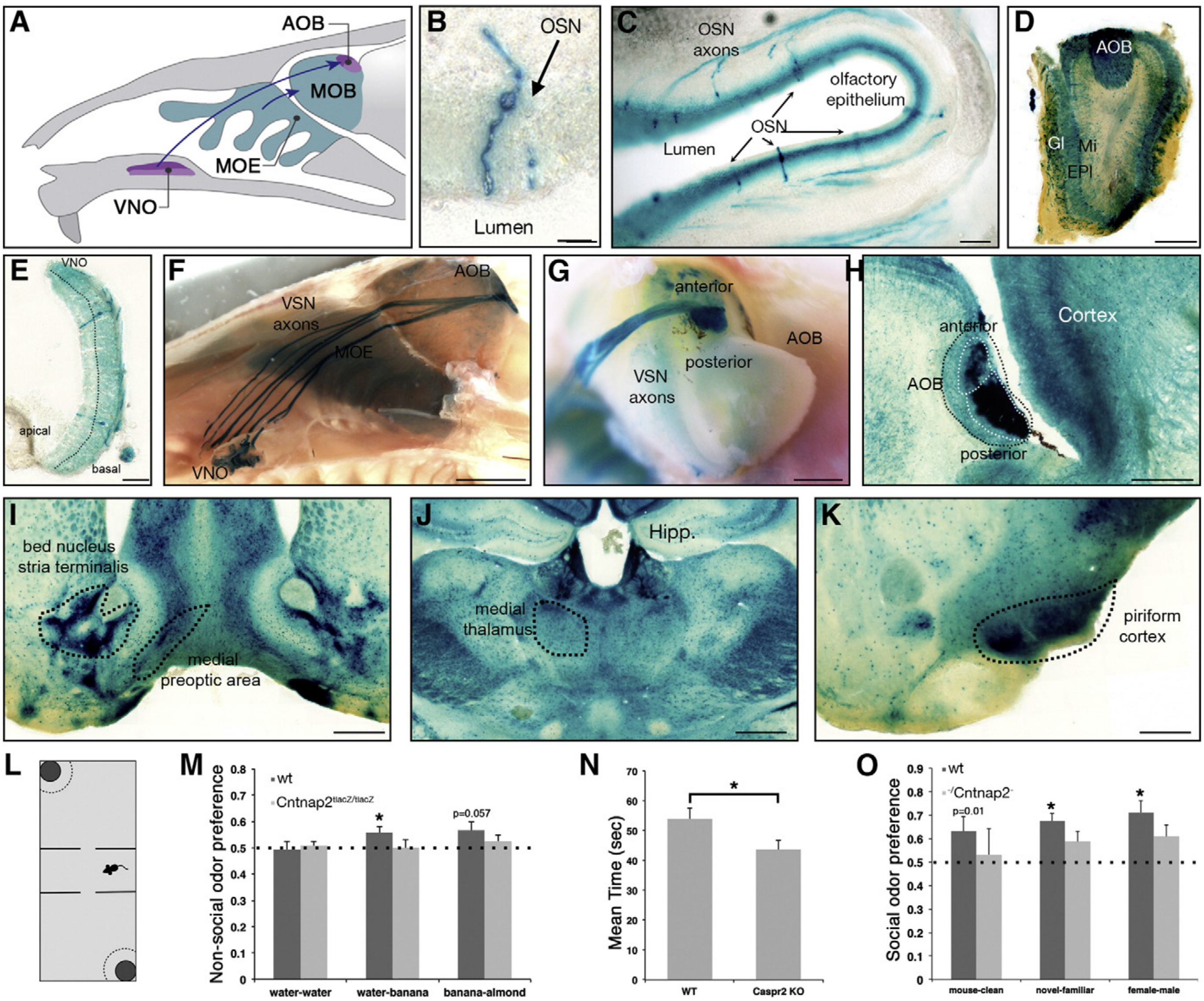

Fig. 7. Caspr2 is expressed in both main and accessory olfactory systems and is involved in sensory processing. A. A scheme of the mouse olfactory system. The main olfactory system is composed of the main olfactory epithelium (MOE), which sends processes into the olfactory bulb (OB). The accessory olfactory epithelium is composed of the vomeronasal organ (VNO), which sends processes to the accessory olfactory bulb (AOB). B–D. Caspr2 is expressed in the main olfactory system. B. High magnification of olfactory sensory neurons (OSN) shows that they express Caspr2. C. Lower magnification shows OSN axons in the MOE converging away from the olfactory epithelium in P7 mice. D. In the OB Caspr2 is expressed in the glomerular (GL), external plexiform (EPI) and mitral (Mi) layers. E-H. Caspr2 is expressed in the accessory olfactory system. E. Caspr2 is expressed in the VNO predominantly by basal vomeronasal sensory neurons (VSN). F. Whole mount staining reveals Caspr2 expression in the VNO as well as in VSN axons converging on the AOB. Expression can also be seen in MOE. G. Whole mount staining revealed strong expression in the posterior AOB. H. Similarly, a section through the AOB shows that Caspr2 is strongly expressed in the posterior AOB with lower levels of expression in the anterior AOB. I–K. Many brain areas involved in olfactory processing express Caspr2. These areas include the bed nucleus of the stria terminalis and the medial preoptic nerve (I), the medial thalamus (J), and the piriform cortex (K) as seen in coronal sections of Caspr2-Lacz brains. L–O. Caspr2 is involved in both processing of both social and non-social stimuli. L. A scheme of the adapted 3-chamber test used to measure olfactory preference. M. Mice lacking Caspr2 do not show preference for novel non-social olfactory stimuli compared to either no odor (banana-water) or compared to a familiar odor (almond-banana). N. Mice lacking Caspr2 show less attraction to olfactory stimuli. Time spent in the proximity of non-social olfactory stimuli (banana and almond) was averaged over all trials. Mice lacking Caspr2 spent less time in the proximity of the olfactory stimuli as compared to WT mice. O. Mice lacking Caspr2 do not show preference for novel and attractive social olfactory stimuli. WT mice but not mice lacking Caspr2 showed a preference for novel social olfactory stimuli when compared to familiar social stimuli (novel-familiar) nor for female olfactory stimuli when compared to male stimuli (female–male). *$p < 0.05$. Scale bar: B — 20 μm, C, E — 100 μm, and D, G–K — 500 μm, F — 1 mm.

along sensory processing pathways and especially in the primary organs adds another level of complexity. At least some of the sensory symptoms may be explained by abnormalities at these primary organs, which could then be amplified by downstream mechanisms. It should be noted that in the current work we only tested for abnormalities in a single sensory modality (the olfactory system), and that further testing is necessary to determine the presence and extent of abnormalities in other sensory systems.

In conclusion, by generating the *Caspr2*$^{tlacz/tlacZ}$ reporter line we were able to provide a detailed description of the brain regions expressing Caspr2. We report that Caspr2 is highly expressed in al sensory processing pathways and show that Caspr2-null mice have sensory processing abnormalities. We therefore suggest that the Caspr2 mouse can serve as a model to study the sensory deficits in ASD.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mcn.2015.11.012.

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