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Alterations of Perineuronal Net Expression and Abnormal Social Behavior and Whisker-dependent Texture Discrimination in Mice Lacking the Autism Candidate Gene Engrailed 2

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Abstract—GABAergic interneurons and perineuronal nets (PNNs) are important regulators of plasticity throughout life and their dysfunction has been implicated in the pathogenesis of several neuropsychiatric conditions, including autism spectrum disorders (ASD). PNNs are condensed portions of the extracellular matrix (ECM) that are crucial for neural development and proper formation of synaptic connections. We previously showed a reduced expression of GABAergic interneuron markers in the hippocampus and somatosensory cortex of adult mice lacking the Engrailed2 gene (En2-/- mice), a mouse model of ASD. Since alterations in PNNs have been proposed as a possible pathogenic mechanism in ASD, we hypothesized that the PNN dysfunction may contribute to the neural and behavioral abnormalities of En2-/- mice. Here, we show an increase in the PNN fluorescence intensity, evaluated by *Wisteria floribunda* agglutinin, in brain regions involved in social behavior and somatosensory processing. In addition, we found that En2-/- mice exhibit altered texture discrimination through whiskers and display a marked decrease in the preference for social novelty. Our results raise the possibility that altered expression of PNNs, together with defects of GABAergic interneurons, might contribute to the pathogenesis of social and sensory behavioral abnormalities.

Key words: autism, perineuronal nets, brain plasticity, whisker-dependent behaviors, sociability.

INTRODUCTION

Autism spectrum disorders (ASDs) refer to a group of complex neurodevelopment disorders (NDDs) characterized by impaired social interaction and stereotyped behavior (American Psychiatric Association, 2013).

Sensory processing atypicalities are present in about 90% of ASD patients (Robertson and Baron-Cohen, 2017) and are currently recognized as a part of the official diagnostic criteria as reported in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5). Abnormal sensory experiences include sensory seeking as well as hyper- and hypo-reactivity to sensory stimuli, with a particularly abnormal sensitivity to tactile stimuli (Balasco et al., 2020).

Aberrant somatosensory processing has already been described in several syndromic animal models for ASD such as mice carrying mutants for Engrailed 2, Shank3b, Cntnap2, and Fmr1 genes (Zhang et al., 2014; He et al., 2017; Chelini et al., 2019; Orefice, 2020; Balasco et al., 2022a, 2022b).

Engrailed-2 (En2) gene is a candidate gene for ASD (Benayed et al., 2009) that encodes for the Engrailed-2 homeobox transcription factor that is critical for brain development (Gharani et al., 2004; Benayed et al., 2009; Hnoonual et al., 2016). Mice lacking En2 (En2-/- mice) show alteration in the cerebellar patterning and reduced Purkinje cell numbers resembling those reported in ASD subjects (Joyner et al., 1991; Joyner, 1996; DiCicco-Bloom et al., 2006). In addition, En2-/- mice display several

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Abbreviations: ASDs, autism spectrum disorders; CA, cornus ammonis; Cntnap2, contactin associated protein 2; DG, dentate gyrus; E/I, excitation/inhibition; ECM, extracellular matrix; En2, Engrailed2 gene; Fmr1, fragile X messenger ribonucleoprotein 1; GABA, gamma-aminobutyric acid; Mecp2, methyl-CpG binding protein 2; NDDs, neurodevelopment disorders; OF, open field; PFC, prefrontal cortex; PNNs, perineuronal nets; PrL-PFC, prelimbic prefrontal cortex; PV, parvalbumin; S1, primary somatosensory cortex; Shank3b, SH3 and multiple ankyrin repeat domains 3; tNORT, textured novel object recognition test; WFA, Wisteria floribunda agglutinin.

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ASD-like behaviors including increased seizure susceptibility, reduced sociability, and impaired spatial learning (Tripathi et al., 2009; Cheh et al., 2006; Brielmaier et al., 2012; Provenzano et al., 2014). We previously reported a significantly reduced synchronization in somatosensory-auditory/associative cortices and dorsal thalamus in these mutants, suggesting the presence of aberrant somatosensory processing (Chelini et al., 2019). This reduced functional connectivity in the somatosensory cortex was paralleled by over-reactivity to repeated whisker stimulation, reduced activation of the somatosensory cortex, and increased activation of the basolateral amygdala in response to repeated whisker stimulation.

The disruption of social and sensory processing in ASD is likely due to impaired brain plasticity mechanisms as well as its relationship to excitatory/inhibitory imbalance (Gogolla et al., 2009; Hansel, 2019). Studies from our laboratory showed an altered expression of GABAergic interneuron markers parvalbumin (PV) and somatostatin (SST) in the forebrain of young and adult En2-/- mice, suggesting that GABAergic neurotransmission is profoundly compromised during both adolescence and adulthood (Sgadò et al., 2013; Provenzano et al., 2020). Abnormalities in GABAergic interneuron functions might represent the anatomical substrate of an unbalanced ratio of excitation/inhibition in sensory and social systems, which has been considered a pathophysiological mechanism in many neuropsychiatric disorders including ASD (Bozzi et al., 2018). Many studies have provided evidence that impaired maturation of the GABAergic circuitry could result in an immature structure and function of the cerebral cortex, which may remain more plastic and sensitive to alterations in sensory inputs (Rubenstein and Merzenich, 2003).

PNNs are specialized cartilage-like structures of the extracellular matrix (ECM), that preferentially ensheath PV-expressing interneurons, about 70% of PNNs are around PV+ cells (Lupori et al., 2023). PNNs have been implicated in a wide range of functions including synaptic plasticity and stabilization of neuronal circuits (Karetko and Skangiel-Kramska, 2009; Caroni et al., 2012; Fawcett et al., 2022). Genome-wide association studies in patients with ASD uncovered many genes involved in ECM and PNNs formation and regulation (Pantazopoulos and Berretta, 2016; Sorg et al., 2016). PV-interneurons density was found to be reduced in postmortem prefrontal cortex (PFC) tissue from autistic patients, while other studies from postmortem tissue showed a lower density of PNNs in the globus pallidus (Hashemi et al., 2017; Brandenburg and Blatt, 2022). Along with these observations, mice treated with valproic acid (a pharmacological model of ASD), exhibit an increased PNN intensity but a reduced density of PV+ interneurons (Xia et al., 2021). Here, we assessed whisker-dependent and social behaviors in En2-/- mice using textured novel object recognition test (tNORT) and three-chambered social task respectively. Moreover, we investigated the possible neural substrates behind the deficit in somatosensory processing and sociability by quantitative measurements of PNNs in the hippocampus and somatosensory cortex of En2-/- and wild-type (En2+/+) mice.

MATERIAL AND METHODS

Animals

All experimental procedures were approved by the Animal Welfare Committee of the University of Trento and Italian Ministry of Health (projects 949/2015-PR and 847/2018-PR), according to the European Community Directive 2010/63/EU.

Mice were housed in a 12 h light/dark cycle with food and water available *ad libitum*, taking care to minimize the animals' pain and discomfort. Wild-type (En2+/+) and En2−/− littermates (Joyner et al., 1991) were obtained by breeding En2+/− heterozygous animals, as described (Sgadò et al., 2013). A total of 12 mice sex balanced (2–4 months old), corresponding to 6 animals per genotype, were used for Wisteria floribunda agglutinin immunohistochemistry experiments. A subset of these animals (5 En2+/+ and 4 En2-/-) was also used for the colocalization WFA and PV+ cells. A total of 43 age-matched adult littermates (33 En2+/+ and 30 En2-/- mice; 2–4 months old; weight = 25–35 g) of both sexes (properly balanced) were used for textured novel object recognition (tNORT) test. A subset of animals subjected to the tNORT test (10 En2+/+ and 10 En2-/-, >4 months) was then used for the three-chamber social interaction test. All experiments were performed blind to genotype. An operator who did not participate in the experiments assigned a numerical code to each animal and codes were only associated with genotypes during data analysis.

Immunohistochemistry

Immunofluorescence staining for the lectin wisteria floribunda agglutinin allowed for a detailed analysis of the perineuronal nets in subregions of the hippocampus and primary somatosensory cortex. After anesthesia, mice were transcardially perfused with 0.1 M phosphate-buffered saline (1xPBS, Ph 7.4) followed by 4% paraformaldehyde (PFA) in PBS. Brains were dissected and post-fixed overnight in 4% PFA. Coronal brain sections were cut with 30 μm thickness using a vibratome (Leica® VT1200). Four consecutive sections were taken from dorsal hippocampus.

Cortical and hippocampal subregions were identified according to Allen Mouse Brain Atlas and Allen Reference Atlas – Mouse Brain. Briefly, to prevent endogenous peroxidase activity and for antigen retrieval treatment, sections were incubated at 70 °C for 2 h with sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0). All staining experiments were initiated by extensive washing with PBS followed by the blocking of potential non-specific binding sites by treatment for 1 h at room temperature with blocking solution (3% bovine serum albumin in PBS containing 0.2% Triton X-100). Sections were first incubated with biotinylated Wisteria Floribunda Lectin (WFA; Vector Laboratories, cat# B-1355-2; 10 ng/mL in BSA-PBS-T) overnight at 4 °C. On the following day, sections were rinsed 3 times in PBS followed by incubation in Alexa Fluor 488 conjugated streptavidin (1:500; S32354,

Thermo Fisher Scientific, MA, USA) in 5% blocking solution for 2 h at room temperature.

For the colocalization between PNNs and PV+ cells, the slices after WFA staining were incubated with a blocking solution (3% bovine serum albumin in PBS containing 0.2% Triton X-100) for 1 h at room temperature, then rinsed 3 times in PBS followed by incubation with the anti-parvalbumin mouse monoclonal antibody (P3088, Sigma-Aldrich, USA; 1:2000 dilution). Then, sections were rinsed 3 times in PBS and finally incubated for 2 h at room temperature with Donkey anti-Mouse IgG (H + L) Alexa Fluor 594 (1:500; A-21203, Thermo Fisher Scientific, MA, USA) in a 5% blocking solution. After three 5-min washes in PBS, sections were mounted onto Epredia™ SuperFrost Plus™ glass slides (Thermo Fisher Scientific Inc.), air-dried, and coverslipped with mounting medium (Aqua PolyMount®).

Image acquisition and analysis

Images were acquired using a Zeiss AxioImager II upright fluorescence microscope at a 10 × primary magnification at the CIBIO Advanced Imaging Core Facility of the University of Trento.

All quantification was done with the ImageJ (NIH) software. Acquired images were converted to 8-bit (gray-scale), inverted, and processed for background subtraction.

A region of interest (ROI) was created for each image in the dorsal hippocampal subregion CA2, for layers 4 (L4) and L5/6. Mean signal intensity was normalized against the background calculated in L1. In the CA1, CA3, dentate gyrus (DG), and L2/3 regions, cells labeled with WFA as well as PV+ cells that were surrounded by WFA were manually counted, and values were expressed in cell/mm2.

ECM/PNNs enrichment analysis on hippocampal differentially expressed genes

To verify the presence of ECM/PNNs genes in the brains of En2−/− mice, an enrichment analysis was performed using a dataset of hippocampal differentially expressed genes derived from our previous study (Sgadò et al., 2013). The analysis was conducted using the Fisher exact test in R, with a significance threshold set at a P-value cutoff of 0.05, consistent with those used in previous research (Provenzano et al., 2016). Gene lists for assessing the overlaps with ECM-associated genes were compiled from the Molecular Signatures Database (MsigDB, https://www.gsea-msigdb.org/gsea/msigdb/), including terms such as chondroitin sulfate biosynthetic process, heparan sulfate proteoglycan biosynthetic process regulation of extracellular matrix organization, naba ECM affiliated, naba core matrisome, ECM proteoglycans, the human gene set module_47 (ECM and collagen genes), matrix metalloproteinases (WikiPathways 441).

Textured novel object recognition test (tNORT)

Whisker-mediated texture discrimination was assessed as previously described (Wu et al., 2013; Balasco et al., 2022a, 2022b). Briefly, all the experiments were carried out in a standard open field arena (40 cm × 40 cm × 40 cm), containing two cylinder-shaped objects covered in garnet sandpaper (1.5 cm radius base × 12 cm height). The familiar object was covered with 150 G sandpaper (i.e., fine texture), while the novel object was covered with 60 G paper (i.e., coarse texture, according to Wu et al., 2013; Balasco et al., 2022a, 2022b).

Many copies of the cylinder-shaped objects were created to avoid olfactory recognition. To avoid visual confounds due to the very low visual acuity of adult mice (Schmucker et al., 2005), the test was performed in the penumbra (4 lx). Before the test, mice were habituated to the arena for 20 min for 2 days. On the third day, the Textured novel object recognition test (tNORT) was performed. In the first test session (learning phase), the animals were placed in the testing arena, facing two identically textured objects (objects A and B, 150 G) placed in the center of the arena and equidistant from each other and the walls. Mice were allowed to investigate the objects freely for 5 min. This short time was selected to favor the investigation through whiskers. The animals were then removed and held in a separate cage for 5 min. This intertrial duration was selected to minimize hippocampal-mediated learning (Wu et al., 2013; Balasco et al., 2022a, 2022b). In the second session, the testing phase, a familiar object (identical to objects A and B) and a new object (novel, 60 G) were placed in the arena. The position of the novel versus the familiar object was counterbalanced and pseudo-randomized (i.e., the position of the two objects was exchanged between each mouse and the following one). Mice were allowed to explore for 5 min. Between sessions and between animals, the testing arena was cleaned with 70% ethanol to avoid olfactory cues. The time spent by each animal actively investigating each object was assessed during both the learning and testing phases. The activity of the mice was recorded with a video camera (Basler) centered above the arena and automatically tracked using EthoVisionXT (Noldus).

Investigation through whiskers was defined as directing the nose towards the object with a distance of less than 2 cm or touching the object with the nose.

Resting, grooming, and digging next to, or sitting on, the object was not considered investigation. The performance of the mice in the tNORT was expressed by the preference index. The preference index is defined as the ratio between the amount of time spent exploring any one of the two objects in the learning phase (objects A or B) or the novel one in the testing phase and the total time spent exploring both objects, expressed as a percentage [i.e., A/(B + A) × 100 in the learning session and novel/(familiar + novel) × 100 in the testing session].

Three-chambers social task

Adult sociability was tested in a rectangular arena divided into three interconnected chambers (left, center, right) of odor-resistant materials like Plexiglas or acrylic plastic using methods previously described (Nadler et al., 2004). The partitions have openings in the middle that

allow the mouse to move freely between compartments and can be closed by small sliding doors. It is important that the surrounding environment is not affected by visual, olfactory, and auditory cues, that may induce innate preferences in the subject mouse. For this reason, lights were kept dim and even, and the apparatus was located on a table where the subject mice could not be influenced by the surrounding environment. A camera was installed one meter above the apparatus to automatically record the number of entries and time spent in each of the three chambers using EthoVision® XT (Noldus, Wageningen, Netherlands) software tracking. The subject mouse was acclimated to the apparatus before sociability testing, allowing it to explore the empty central compartment for 5 min, followed by 10 min of habituation to all three empty chambers. Exploration was tracked in order to spot possible preferences that could invalidate sociability and social novelty tasks. After the habituation, the subject mouse was briefly confined in the center chamber, while an empty wire cup or a wire cup containing a target stranger mouse (Mouse 1) was positioned in the lateral chambers to start the sociability task. When the doorways opened simultaneously, the camera started tracking for 10 min. In previous testing, target mice were habituated to the cylindric cage for two 10 min sessions distributed over 2 days. Mice that showed inadequate behaviors (barbiting, excessive self-grooming, circling, or clinging to the sidebars with all four paws) were excluded from the following test; in this way, it was assured that target mice displayed a collaborative and sociable behavior toward the subject mice during the test phase. To test social novelty, the subject mouse was isolated in the center chamber again, and two caged mice were positioned in the lateral chambers. The familiar target mouse (Mouse 1) was switched in position after the social task, while a new novel mouse (Mouse 2) was placed in the opposite chamber. The software EthoVisionXT (Noldus) evaluated the number of times the subject mouse entered the lateral chambers and how much time it spent in it, based on its center-point. The software was also set to measure the quantity of time the mouse performed active sniffing toward the two conditions. This was tracked by measuring the time the nose-point mark entered a boundary zone of 15 cm in diameter around the cages. The performances were expressed by preference index, a parameter used to express the time the subject mouse spent in the boundary area, relative to the time spent in both boundary areas, expressed as a percentage. Cumulative time was also scored by an observer blinded to the genotype. The arena was cleaned with soap and water between subjects.

Statistical analysis

Statistical analyses of behavioral and immunohistochemistry data were performed with Prism software (GraphPad 9.0), with the level of significance set at $P < 0.05$ and results are shown as the mean ± SEM.

For behavioral experiments, statistical analysis was performed by Mann-Whitney test or two/three-way ANOVA followed by Tukey's or Bonferroni's post-hoc multiple comparisons, as appropriate. Statistical analysis from immunofluorescence experiments was performed by unpaired t-test.

RESULTS

Increased perineuronal nets density in En2-/- mice

PNNs are found primarily around inhibitory GABAergic neurons expressing parvalbumin and are important regulators of neural plasticity and contribute to the onset of several brain diseases (Xi et al., 2021; Sorg et al., 2016).

En2-/- adult mice show reduced expression of GABAergic markers including PV in the hippocampus and cerebral cortex (Sgadò et al., 2013). To investigate a potential link between the reduced number of PV+ interneurons and impaired neuronal plasticity, a quantification of the PNNs in the primary somatosensory cortex (S1) and hippocampus was performed using *Wisteria floribunda agglutinin* (WFA) immunostaining (Fig. 1(**A,B**)).

A significant increase in the density of PNN-positive neurons was found in the cornus ammonis (CA) 2 and in the dentate gyrus (DG) subregion of the hippocampus of En2-/- mice (Fig. 1(**D,F**); unpaired t-test; En2+/+ vs En2-/-; $P = 0.0077$ and $P = 0.0016$ respectively; $n = 6$ per genotype). No difference in WFA-positive cells was observed between the two genotypes in the CA1 (Fig. 1(**C**); unpaired t-test; EN2+/+ vs En2-/-; $P = 0.1118$; $n = 6$ per genotype) and CA3 subregion (Fig. 1(**E**); unpaired t-test; EN2+/+ vs En2-/-; $P = 0.0909$; $n = 6$ per genotype).

Furthermore, no differences were detected in WFA-positive cells and WFA signal intensity in L2/3 (Fig. 1 (**G**); unpaired t-test; EN2+/+ vs En2-/-; $P = 0.9085$; $n = 6$ per genotype) and L4 respectively (Fig. 1(**H**); unpaired t-test; EN2+/+ vs En2-/-; $P = 0.1118$; $n = 6$ per genotype).

However, a significant increase in the WFA signal intensity was identified in layers 5/6 of the primary somatosensory cortex of En2-/- mice (Fig. 1(**I**); unpaired t-test; EN2+/+ vs En2-/-; $P = 0.0225$; $n = 6$ per genotype). These findings indicate that under baseline conditions En2-/- mice display an increased density of PNNs in both the hippocampus and primary somatosensory cortex, indicating a possible shift toward a premature decrease of neuronal plasticity.

To further characterize PNN alterations in the En2-/- mouse model, double immunostaining with WFA and PV was performed (Fig. 2(**A**)). A higher number of PV+ neurons surrounded by WFA was found in CA3 and DG of En2-/- mice compared to their control littermates (Fig. 2(**C,D**); unpaired t-test; En2+/+ vs En2-/-; $P = 0.0475$ and $P = 0.0019$; $n = 5$ and $n = 4$ per genotype). No significant changes of PV+ interneurons enwrapped by WFA were observed in CA1 and L2/3 (Fig. 2(**B,E**); unpaired t-test; EN2+/+ vs En2-/-; $P = 0.2981$ and $P = 0.1428$; $n = 5$ and $n = 4$ per genotype).

The observed rise in the number of PV+ interneurons encased by PNNs within the hippocampus, not only suggests a diminution of neural plasticity but also

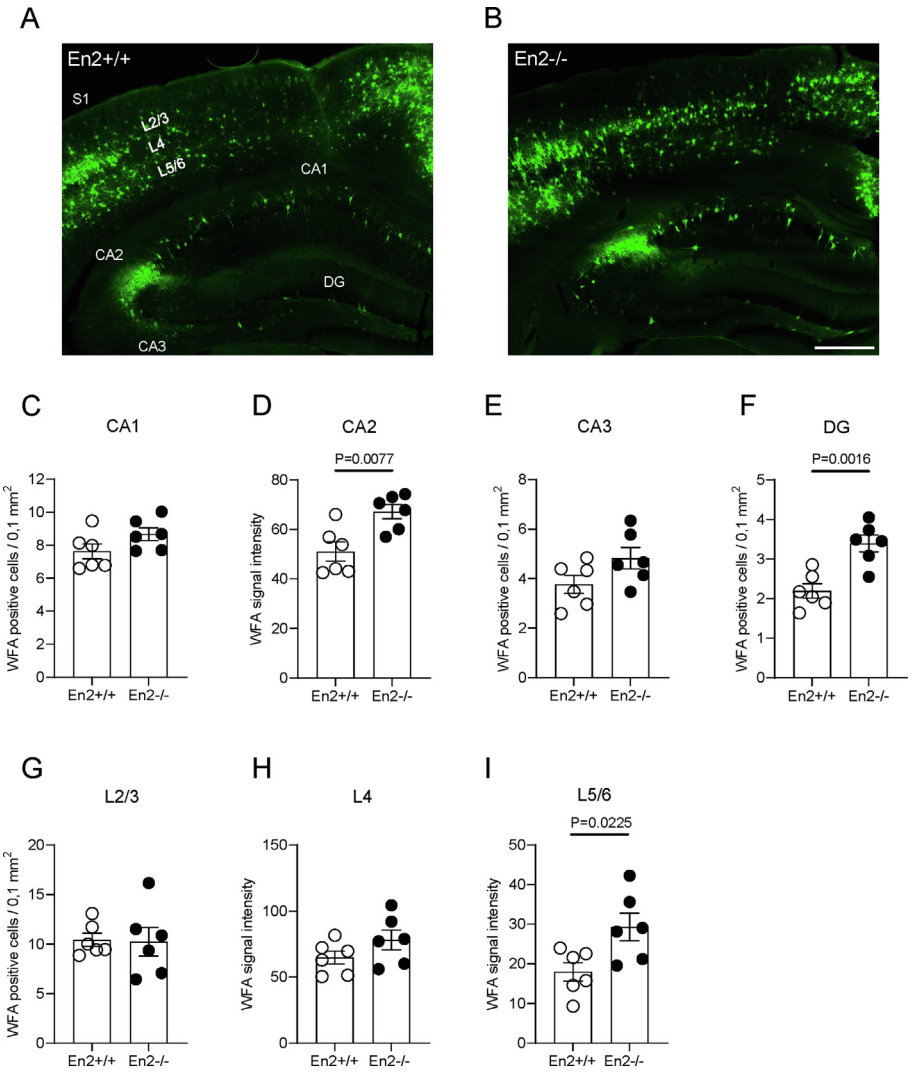

Fig. 1. Increased perineuronal nets in CA2, DG, and L5/6 in EN2-/- mice. (**A, B**) Representative images of WFA-positive PNNs in S1 and dorsal hippocampus of En2+/+ and En2−/− mice. Scale bar = 300 μm. (**C-G**) Quantification of WFA-positive cells in CA1 (**C**), CA3 (**E**), and DG (**F**) regions of the hippocampus and L2/3 (**G**) of the S1. Values are expressed as the mean number (±SEM) of positive cells per area (0.1 mm2) per group; (**D, H, I**) Quantification of WFA fluorescence intensity in CA2 hippocampal region (**D**), L4 (**H**), and L5/6 (**I**) of the S1. Values are expressed as the mean signal intensities (±SEM). Unpaired t-test; En2+/+ vs En2−/− (n = 6 mice for each group). Abbreviations: CA1/2/3, cornu ammonis regions; DG, dentate gyrus; L2–6, S1 cortical layers; S1, primary somatosensory cortex.

highlights the critical influence of PV+ interneurons in regulating the dynamics of neural circuits under pathological conditions.

ECM/PNNs genes enrichment in the hippocampal DEGs of En2-/- mice

Our investigation also sought to determine whether the increased density of PNNs in En2 mice was paralleled by deregulation of genes encoding for components or enzymes involved in the synthesis or degradation of the ECM, with a particular focus on PNNs.

Using differentially expressed genes (DEGs) from En2-/- mice, identified in our previous work (Sgadò et al., 2013), and ECM-related genes from the Molecular Signatures Database (see material and methods section), we performed a comprehensive enrichment analysis.

This analysis revealed a significant enrichment of ECM/PNNs associated genes within the hippocampal DEGs of En2−/− mice (P = 0,010). The enrichment includes 31 genes: Acta2, Agt, Amelx, Anxa8, Aqp1, Calu, Cdkn1a, Chst11, Clec2d, Cthrc1, Elfn2, Fbln1, Fgfr1, Fscn1, Gpc4, Kcnk2, Meg3, Nell2, Ntng1, Prg4, Rgs16, Rgs5, Rspo1, Sema3e, Sema6d, Serpine1, Svep1, Tcf7l2, Thsd4, Tpm1, and Tpm1. This significant finding reinforces the potential complex role of the ECM and PNNs in the genesis of brain, behavioral and functional alterations in En2-/- mice.

En2-/- mice exhibit altered texture discrimination through whiskers

Alterations in whisker-dependent behaviors in En2-/- mice were already observed by Chielini and colleagues (Chielini et al., 2019). To further dissect somatosensory dysfunction, En2 mutant mice and control mice were tested in a whisker-mediated version of tNORT, using sandpaper-wrapped cylinders with different textures (smooth or rough; see Materials and Methods; Fig. 2(**A**)).

Mice have an innate preference for novel stimuli, therefore if the animal is able to discriminate between different textures, it will spend more time investigating the novel textured object, whereas if the animal cannot discriminate between textures, it is expected to spend the same amount of time investigating both objects.

To characterize general locomotor activity and novelty-induced anxiety behavior of En2-/- mice, open field test (OF) was performed for two consecutive days.

During both days En2-/- mice did not show any difference in the total distance traveled compared to controls (Fig. 3(**B**); two-way ANOVA, En2+/+ vs. En2−/−; main effect of genotype F (1, 82) = 2.186; P = 0.1431), but both genotypes showed a significant reduction of distance traveled, implying habituation to the novel environment and similar exploratory behavior (Fig. 3(**B**); two-way ANOVA, main effect of testing days

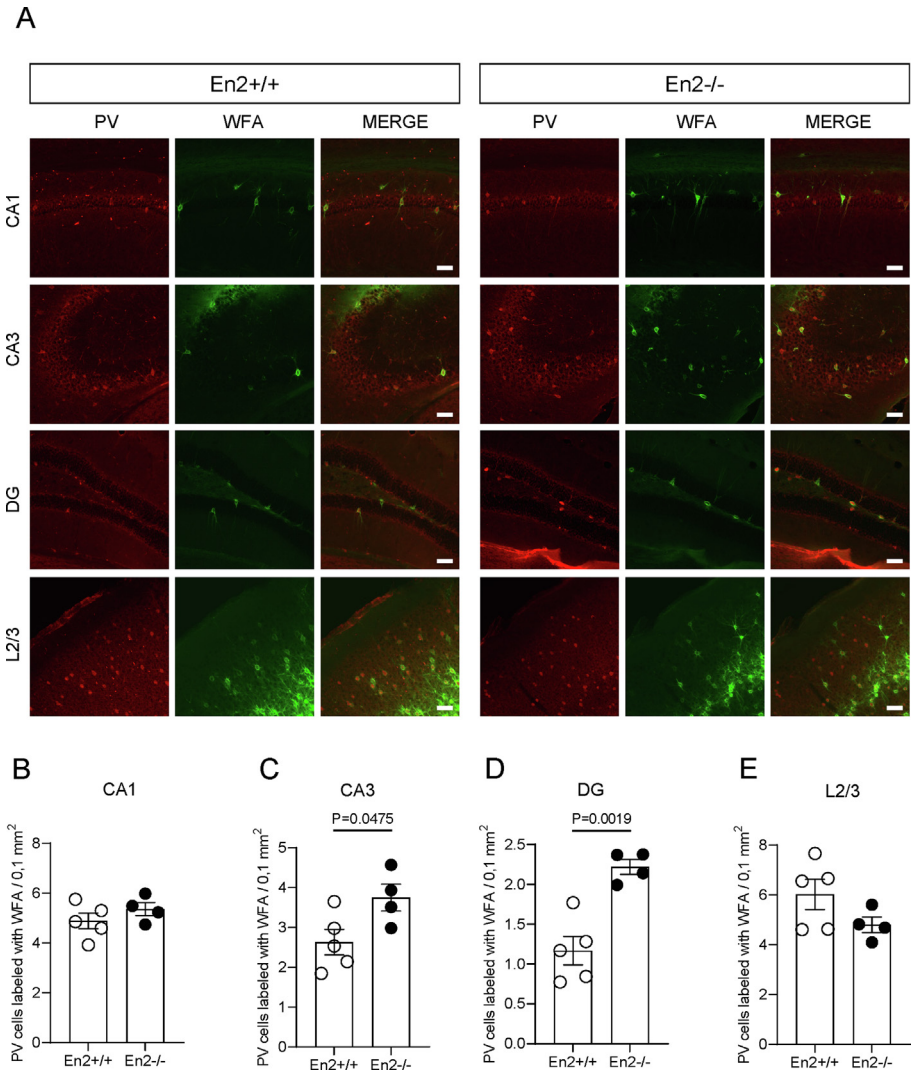

Fig. 2. Density of parvalbumin (PV+) neurons and Wisteria floribunda agglutinin (WFA) perineuronal nets (PNN) is increased in En2-/- mice. Representative immunofluorescence images from layer 2/3 (L2/3) of the S1, and CA1, CA3 and DG from the dorsal hippocampus of En2+/+ and En2−/− mice, respectively. Scale bar = 100 μm (**A**). Quantification of PV+ neurons labeled with WFA (**B-E**). Values are expressed as mean signal intensities (±SEM). Unpaired t-tests; En2+/+ vs En2 (n = 5 and n = 4 mice for each group). Abbreviations: CA1/2/3, cornu ammonis regions; DG, dentate gyrus; S1 cortical layers; S1, primary somatosensory cortex.

F (1, 82) = 19.33, $P < 0.0001$; post hoc Tukey's test, En2+/+ day 1 vs. En2+/+ day 2, $P = 0.0252$, En2−/− day 1 vs. En2−/− day 2, $P = 0.0072$). Furthermore, in the 2 days of habituation, En2-/- mice did not display any difference compared to control mice in the time spent in the center and border of the OF arena (Fig. 3(**C**) three-way ANOVA, En2+/+ vs. En2−/−; main effect of genotype F (1, 164) = 0.001050, $P = 0.9742$; main effect of days F (1, 164) = 0,003409, $P = 0.9535$; main effect of arena regions F (1, 164) = 12.72, $P < 0.0005$). In detail, on the second day of habituation, both En2-/- and control animals exhibited a preference for borders regions of the arena, thus indicating a similar anxious behavior in both genotypes (Fig. 3(**C**); Tukey's post hoc following three-way ANOVA, center vs. borders within En2+/+ and center vs. borders within En2−/−; $P < 0.0001$).

In the learning phase of the tNORT, both genotypes did not show any preference for one of the two identical objects with the same texture (Fig. 3(**D**); two-way ANOVA, main effect of objects F (1, 82) = 3,260, $P = 0.0747$).

Additionally, in both the learning and testing phases, En2-/- and the control group, spent a comparable total amount of time examining the two objects (Fig. 3(**E,G**); unpaired t-test, En2+/+ vs En2-/-, $P > 0.05$), suggesting that mutant mice have preserved whisker-based exploration.

In the testing phase, wild-type mice spent a greater amount of time investigating the novel object. On the contrary, En2-/- mice did not show any difference in the time spent exploring the familiar and novel object (Fig. 3(**F**); Tukey's post hoc following two-way ANOVA, familiar object vs novel object within En2+/+, $P < 0.0001$ and En2-/-, $P = 0.9405$). Altogether, these results indicate that En2-/- mice have impaired whisker-dependent texture discrimination.

Adult En2-/- mice display reduced sociability

A subset of En2-/- and En2+/+ mice used for tNORT were then subjected to the three-chambered social interaction test, which consists of two test sessions (one for sociability and the subsequent one for preference for social novelty). The sociability task is designed to test whether the subject mouse prefers spending more time with a non-familiar mouse, demonstrating social skills typical of rodents, or with an inanimate object. In the tested cohort, En2-/- mice did not show preferences for the novel mouse as compared to the novel object, on the contrary, En2+/+ mice spent more time sniffing the novel mouse than the novel object (Fig. 4(**C**); Sidak's test following two-way ANOVA, novel object vs novel mouse within En2+/+, $P < 0.0001$, and En2-/-, $P > 0.05$). These results perfectly align with previous work (Brielmaier et al., 2012), confirming and validating En2-/- as a mouse model of impairments in social behavior even in late adulthood.

The social novelty task assesses whether the subject mouse prefers spending more time with a novel mouse or a familiar one. As expected, En2+/+ mice spent significantly more time in the chamber with novel target mice, while En2-/- mice did not show a significant

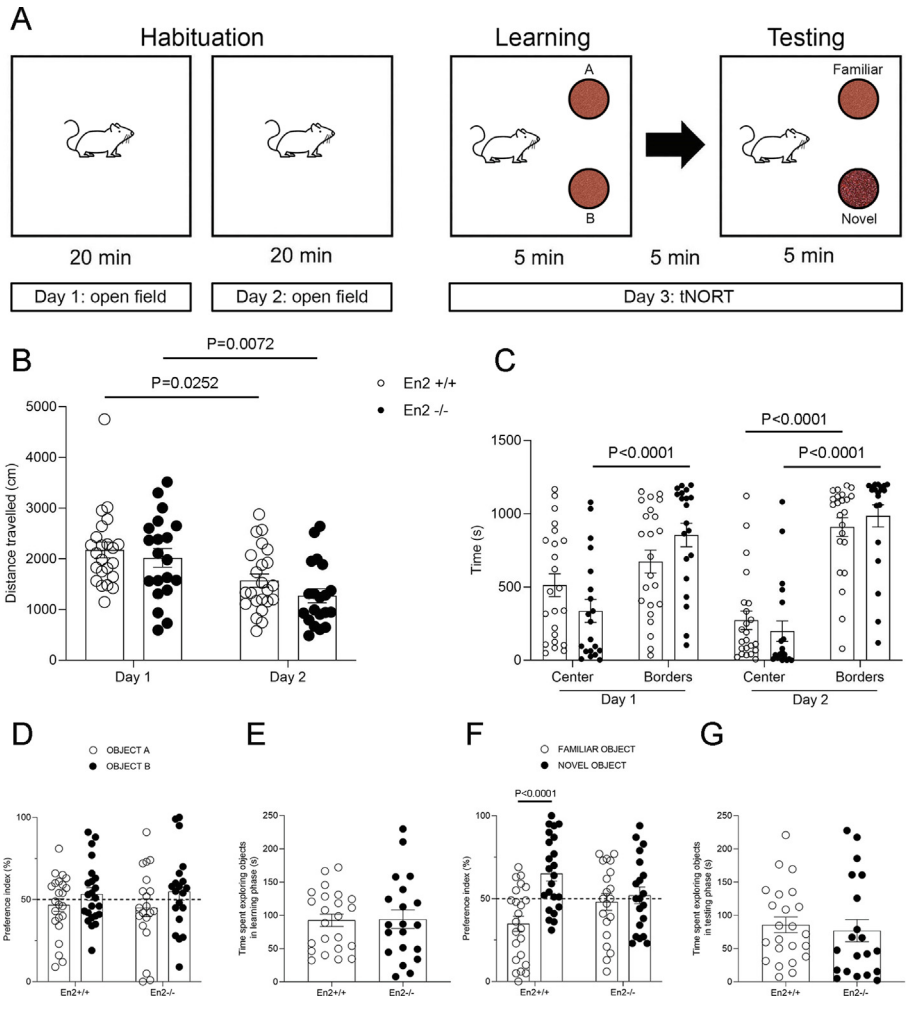

Fig. 3. En2-/- mice display deficits in texture discrimination. (**A**) Schematic of open field and the texture discrimination task (tNORT). (**B** and **C**) Measurement of open field performance by En2+/+ and En2−/− mice. No difference between genotypes in the total distance traveled compared to controls (B; $P > 0.05$ two-way ANOVA). Both genotypes traveled a significantly shorter distance on the second day of the test (B, Tukey's test following two-way ANOVA). En2+/+ and En2−/− mice spent significantly more time in borders than the center of the arena on the second day of habituation (**C**, Tukey's test following three-way ANOVA). (**D**–**G**) tNORT performance quantification for En2+/+ and En2−/− mice. The preference index (%) for objects A and B (D) and the time spent exploring objects in the learning phase (**E**) did not differ between En2+/+ and En2−/− mice ($P > 0.05$, Tukey's test following two-way ANOVA). In the testing phase, En2+/+ mice showed a significantly higher preference index (%) for the novel object as compared to the familiar object (**F**, Tukey's test following two-way ANOVA). En2-/- mice showed no difference in the time spent exploring the familiar and novel objects while spending the same time as controls in exploring objects (**G**, $P > 0.05$, Tukey's test following two-way ANOVA). In D and F, the dashed line represents 50% in the preference index, meaning equal preference for both objects. See Materials and Methods for details about preference index calculation. All plots report the mean values ± SEM; each dot represents one animal. Genotypes are as indicated (n = 23 En2+/+ and 20 En2−/− for open field and tNORT).

preference (Fig. 4(**D**); Sidak's test following two-way ANOVA, familiar mouse vs novel mouse within En2+/+, $P = 0.0366$, and En2-/-, $P = 0.1470$), suggesting impaired social memory.

During the 10 min of habituation, subject mice did not develop a preference for any of the three chambers: both genotypes are inclined to investigate equally, and no biases were detected, confirming optimal conditions for the following test phases (Fig. 4(**A**)). Moreover, all the three phases of the test mice of both genotypes did not show any difference in total distance traveled, implying a preserved locomotor activity (Fig. 4(**B**)).

In conclusion, En2-/- mice displayed behavioral abnormalities in sociability, confirming previous results (Brielmaier et al., 2012), and, in addition, they showed social novelty deficits, a feature never assessed before in this model.

DISCUSSION

In this study, we show that En2-/- mice display increased PNNs intensity in the CA2 and DG subregions of the hippocampus and layer 5/6 of the primary somatosensory cortex. Along with this increased intensity of PNNs, we also observe a higher number of PV+ interneurons encased by PNNs in the CA3 and DG regions of En2-/- mice. We next asked whether En2-/- mice exhibit impaired whisker-dependent texture discrimination and deficits in sociability tasks.

PNNs are extracellular matrix structures that surround preferentially inhibitory (PV+) interneurons regulating their activity by reinforcing the synaptic structure, contributing to synaptic stability and plasticity (Balmer, 2016; Sorg et al., 2016; Bosiacki et al., 2019; Christensen et al., 2021).

An increase in PNNs is associated with a reduced number of inhibitory GABAergic PV+ neurons in the valproic acid-induced mouse model of ASD (Xia et al., 2021). Administration with bacterial enzyme chondroitinase ABC restores PV+ neuronal population in the prelimbic prefrontal cortex (PrL-PFC) (Xia et al., 2021). Previous studies from our laboratory showed that the deletion of En2 results in the partial loss of PV+ interneurons in the hippocampus and the superficial layers of the somatosensory cortex role (Sgadò et al., 2013).

This reduction in PV+ interneurons might contribute to an excitation/inhibition (E/I) imbalance in brain areas involved in the complex behavioral phenotypes observed in En2-/- mice, as also reported by others in different mouse models for ASDs (Gogolla et al., 2009; Sgadò et al., 2011; Lee et al., 2017). In line with these findings, we observed an increased PNNs intensity in brain regions involved in sensory responses and social behaviors. Particularly in layer 5/6 of the somatosensory

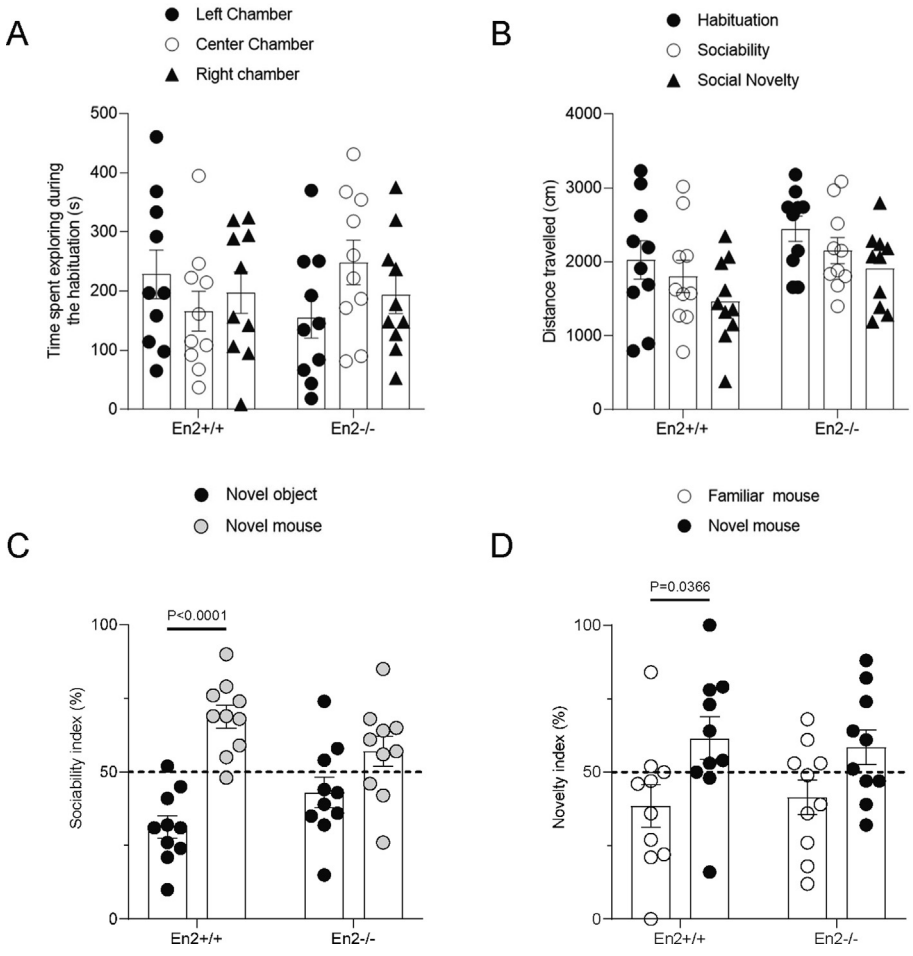

Fig. 4. En2-/- mice show no preference for sociability and social novelty. (**A**) Quantification of the time spent in the left, center, and right chamber of the three-chambered social task during the habituation phase ($P > 0.05$ two-way ANOVA). (**B**) Measurements of the total distance traveled compared to controls during the three phases of the test ($P > 0.05$ two-way ANOVA). (**C**) Quantification of the sociability index for En2+/+ and En2−/− (Sidak's test following two-way ANOVA). (**D**) Quantification of the novelty index for both genotypes (**D**, Sidak's test following two-way ANOVA). See Materials and Methods for details about sociability and novelty index calculation. All plots report the mean values ± SEM; each dot represents one animal. Genotypes are as indicated (n = 10 En2+/+ and 10 En2−/−).

cortex, an area involved in processing sensory stimuli, the increased intensity of the PNNs might correlate with the observed behavioral deficit in texture discrimination via whiskers (Fig. 1). Mice harboring genetic modifications of ASD-associated genes frequently exhibit sensory impairments (Balasco et al., 2020). Several studies have recently focused on tactile abnormalities in ASD mice models (He et al., 2017; Chelini et al., 2019; Chen et al., 2020; Orefice, 2020; Pizzo et al., 2020; Balasco et al., 2022a, 2022b), indicating aberrant somatosensory processing at molecular, behavioral, and functional level. We therefore further characterized the whisker-mediated behaviors using a version of tNORT specifically designed to favor whisker-mediated object exploration (Wu et al., 2013; Balasco et al., 2022a, 2022b). Despite spending the same time examining the two objects with identical textures, En2 mutant mice were unable to distinguish between objects with different textures (Fig. 3). These findings suggest that En2-/- mice have diminished whisker texture discrimination abilities. Excitatory neurons constantly arise in the DG gyrus throughout the lifespan, and DG granule cell firing is increased by somatosensory stimulation (Bellistri et al., 2013; Tuncdemir et al., 2019). Therefore, the observed increase in PNNs density in DG subregion could be linked to reduced adult neurogenesis, that may contribute to the diminished discrimination abilities via the mice whiskers.

The fluorescence intensity of the PNNs was significantly increased also in the CA2 subregion of the hippocampus, an area crucially involved in socio-cognitive memory processing (Hitti and Siegelbaum, 2014). Recent research has shown that a rise in PNNs, specifically in the CA2 region of adult mice, is sufficient to induce social memory dysfunctions (Huang et al., 2023).

The increased intensity and number of PNNs within the CA2 and DG regions, respectively, hint at a potential mechanism for the social deficits observed in En2-/- mice. This finding aligns with the well-documented dysfunction of the DG-CA3-CA2 circuit in both NLG3R451C knock-in (KI) and NLG3 knockout (KO) mice that are associated with impaired social stimuli discrimination (Etherton et al., 2011; Modi et al., 2019).

Social deficits were previously reported in young adult En2−/− mice (8–10 weeks; Cheh et al., 2006; Brielmaier et al., 2012), thus we decided to test a cohort of older adult En2-/- mice (>4 months) and age- and sex-matched control groups by using the three-chambered social approach to verify whether the social deficits are persistent throughout the lifespan. Our findings revealed that En2-/- did not display sociability, as indicated by the lack of preference between the inanimate object and the novel mouse. In addition, En2 mutant mice did not significantly spend time with the novel mouse compared to the familiar one, showing social novelty deficits (Fig. 4). As far as we can tell, this is a feature that has never been examined before in this mouse model.

Our results, obtained on an independent colony of En2 mice (though generated from the same original En2tm1Alj strain, and maintained on a similar genetic background), are in good accordance with previous studies, thus confirming that the lack of sociability is a specific behavioral trait in En2-/-, which is retained in late adulthood. In the barrel cortex, PNNs are generated

in an activity-dependent manner rather than during early postnatal development (Nakamura et al., 2009). Conversely, the alterations of PNNs in the hippocampus could manifest during critical period plasticity due to the reduced firing of GABAergic interneurons, altering the right neural circuits balance in En2-/-. This hypothesis was tested in the Mecp2-null mice model for Rett syndrome, in which it has been described an increased expression of PNNs in the hippocampal area CA2 during development before the onset of behavioral deficits (Carstens et al., 2021).

In our study, we observed a significant increase in WFA-positive PV neurons within the CA3 and DG regions of the hippocampus in En2-/- mice (Fig. 2). This observation raises intriguing questions about the functional implications of such an increase, especially, given the overall decrease in PV neuron populations previously described in En2-/- mice (Sgadò et al., 2013; Provenzano et al., 2020).

The presence of a higher number of WFA-positive PV + neurons, despite the overall reduction in PV+ neurons in En2 -/- mice, suggests a differential regulatory mechanism at play, potentially indicative of a compensatory response aimed at stabilizing neural circuits in critical hippocampal areas. Given the critical role of PV+ neurons in regulating neural circuitry and the importance of PNNs in modulating synaptic plasticity, such a compensatory increase in PNNs might be an attempt to maintain neural circuit stability in the face of reduced PV neuron populations.

However, this increase in PNN levels in PV+ neurons might also imply greater maturation or stabilization of these neurons, along with a reduction in synaptic plasticity within CA3 and DG regions. This nuanced interplay suggests that while PNN formation around PV + interneurons might serve to protect and stabilize neural circuits, it could concurrently diminish the brain's adaptability, impacting cognitive and social memory processes. The complexity of how En2 deletion affects PNN dynamics, potentially leading to altered cognitive and social behaviors, underscores the need for further investigation into the molecular and cellular mechanisms underlying these changes.

The molecular mechanisms underlying En2′s role in PNN maturation and neural plasticity are complex and not well understood. The increase in PNN quantity and density in En2-/- mice may be attributed to En2′s activity as a transcription factor. Research has previously demonstrated that engrailed proteins primarily act as transcriptional repressors (Tolkunova et al., 1998), yet they have also been identified to influence translation via interactions with the eukaryotic translation initiation factor 4E (Nédélec et al., 2004). Therefore, the lack of En2 might result in altered expression levels of key structural elements and enzymes crucial for PNN assembly or formation.

In our study, we also explored the effect of En2 loss on genes associated with the ECM and PNNs through an enrichment analysis, leveraging the list of hippocampal DEGs identified in En2-/- mice from our previous study (Sgadò et al., 2013). This analysis revealed a significant overrepresentation of these genes, indicating that En2 could play a crucial role in regulating the expression of genes essential for the synthesis and regulation of PNN components, as well as enzymes that participate in their synthesis or degradation.

Among the downregulated DEGs, Chst11, Fgfr1, and Sema3e emerge as particularly significant. Sema3e plays a role in axonal guidance and the organization of ECM components surrounding neurons (Bribián et al., 2014), potentially affecting the spatial arrangement of PNNs and their influence on synaptic plasticity. Chst11 is a gene that encodes an enzyme responsible for the 4-O-sulfation of chondroitin sulfate polysaccharides, which are critical components of PNNs. In a recent study, the brain-specific deletion of Chst11 in mice has been shown to lead to a marked increase in PNNs especially around PV+ neurons, within the hippocampal CA2 region. This increase underscores the critical role of Chst11 in regulating the extracellular matrix composition that influences PNN formation, highlighting its importance in neural plasticity and social cognitive dysfunction (Huang et al., 2023).

Disrupted expression of Fgfr1 in mouse neuronal cells upregulates both the expression of genes encoding members of the ECM pathway and the transcription factor Sox9 (Moon and Zhao, 2022). Intriguingly, conditional ablation of Sox9 leads to reduced levels of chondroitin sulfate proteoglycans (CSPGs) within PNNs, highlighting its crucial role in their formation (McKillop et al., 2016).

Among the upregulated genes identified in our analysis, Serpine1 emerges as particularly noteworthy, functioning as a crucial downregulator of fibrinolysis and ECM degradation (Rijken and Sakharov, 2001). Specifically, Serpine1, a serine protease, acts as the primary inhibitor of the plasminogen activator (Plat). Intriguingly, the conditional deletion of Plat in PV+ interneurons *in vivo* leads to an increased density of PNNs in the somatosensory cortex, suggesting its significant role in regulating PNN plasticity (Lépine et al., 2022).

Future studies aimed at deciphering the transcriptional functions of En2, particularly through the use of chromatin immunoprecipitation-sequencing (ChIP-seq) to identify direct En2 target genes, will be instrumental in yielding deeper insights into how En2′s transcriptional activities influence the regulation of PNNs and, consequently, the broader spectrum of synaptic plasticity and neural network dynamics.

Central to the architecture of PNNs are CSPGs, which are essential for their integrity and functionality. Notably, CSPGs engage in critical interactions with signaling molecules, such as orthodenticle homeobox 2 (Otx2), through their chondroitin sulfate glycosaminoglycan (CS-GAG) chains (Carulli and Verhaagen, 2021). Otx2′s selective uptake by PV+ interneurons, through interactions with surrounding PNNs, during postnatal cortical development not only regulates the maturation of the PNNs around PV-cells but also signals both the onset and the closure of critical periods for synaptic plasticity (Bernard and Prochiantz, 2016). These interactions could potentially be shared by other homeoproteins, including En2, given their common structural motifs for cell signal-

ing and internalization (Lee et al., 2019; Carulli and Verhaagen, 2021).

A recent study (Cardon et al., 2023) highlighted the complex interactions between En2 and glycosaminoglycans such as heparan sulfate (HS), emphasizing the intricate mechanism of En2′s internalization that could indirectly influence neural development and plasticity. HS is a key component of the basal lamina, which itself embeds PNNs. Given the role of HS in the extracellular matrix and its capacity to bind to a variety of signaling molecules, the interaction between En2 and HS might influence the extracellular environment, potentially affecting the assembly and stability of PNNs. Through such interactions, HS could influence not only the physical scaffolding provided by the ECM but also the biochemical signals essential for neural development and plasticity. Future research might aim to delineate these indirect effects more clearly, exploring how changes in the extracellular matrix composition, prompted by HS-En2 interactions, contribute to neural plasticity and the dynamic regulation of PNNs.

Restoring normal levels of PNNs via enzymatic degradation has been shown to rescue the abnormal level of PV+ neurons in PrL-PFC *in-vivo* and synaptic plasticity in CA2 *in-vitro* (Carstens et al., 2021; Xia et al., 2021), making PNNs a potential therapeutic target for neurodevelopmental disorders.

In conclusion, our results suggest that the increase in PNNs intensity and density together with a reduced number of PV+ interneurons may contribute to the sensory and social abnormalities that occur in En2-/- mice. The proper formation of synaptic connections and the development of stable neuronal networks may be a result of normal PNN interactions with PV interneurons, in particular fast-spiking PV cells (Wen et al., 2018).

Structural and functional abnormalities of perineuronal nets may thus underlie an improper functioning of the forebrain, which remains less plastic and sensitive to alterations in sensory and social inputs.

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CONFLICT OF INTEREST

The authors declare there are no competing financial interests.

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