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ORIGINAL PAPER

Atypical Auditory Brainstem Response and Protein Expression Aberrations Related to ASD and Hearing Loss in the *Adnp* Haploinsufficient Mouse Brain

Gal Hacohen-Kleiman[1] · Ofer Yizhar-Barnea[2] · Olga Touloumi[3] · Roza Lagoudaki[3] · Karen B. Avraham[2] · Nikolaos Grigoriadis[3] · Illana Gozes[1]

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Abstract

Autism is a wide spread neurodevelopmental disorder with growing morbidity rates, affecting more boys than girls worldwide. *Activity-dependent neuroprotective protein* (*ADNP*) was recently recognized as a leading gene accounted for 0.17% of autism spectrum disorder (ASD) cases globally. Respectively, mutations in the human *ADNP* gene (*ADNP* syndrome), cause multi-system body dysfunctions with apparent ASD-related traits, commencing as early as childhood. The *Adnp* haploinsufficient ($Adnp^{+/-}$) mouse model was researched before in relations to Alzheimer's disease and autism. $Adnp^{+/-}$ mice suffer from deficient social memory, vocal and motor impediments, irregular tooth eruption and short stature, all of which corresponds with reported phenotypes in patients with the *ADNP* syndrome. Recently, a more elaborated description of the *ADNP* syndrome was published, presenting impediments such as hearing disabilities in > 10% of the studied children. Irregular auditory brainstem response (ABR) has been connected to ASD-related cases and has been suggested as a potential hallmark for autism, allowing diagnosis of ASD risk and early intervention. Herein, we present detriment hearing in the $Adnp^{+/-}$ mice with atypical ABR and significant protein expression irregularities that coincides with ASD and hearing loss studies in the brain.

Keywords *Activity-dependent neuroprotective protein ADNP* · *ADNP* syndrome · Hearing loss · Developmental delays · Auditory brainstem response ABR · *Adnp* haploinsufficient $Adnp^{+/-}$

Introduction

Autism spectrum disorder (ASD) affects ~ 1.5% children worldwide with higher prevalence in boys [almost fives times more common among boys (1 in 42) than among girls (1 in 189)] with ~ 50% deficient or borderline intellectual function in the US [1]. With time, ASD prevalence keeps rising, as a recent paper reported an outgrowth of ~ 30% in the US, compared with records given by the Center for Disease Control in 2012 [2]. ASD is of heterogenic background and etiology remains unclear [3, 4]. Today, there is growing evidence that single gene rare mutations may be sufficient to cause ASD and are a significant tool in deciphering the etiology of the disease [4–8]. Activity-dependent neuroprotective protein (*ADNP*), discovered and extensively studied by our laboratory (Gozes), is crucial for brain development and mental function in mice and humans [9–11]. Previous studies in mice show *Adnp* is expressed as early as during the second week of the pregnancy [10], regulating > 400 genes

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✉ Illana Gozes
igozes@tauex.tau.ac.il
https://english.tau.ac.il/profile/igozes
https://www.adnpkids.com/illana-gozes.html

[1] The Lily and Avrahamo Gildor Chair for the Investigation of Growth Factors; The Elton Laboratory for Neuroendocrinology; Department of Human Molecular Genetics and Biochemistry, Sackler Faculty of Medicine, Sagol School of Neuroscience and Adams Super Center for Brain Studies, Tel Aviv University, 69978 Tel Aviv, Israel

[2] Department of Human Molecular Genetics and Biochemistry, Sackler Faculty of Medicine and Sagol School of Neuroscience, Tel Aviv University, Tel Aviv, Israel

[3] Department of Neurology, Laboratory of Experimental Neurology, AHEPA University Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece

important for brain and cardiovascular systems development [12]. *ADNP* is highly conserved in vertebrates ranging from fish to human [13] and has been identified as responsible for ≥0.17% of known ASD cases [14, 15]. In children, *ADNP* mutations cause an autistic-like disorder (Helsmoortel-Van der Aa syndrome; *ADNP* syndrome) depicting mild to severe intellectual, motor, social and speech delays/disabilities [14–16]. Similarly, in mice, *Adnp*-deficiency ($Adnp^{+/-}$) leads to social and vocal impediments, motor delays and learning and memory deficits [17–19]. Further biochemical evidence emerged, showing *ADNP* regulates ion channels genes (e.g. calcium channels) and potentially regulates the protein translation process through eukaryotic initiation factor 4E (eIF4E)-binding, both tightly associated with autism [17, 19, 20]. This was also coupled with significant sex-dependent disruption of synaptic formation (hippocampus and motor cortex) and axonal transport in $Adnp^{+/-}$ mice [18, 19]. In a recent study, which presented full clinical characterization of the *ADNP* syndrome, high resemblance was found to other ASD-related syndromes, including mild hearing loss (11.7% out of a 78-study cohort) [21]. Commonly, auditory brainstem response (ABR) is tested in suspicion of potential hearing loss or other developmental aberrations [22, 23]. As prolonged ABR latencies were reported in young children with autism (as early as infancy) and in some cases with normal hearing [22, 24–26], ABR was suggested as a discrimination tool for early diagnosis and intervention in autism [22, 27]. To determine $Adnp^{+/-}$ mouse model relevance to hearing loss studies and potential correlation with additional symptoms in the *ADNP* syndrome, we set out to test neurotransmitter pathways in the auditory cortex and added cerebellum and hippocampus for additional reference.

Methods

Animals

Animals: The $Adnp^{+/-}$ mice generation was previously described [10, 17–19, 28]. Animals were housed in a 12-h light/12-h dark cycle animal facility, with free access to rodent chow and water. At the age of 21–30 days, male and female mice were separated and *Adnp* genotype ($Adnp^{+/+}$ and $Adnp^{+/-}$) was confirmed using Transnetyx services (Memphis, TN, USA). ABR hearing test and ultrasonic vocalization (USV) recordings were performed on 2.5-month-old and 8-day-old *Adnp* mice respectively, both ideal ages for the machinery and settings at our disposal.

Mouse Hearing Test

Auditory brainstem response (ABR) recording was used to evaluate the inner ear cochlear functionality as was previously described [29, 30]. 2.5-month-old $Adnp^{+/+}$ and $Adnp^{+/-}$ male and female mice (n = 5–7) were anesthetized (ketamine/xylazine) and placed in a sound attenuating chamber connected to the ABR auditory workstation (Tucker Davis Technologies). Body temperature was maintained at approximately 37 °C using a heating pad (Braintree Scientific). System calibration was maintained throughout the day when measurements were made. Subdermal needle electrodes were placed midline above the frontal bone (positive) and behind the left pinna, with a ground electrode in the left thigh. ABR thresholds (dB) and ABR Hearing latency (sec) were measured in response to tone bursts at 6, 12, 18, 24, 30, and 36 kHz [29, 31]. Tone burst stimuli of alternating polarity were repeated at 75-ms intervals in 10-dB increments starting at 100dB (90dB in click test) and decreasing to 20dB. ABR were recorded over 40 msec and averaged at each intensity level for 1024 presentations. Mice were not euthanized at the end of an ABR measurement, but rather returned to a recovery cage.

Ultrasonic Vocalization (USV)

USV recordings were performed on naïve $Adnp^{+/+}$ and $Adnp^{+/-}$ male and female pups (n = 10–14) as previously described [19]. In short, on day 8 (P8), each pup was separated from the dam and placed in an empty cage for 6 min. USVs (40–70 kHz) were recorded using an ultrasonic vocalization detector ANL-973-1 microphone. At the end of each trial, the pup was marked with a tattoo for identification (Ketchum, Canada), and reunited back with its dam, placed in the far corner of the box, allowing the mother to retrieve the pup [19, 32]. Data were analyzed using Med USV viewer SOF-937-1 (Med associates, Vermont, USA). The minimal time distance between calls was set to 0.04 s and the volume threshold was set to 40dB. Results are presented as mean number of ultrasonic vocalizations recorded per minute of the trial, compared with $Adnp^{+/+}$ pup littermates (control).

Immunohistochemistry

P0 $Adnp^{+/+}$ and $Adnp^{+/-}$ mice (n = 2–3, undetermined sex) were euthanized according to the ethical guidelines, afterwards both inner ear of each mouse was taken out rapidly. The cochlear oval window was exposed and punctured prior to placing the entire inner ear in paraformaldehyde (4% v/v in PBS) for 2 h at room temperature. Samples were rinsed in phosphate buffered saline (PBS), and blocking was achieved with normal goat serum (10% v/v in PBS, 0.5% Triton X-100). The samples were then incubated with primary antibodies at 4 °C overnight (Table 1). Samples were washed with PBS three time and counterstained with fluorescent conjugated secondary antibody if needed (Table 1) [33]. Samples were visualized using a Leica TCS

Table 1 Summary of antibodies and reagents used in this study

Antibody	Type	Code	Source
Myosin-VIIa	Rabbit polyclonal antibody	PTS-25-6790-C050	Enzo life sciences, USA
Phalloidin-iFluor 488 reagent [F-Actin]	iFluor 488 dye	CAT#ab176753	Abcam, UK
DRAQ5™ fluorescent probe solution	probe solution	CAT# 62251	Invitrogen, USA
Alexa fluor 568- goat anti-rabbit	Secondary antibody	CAT#A-11011	Invitrogen, USA
ChAT	Rabbit polyclonal antibody	AB144-P	Millipore
GAD67	Mouse monoclonal antibody	ab 26116	Abcam, UK
Parvalbumin (PVALB)	Rabbit polyclonal antibody	ab 11427	Abcam, UK
VGLUT2	Mouse monoclonal antibody	ab 79157	Abcam, UK
CX32	Rabbit polyclonal antibody	34-5700	Invitrogen, USA
Serotonin transporter (Slc6a4)	Rabbit polyclonal antibody	AB 9726	Millipore
GAD65	Rabbit polyclonal antibody	PA5-22260	Thermo Fisher Scientific
VGLUT1	Goat polyclonal antibody	sc-13320	Santa Cruz Biotechnology, Inc
FOXP2	Rabbit polyclonal antibody	ab 16046	Abcam, UK
Goat anti-rabbit IgG	Secondary antibody	BA-1000	Vector
Goat anti-mouse IgG	Secondary antibody	sc-2039	Santa Cruz Biotechnology, Inc
Rabbit anti-goat IgG	Secondary antibody	AP-106B	Chemicon

Stimulated emission depletion (STED) confocal microscope. Images were taken from n = 3 $Adnp^{+/-}$ pups and n = 1 $Adnp^{+/+}$ pup, per region.

For brain immunohistochemical staining, 5-month-old $Adnp^{+/+}$ and $Adnp^{+/-}$ male and female mice (n = 2–3) were perfused transcardially under deep anesthesia with 4% PFA in 0.1M PBS, PH 7.4. Brains were removed, post-fixed in the same fixative and embedded in paraffin. Brains were cut to 6 µm serial brain sections that were then deparaffinized and hydrated in xylene and alcohol solutions and rinsed with TBS. Citrate buffer was used for antigen retrieval and the endogenous hyperoxidase was blocked with 3% H2O2 in methanol. After incubation in blocking buffer (FBS 10%), the sections were treated with primary antibodies overnight (see Table 1). Secondary antibodies were used appropriately (see Table 1), immunoreactions were visualized using DAB as chromogen. Counterstaining was performed with hematoxyline to show nuclear staining. The evaluation was performed on the auditory cortex, cerebellum and hippocampus (CA1).

Bioinformatics

The STRING protein–protein interaction network tool [34] (https://string-db.org) was utilized on proteins, which significantly changed in expression due to the *Adnp* haploinsufficient genotype in mouse auditory cortex, cerebellum and hippocampus.

Statistics

Results are presented as means ± standard error of the mean (SEM). Data were checked for normal distribution by normality test using the Shapiro– Wilk test. For two different categorical independent variables, two-way analysis of variance (ANOVA) or two-way repeated measures ANOVA followed by Holm–Sidak method or Tukey post hoc test were utilized. Student's *t*-test analysis was performed when needed. P values smaller than 0.05 were considered significant. All tests were two-tailed. Statistical analyses were conducted using either SigmaPlot software version 11 Inc. for Windows (Chicago, IL, USA) or GraphPad Prism 5.0 software (immunohistochemistry). For immunohistochemistry, data were analyzed using either one-way ANOVA or Kruskal–Wallis test followed by Dunn post-hoc test.

Results

Abnormal ABR and Decreased Vocalization in $Adnp^{+/-}$ Mice

The *Adnp* haploinsufficient mouse model was previously generated and presents many similarities to the *ADNP* syndrome symptoms including significant vocal, motor and cognitive impediments, compared with intact littermates [19]. In a recent paper, children with the *ADNP* syndrome were diagnosed with hearing impairment, with two (out of

78) children using hearing aids as a result of sensorineural hearing loss and > 10% suffering from hearing loss [21]. Thus, auditory brainstem response (ABR) hearing test was applied on *Adnp* mice. 2.5-month-old *Adnp* mice ABR were analyzed for threshold and latency of auditory response (Fig. 1a–e). The ABR test provides information regarding the inner ear (cochlea) and the central pathways for hearing. The stimuli response is measured electronically and therefore reflects issues with hearing perception. The data is registered at the endpoint reflecting the mis-conduction of normal electrical signaling from the cochlea to the recipient brain section (the auditory nuclei) [35, 36]. Results showcased an increase in hearing thresholds as well as prolonged latency in auditory brain response in the $Adnp^{+/-}$ mice (males and females) compared with $Adnp^{+/+}$littermates ($p < 0.05$) (Fig. 1a–d). Click hearing thresholds are presented in Fig. 1e. Male $Adnp^{+/-}$ exhibited significant hear loss ($p < 0.05$) with increased hearing threshold compared

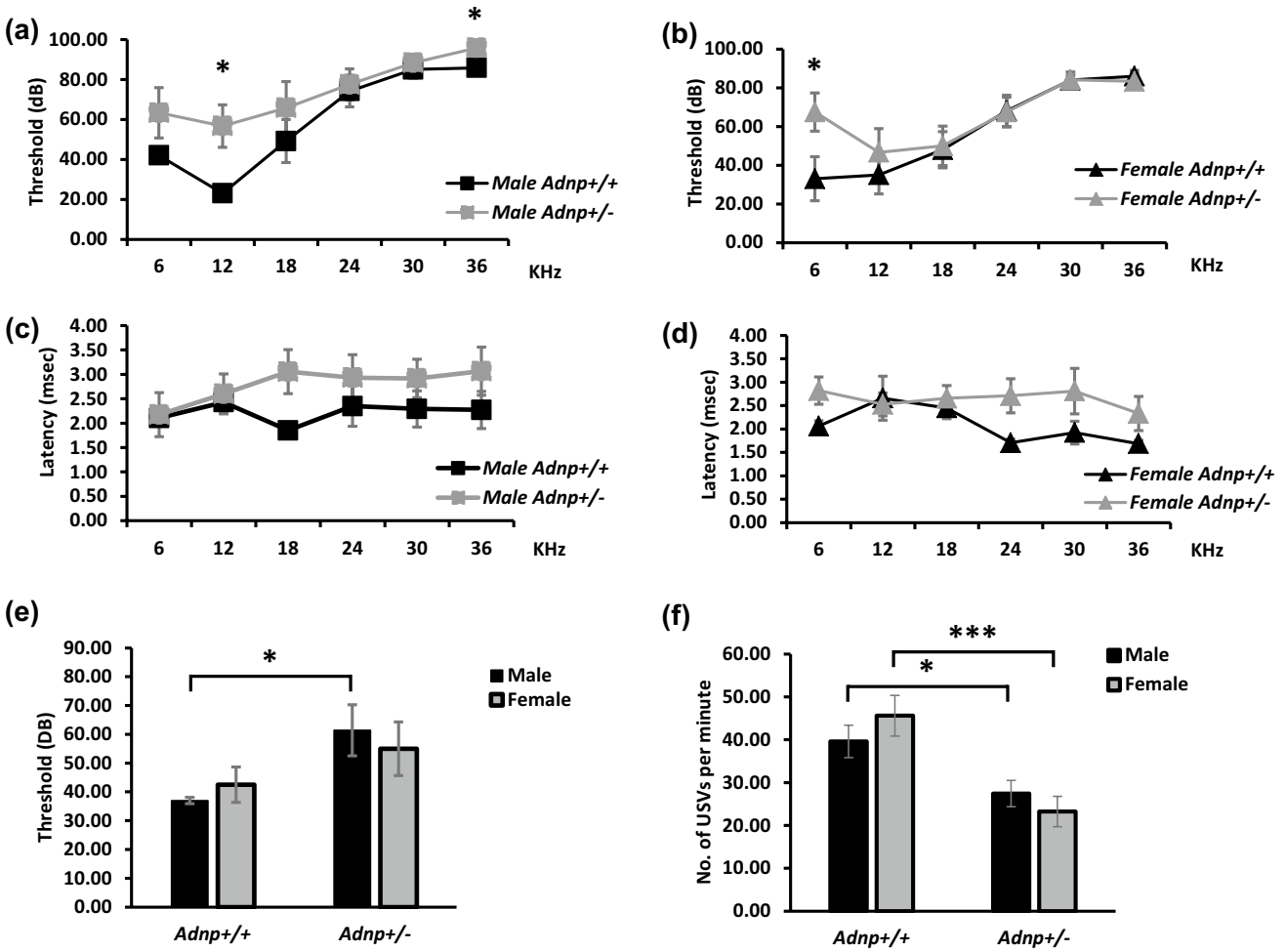

Fig. 1 *Adnp* $^{+/-}$ mice present increased thresholds and latency of auditory evoked responses coupled with a decrease in number of USV calls. ABR was recorded in 2.5 months old $Adnp^{+/-}$ mice in response to pure tone bursts (kHz) at 6, 12, 18, 24, 30, and 36 kHz [29, 31]. For thresholds and latency determination, two-way ANOVA repeated measures followed by Holm-Sidak method were performed. **a, b** For auditory threshold, main kHz effect was found for both sexes ($F(5,48) = 27.268$, $p < 0.001$ in males; $F(5,45) = 21.315$, $p < 0.001$ in females) as well as a statistically significant interaction between Sex and Genotype and kHz in females ($F(5,45) = 2.977$, $p = 0.021$). Significantly higher thresholds were found for male $Adnp^{+/-}$ in 12 and 36 kHz compared with $Adnp^{+/+}$ (*$p < 0.05$, Student's t-test). Similarly, significantly higher threshold was observed in 6 kHz for female $Adnp^{+/-}$ compared with $Adnp^{+/+}$ (*$p < 0.05$). **c, d** For latency of auditory evoked responses, no statistically significant factors were found. **e** For click measurement, two-way ANOVA followed by Holm-Sidak method were applied. Main Genotype effect was found ($F(1,20) = 6.108$, $p < 0.05$). $Adnp^{+/-}$ males exhibit significant increase in hearing threshold compared with littermates (*$p < 0.05$). Male $Adnp^{+/+}$ ($n = 6$); Male $Adnp^{+/-}$ ($n = 6$–7); female $Adnp^{+/+}$ ($n = 5$–6); Female $Adnp^{+/-}$ ($n = 6$). **f** For USV analysis, two-way ANOVA with Tukey post hoc test was performed. Main genotype effect ($F(1,277) = 19.192$, $p < 0.001$) was found. Both male and female naïve $Adnp^{+/-}$ pups (P8) produced significantly less USVs per minute, compared with $Adnp^{+/+}$ (*$p < 0.05$, ***$p < 0.001$). Results are presented as mean (±SEM) USV per minute (males: $Adnp^{+/+}$ $n = 14$; $Adnp^{+/-}$ $n = 11$; females: $Adnp^{+/+}$ $n = 12$; $Adnp^{+/-}$ $n = 10$, 6 USV calls per mouse)

with $Adnp^{+/+}$ littermates. Females presented a similar trend ($p = 0.247$). To further the potential correlation between *Adnp* haploinsufficient mice and the *ADNP* syndrome, vocal communication was tested on naïve *Adnp* mouse pups. Pup USV calls (P8) were induced following dam separation (40–70 kHz) and recorded as previously described [19, 32, 37]. A significant decline in the number of USVs per minute was observed in both male ($p < 0.05$) and female (twofold, $p < 0.001$) $Adnp^{+/-}$ pups, compared with $Adnp^{+/+}$ littermates (Fig. 1f). Significant p-values appear in asterisks.

Normal Hair-Cell Morphology and Structure

ADNP has been known to be associated with the cytoskeleton, implicated in synaptic plasticity, microtubule-dependent axonal transport and dendritic spine formation [18, 19, 38, 39]. To further investigate the origin of hearing loss in $Adnp^{+/-}$ mice, $Adnp^{+/+}$and $Adnp^{+/-}$ mouse pup (P0) cochlea were prepared and stained for myosin and actin (cytoskeleton) (Fig. 2) [33]. $Adnp^{+/-}$ hair-cell morphology was normal (Fig. 2a) with no visible changes compared with $Adnp^{+/+}$ stains (Fig. 2b).

Significant Protein Expression Alterations in $Adnp^{+/-}$ Mouse Auditory Cortex, Cerebellum and Hippocampus

Several autism and auditory related proteins were examined for expression patterns in the $Adnp^{+/-}$ mouse brain. A closer look into the auditory cortex (Fig. 3a) revealed significant changes in two important proteins: Choline O-Acetyltransferase (ChAT) and Glutamate Decarboxylase 1 (GAD67), previously studied in association with hearing loss [40–42]. Specifically, ChAT was significantly decreased (> twofold) in male $Adnp^{+/-}$ ($p < 0.05$) while GAD67 significantly increased (threefold, $p < 0.05$) in female $Adnp^{+/-}$ compared with littermates. The cerebellum was also previously related with the auditory circuitry, more specifically to the

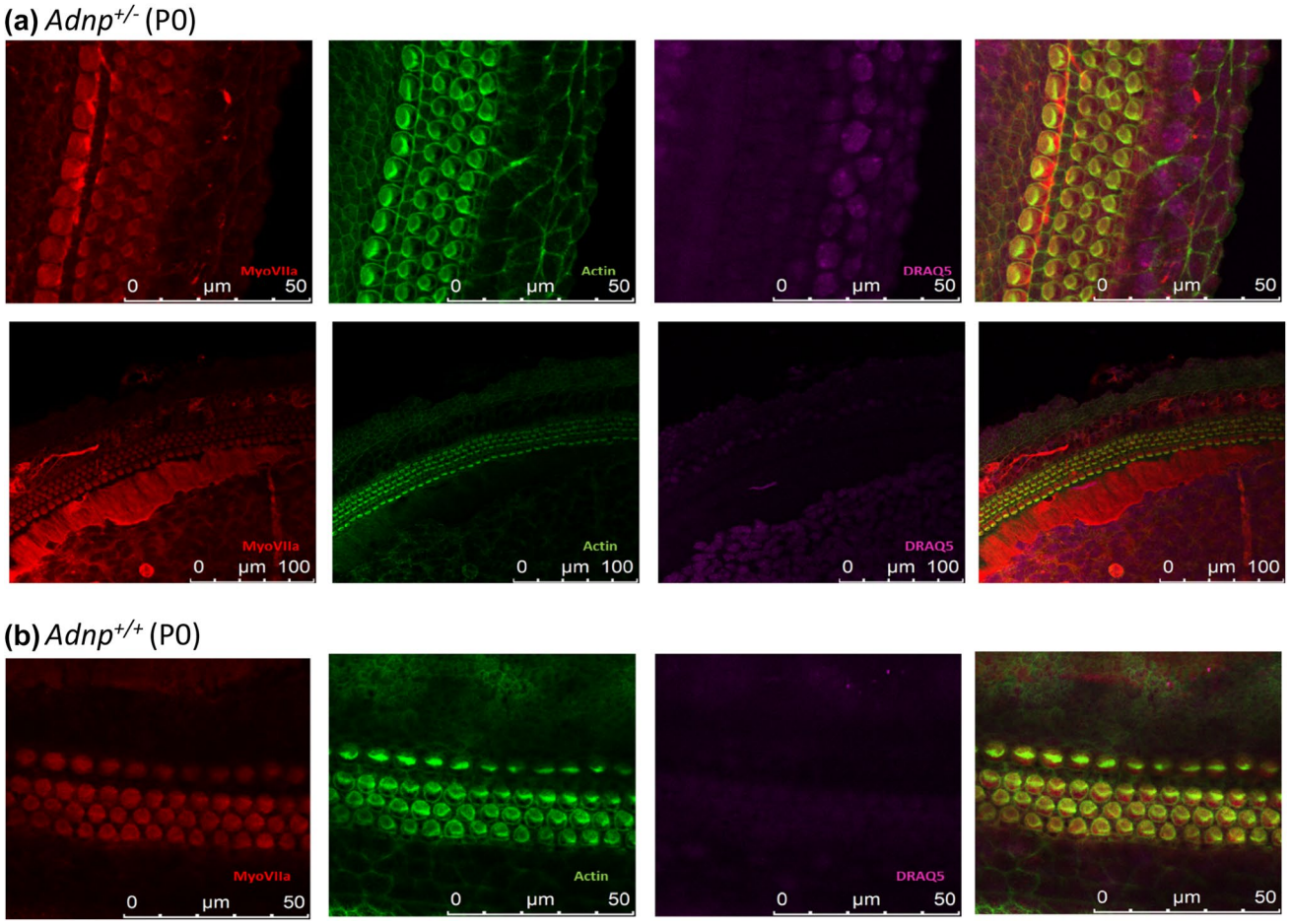

Fig. 2 Normal hair cell morphological appearance in the $Adnp^{+/-}$ mouse ear. Representative images of hair cell phenotypes in the basal and middle turns at P0 for **a** $Adnp^{+/-}$ and **b** $Adnp^{+/+}$ mice stained for Myosin (red), Actin (green) and cell nuclei (purple). Images show normal morphological appearance in $Adnp^{+/-}$ and $Adnp^{+/+}$ mice. Images were taken from n = 3 $Adnp^{+/-}$ pups and n = 1 $Adnp^{+/+}$ pup, per region. Scale bars: 0–50 µm or 0-100 µm

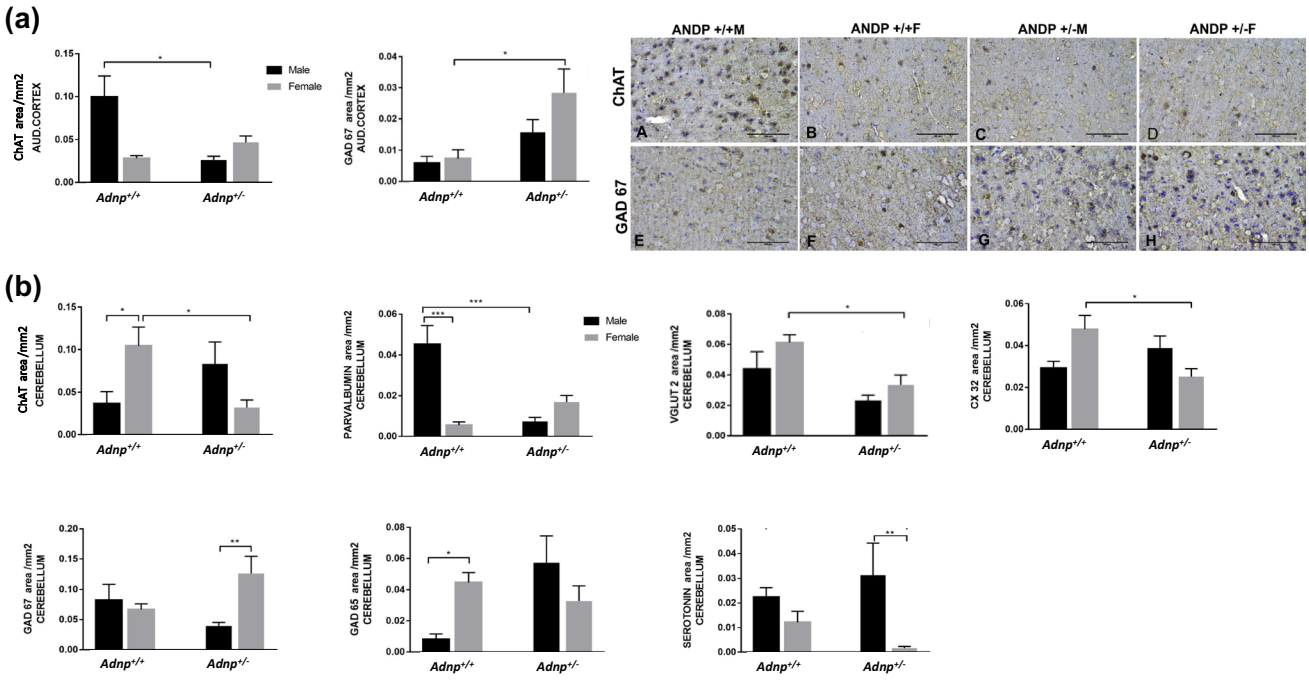

Fig. 3 Significant protein expression abnormalities related to autism and hearing loss in the *Adnp*$^{+/-}$ mouse auditory cortex and cerebellum. Brain sections from 5-month-old mice were stained for potential auditory-related abnormalities (Table 1) within the **a** auditory cortex, **b** cerebellum. Bar graphs represent means (cells per mm2) ± SEM. Representative photos of ChAT (A,B,C,D) and GAD67 (E,F,G,H) expression patterns in the auditory cortex, using optical microscopy. DAB (brown color) and hematoxylin (blue color), magnification x40. Data were analyzed using either one-way ANOVA or Kruskal–Wallis test with Dunn post-hoc test. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). *Adnp*$^{+/+}$ male (n = 2); *Adnp*$^{+/+}$ female (n = 3); *Adnp*$^{+/-}$ male (n = 3); *Adnp*$^{+/-}$ female (n = 3)

auditory cortex and cochlea [43], as well as to autism [44] (Fig. 3b). Parvalbumin (PVALB), a high affinity calcium ion-binding protein, was found to dramatically decrease in male *Adnp*$^{+/-}$ cerebellum (fourfold, $p < 0.001$) compared with *Adnp*$^{+/+}$. *Adnp*$^{+/+}$ Female expression levels were also significantly lower compared with males ($p < 0.001$). Solute Carrier Family 17 Member 6 (VGLUT2), Gap Junction Protein Beta 1 connexin 32 (CX32) and ChAT were downregulated in female *Adnp*$^{+/-}$ ($p < 0.05$), compared with *Adnp*$^{+/+}$. Interestingly, ChAT expression levels were significantly lower in *Adnp*$^{+/+}$ male cerebellum than in female *Adnp*$^{+/+}$ ($p < 0.05$). Statistically significant sex differences were also found for Serotonin transporter, solute carrier family 6 member 4 (Slc6a4) [18] and GAD67 in *Adnp*$^{+/-}$ mice ($p < 0.01$) and for Glutamate Decarboxylase 2 (GAD65) in *Adnp*$^{+/+}$ ($p < 0.05$). ADNP has been extensively studied in the hippocampus giving rise to hippocampal-specific gene/protein expression changes associated with autism and Alzheimer's disease (AD) [17–19]. These results corroborate previous reports of *Adnp*$^{+/-}$ affecting learning and memory (in-vivo) and tau hyperphosphorylation (in-vitro), further linking ADNP to Alzheimer's disease and taupathy [28]. Therefore, we have chosen to test these important proteins in the hippocampus (Fig. 4). For ChAT, results in the hippocampus present similar expression patterns to the auditory cortex, with a significant decrease in male *Adnp*$^{+/-}$ ($p < 0.05$) compared with male littermates. Similarly, PVALB expression levels were decreased in male *Adnp*$^{+/-}$ and female *Adnp*$^{+/+}$ compared with male *Adnp*$^{+/+}$ ($p < 0.0001$), parallel to cerebellar results. Expression levels for male hippocampal CX32 and Forkhead box protein P2 (FOXP2) were significantly altered as well. CX32 expression decreased due to *Adnp* haploinsufficiency while FOXP2 increased ($p < 0.05$). In females, hippocampal GAD67 expression levels significantly increased *Adnp*$^{+/-}$, compared with female *Adnp*$^{+/+}$ ($p < 0.0001$). These results correspond with GAD67 results in the auditory cortex. Statistically significant sex differences were also found for Solute Carrier Family 17 Member 7 (VGLUT1) in *Adnp*$^{+/-}$ mice ($p < 0.001$) and for GAD65 in *Adnp*$^{+/+}$ ($p < 0.05$).

Potential Auditory-Related ADNP Mechanism in the Brain

The STRING protein–protein interaction tool was implemented for potential auditory-related mechanism and function enrichment of biological processes related to *Adnp* deficiency (Fig. 5) [19]. The analysis was performed on all proteins that changed as a result of the *Adnp*$^{+/-}$ genotype

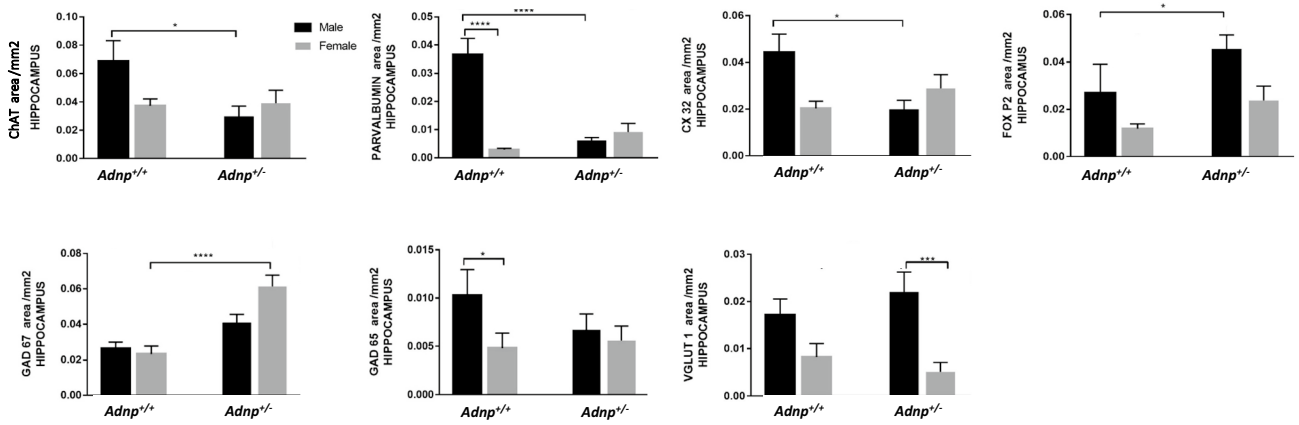

Fig. 4 ASD and inflammation potential markers in the *Adnp*$^{+/-}$ mouse hippocampus. Brain sections from 5-month-old mice were stained for potential hippocampal abnormalities (Table 1) within the hippocampus (CA1). Bar graphs represent means (cells per mm2) ± SEM. Data were analyzed using either one-way ANOVA or Kruskal–Wallis test with Dunn post-hoc test. (*p < 0.05; ***p < 0.001; ****p < 0.0001). *Adnp*$^{+/+}$ male (n = 2); *Adnp*$^{+/+}$ female (n = 3); *Adnp*$^{+/-}$ male (n = 3); *Adnp*$^{+/-}$ female (n = 3)

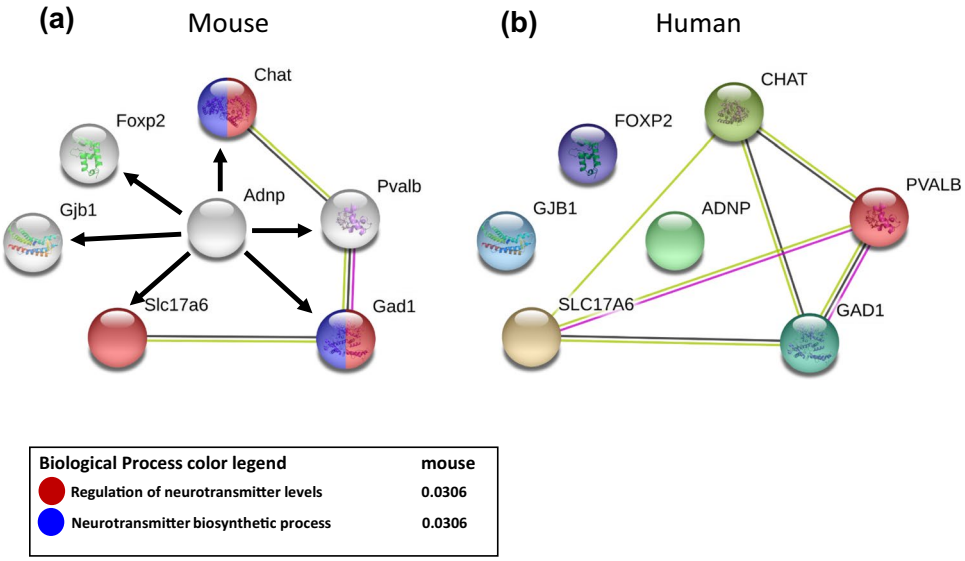

Fig. 5 Function enrichment and network analysis for proteins regulated by the *Adnp* genotype. STRING protein–protein interaction network [19, 34] (https://string-db.org) was performed on proteins that were found to be regulated by the *Adnp* haploinsufficient genotype in the brain immunohistochemistry (Figs. 3, 4). Proteins were compared with either mouse (**a**) or human databases (**b**). **a** Two biological processes were found to be enriched in mouse and marked on the network according to the color legend. False discovery rates are also specified. The suggested newly-found connections between Adnp and other proteins are marked with black arrows. **b** Results from the human database present a similar protein network with an additional connection between VGLUT2 and ChAT proteins with no enriched biological processes found. Proteins are presented in accordance to genome databases as follows: ADNP, FOXP2, PVALB, ChAT (CHAT), GAD67 (GAD1), VGLUT2 (SLC17A6) and CX32 (GJB1)

in the mouse auditory cortex, cerebellum and hippocampus. Enriched biological processes were found when analyzed according to the mouse genome, encompassing regulation of neurotransmitter levels and neurotransmitter biosynthesis (Fig. 5 enclosed table).

Discussion

As autism prevalence rises [2], early intervention is needed. There is a noted difficulty in early treatment onset as most children with ASD are diagnosed only at the age

of 2–4 years [45]. The reported children with the *ADNP* syndrome show many disorder manifestations including ASD symptoms, facial features and tooth eruption abnormalities, severe motor and speech delay, hearing loss and neuronal and behavioral problems, with all children presenting intellectual disabilities [14, 16, 21, 46]. The known *ADNP* mutations result in a truncated ADNP protein, excluding important functional properties, which may mimic the phenotype of the $Adnp^{+/-}$ mouse model. In a recent paper, NAP (CP201) therapeutic effects were demonstrated with tremendous impact on the development of postnatal mice, treated as early as one day of age [19]. NAP treatment was able to abolish motor developmental delays, vocal detriments and social behavior deficits by affecting the trajectory of intrinsic synapse formation and key gene expression patterns [19]. This further supports the need for early diagnosis of the *ADNP* syndrome, in particular, and ASD in general. As > 10% of children with the *ADNP* syndrome were recently reported suffering from hearing loss, the ABR hearing test was applied on the $Adnp^{+/-}$ mouse model. Importantly, the ABR hearing test was also suggested as a potential early marker for ASD risk, paving the path for prodromal diagnosis in infants [45]. The $Adnp^{+/-}$ mouse results corroborate the potential of the ABR test for an early diagnosis as we demonstrate aberrant click test results and higher hearing thresholds in $Adnp^{+/-}$ mice compared with littermates (Fig. 1A-B). Prolonged hearing latencies were also apparent but were non-significant (Fig. 1C). As there were no known indications that ADNP may cause outer or middle ear deformations nor acute middle ear chronic inflammation, the ABR registered impairments were plausibly associated with insults located along the inner ear-auditory nerve-brain axis, as observed here by immunohistochemistry and gene expression analysis.

ADNP functions as an important transcription factor, regulating over 400 genes during development, vital for neural tube closure and brain development [10, 12]. This corresponds with evidence both in mice and humans showcasing extensive physical deficits and abnormalities as early as infancy [14–16, 21, 46]. Thus, we tested the integrity of P0 $Adnp^{+/-}$ mouse ear hair cells for potential developmental irregularities. Immunohistochemistry in ear hair bundle cells in P0 $Adnp^{+/-}$ mice revealed normal morphological appearance and therefore probably is not part of the pathophysiology found in the $Adnp^{+/-}$ auditory system function (Fig. 2). To further investigate the ADNP- hearing loss deficit etiology, we have focused on two important auditory-related brain regions, the auditory cortex and the cerebellum, for potential significant changes in neurotransmitter/synapse protein expression. Additional immune-staining was performed on the hippocampus as both the hippocampus and the cerebellum possess great significance in ASD research. Several meaningful changes were reported. For ChAT, PVALB, VGLUT2, CX32, a significant decrease in expression levels was observed in association with the $Adnp^{+/-}$ genotype (Figs. 3, 4).

Acetylcholine (Ach) involvement in cortical sensory processing has been previously studied and found to play a major role in learning-induced plasticity as well as spontaneous firing rates in the auditory cortex [47, 48]. More specifically, ChAT activity has been linked to intense sound exposure and significantly increased in the anteroventral cochlear nucleus granular region and the lateral superior olivary nucleus following intense sound exposure [42]. Interestingly, no such effect was found in auditory cortex, although ChAT activity was reported relatively high in auditory cortex of control hamsters [42]. A reduction in ChAT expression levels was associated with impairments in spatial learning and memory in hippocampus and frontal cortex of the Japanese encephalitis rat model [49] as well as in the hippocampus of senile dementia of Alzheimer's type [50]. This corresponds with our findings of a significant decrease in ChAT levels in all three tested brain regions in the $Adnp^{+/-}$ mouse.

PVALB is a calcium binding protein involved in Ca^{2+} homeostasis [51]. As previously stated, there is strong evidence of relations between synaptogenesis, calcium-signaling and ASD [18]. PVALB heterozygous and knockout mouse models exhibited significant behavioral ASD traits and neurodevelopment brain pathologies linked to alterations in inhibitory and excitatory synaptic transmission [52]. One of the major anatomical brain pathologies observed by magnetic resonance imaging (MRI) was cerebellar hypoplasia in both $PVALB^{-/-}$ and $PVALB^{+/-}$ mice [52]. PVALB was also connected to age-related hearing loss (presbycusis) C57bl/6 mouse model, whereas there was a significant reduction in parvalbumin expression in the primary auditory cortex (layers 1–5) with age [53]. In hippocampus PVALB deficiency enhanced inhibition, which in turn is suggested to affect cognitive functions related with gamma oscillations such as sensory information processing and perhaps learning and memory [51, 54]. Although PVALB was not shown to decline in response to $Adnp^{+/-}$ in the auditory cortex, it was decreased in both cerebellum and hippocampus, specifically in $Adnp^{+/-}$ males. With previous data advocating PVALB importance to cognition and hippocampus, it is possible that PVALB is related to the mechanism underlying the learning and memory deficiency in $Adnp^{+/-}$ males. ADNP was already proposed to affect voltage-dependent calcium channel expression both on transcriptional and translational levels [18]. This also aliens with the belief that in case of $Adnp^{+/-}$ mice, the males are more affected by the *Adnp* haploinsufficiency in comparison with females [17–19].

Positive regulation of glutamatergic synaptic transmission was presented as one of the most enriched modified functions affected by $Adnp^{+/-}$, NAP treatment or sex differences

[19]. VGLUT, critical modulator of synaptic function and dendritic structure is also considered as the most reliable marker for glutamatergic neurons to-date [55, 56]. In our study, VGLUT2 levels were significantly decreased specifically in the *Adnp*$^{+/-}$ female brain. Similarly, VGLUT2 was previously shown to decrease in the cerebellum of lysosomal storage disorders coupled with a significant delay in the acquisition of complex motor abilities [57]. Interestingly, our model also portrays similar motor delays as well as sex dependent gait impairments [19]. Correspondingly, VGluT1 levels were also reported to significantly reduced, in whole mouse brain modeling Rett syndrome [55]. We did not observe significant changes for VGLUT1between *Adnp*$^{+/-}$ and *Adnp*$^{+/+}$ littermates.

CX32 is a gap junction crucial for myelin function and structure in the peripheral nervous system (PNS) as well as implicated in myelin radial diffusion. Its expression in Schwann cells is regulated during development. Loss of CX32 function (mutations in GJB1 gene on chromosome X) is attributed to the manifestation of the X-linked Charcot-Marie-Tooth disease (CMTX) causing sensory and motor peripheral neuropathy, affecting males more severally due to X-chromosome inactivation in females [58]. Cx32 is also expressed in the regenerative nerve and has been connected with seizures and epilepsy as well as potentially implicated in the pathophysiology of Rett syndrome [59, 60]. Neuroinflammation is known to be a hallmark in neuronal diseases such as Alzheimer's disease and epilepsy [61]. ADNP has also been previously linked to inflammation [62] and potential peripheral blood inflammation biomarkers, ameliorated by NAP treatment in both *Adnp*$^{+/-}$ mice and the *ADNP* syndrome [19]. Here, we show a reduction in CX32 levels in both the cerebellum and hippocampus of *Adnp*$^{+/-}$ mice in a sex-dependent manner. This corroborated past finding of potential inflammation in the two regions. In the cerebellum, lack of CX32 together with CX47 was reported to cause an extrusive immune response [63]. In the hippocampus, CX32 known to be expressed in the oligodendrocytes and neuronal subpopulations [61] and was shown to decrease in case of inflammation in the mouse following Exposure to high levels of glucocorticoids [64].

In contrary, GAD67 and FOXP2 were increased in the *Adnp*$^{+/-}$ mouse model (Figs. 3, 4). GAD67 has been previously associated with partial hearing loss and increased spontaneous neuronal activity in the guinea-pig brainstem [40]. Here, GAD67 was increased in the *Adnp*$^{+/-}$ female auditory cortex. Respectively, in total sensorineural hearing loss condition, an increase in GAD67/65 protein levels was reported in the GABAergic presynaptic terminals in the auditory cortex, associated with increased activity and lower inhibition [41]. It was potentially suggested as a compensatory response for the ineffective inhibitory synapses [41]. In a valproic acid rat model for autism, GAD67 expression levels were decreased in hippocampus and cerebellum and may underlay alterations in the excitatory-inhibitory balance in the cerebral cortex, hippocampus and cerebellum [65]. Surprisingly, in our model, GAD67 levels were increased in the *Adnp*$^{+/-}$female hippocampus compared with *Adnp*$^{+/+}$ and in the *Adnp*$^{+/-}$ female cerebellum compared with male *Adnp*$^{+/-}$. GAD65 expression patterns also showing sex-dependent significant differences in both the cerebellum and the hippocampus of *Adnp*$^{+/+}$ mice.

FOXP2 is a gene linked with developmental disorders disrupting speech and language skills in humans, songbirds and rodents [32, 66–68]. FOXP2 was also suggested to be associated in the language disturbances found in autism [69]. FOXP2 increases in the presence of paired box protein (PAX6) [70], a protein regulated by Adnp during mouse brain formation [10]. Here, Foxp2 levels were elevated in the hippocampus of *Adnp*$^{+/-}$ mice, which may suit our previous findings of an increase in *Foxp2* mRNA expression levels in the DISC1-mutated mouse hippocampus (schizophrenia mouse model) and a decrease following the ADNP snippet, NAP treatment [71], enhancing endogenous ADNP activity [38]. This may also support the notion that FOXP2 is related to ADNP and cognitive function in the hippocampus more specifically in males that are known to have a more severe autistic phenotype compared with females.

For Slc6a4, increased expression has been linked with ASD and found higher in *Adnp*$^{+/-}$ male hippocampus, compared with *Adnp*$^{+/+}$ [18]. Our present results identify a sex-dependent difference in Slc6a4 expression in the cerebellum with significantly higher expression in male *Adnp*$^{+/-}$ compared with female. Unexpectedly, we did not find similar changes in Slc6a4 in the hippocampus, corroborating past results. This may be explained by the age difference between the animals of both studies (5-months versus 9-months old), which was already demonstrated to have significant impact on Adnp's role as a transcription regulator in mice [19]. More specifically, the age of mice was proven to affect ADNP-related regulation on other members of the Solute carrier family (Slc12a2, Slc9a3) in the hippocampus, cortex and spleen [19].

To establish ADNP-regulated protein networks, the STRING protein–protein interaction tool was applied. Being that ADNP affects ChAT, VGLUT2 and GAD67, linked to neurotransmission, it is not surprising that the enriched functions found in the mouse database were associated with neurotransmission regulation. ADNP was previously shown to regulate synaptogenesis, dendritic spine plasticity, axonal transport and calcium channels [17–19, 38]. FOXP2 and CX32, also regulated by the *Adnp* haploinsufficient genotype, were previously connected with synaptogenesis [72, 73] as well as vocal-motor function (*Foxp2*) [67]. PVALB was suggested to potently modulate short-term synaptic plasticity [74]. Therefore, we hypothesize that ADNP causes

hearing and vocal malfunctions through its effect on synaptogenesis, resulting in fewer mature synapses in the brain cortex (e.g. [19]) that may also occur in other brain regions, such as the cerebellum.

In previous studies we have demonstrated multiple sex-dependent differences in the *Adnp* deficient mouse, which include for example: (1) vocal impediments (for saline-treated mice), (2) motor, (3) gait, (4) axonal transport, (5) dendritic pathology and (6) sex-specific gene alterations [17–19]. Here, ABR test results further advance our hypothesis in which ADNP affects synaptogenesis and brain function that in turn may translate into auditory brain response insults. Once more, the results changed in accordance to the mouse sex, with a more severe hearing malfunction observed in males. Furthermore, we have evaluated USV production in naïve *Adnp* pups (P8), adding on to our previous observations on daily-saline-treated mice [19]. $Adnp^{+/-}$ pups produced significantly less USV calls compared to control pups in both sexes. Surprisingly, in contrary to previous results in saline-treated $Adnp^{+/-}$ males (non-significant trend), the reduction in the number of USV calls in naïve $Adnp^{+/-}$ males was found to be statistically significant [19]. This may be explained by the mandatory daily pup-handling required for treatment. Importantly, both hearing impediments and vocalization impairments were shown here as associated with the *ADNP* syndrome in mice.

In past studies, a major part of the cerebellar connectivity has been identified as non-motor projections connecting to cognitive and affective brain regions. The cerebellum circuits were also found to be highly conserved among vertebrates [44]. It is proposed that the cerebellum plays an important role in the modification and fine tuning of the cortex, during development, in brain regions related to cognition and affect in particular [44]. Moreover, the cerebellum is argued to affect the development and organization of distant connection sites in the brain, which in turn might be disturbed as a cause of cerebellar malfunctions, *developmental diaschisis*. In this manner, the cerebellum has been linked with ASD showing strong expression of ASD susceptible genes in the human cerebellum during the first years of life. It was also classified as one of the most commonly found brain regions disarrayed in ASD patients and may be potentially enough to cause ASD if the impediment appears during early development [44]. Importantly, similar cognitive and affective function irregularities were also described in animal models with cerebellar damage at a young age [44, 75]. Interestingly, the ASD co-expressed susceptible genes were found to be strongly associated with neural circuitry and plasticity [44, 76], much like the *ADNP* gene, which was already found to directly affect the axonal transport as well as regulate dendritic spine formation and synaptic plasticity [18, 19]. Cerebellar lesions were also proven to cause atypical social behavior, more so in young individuals than in adulthood [44]. In this respect, cerebellar lesions were reported to cause language delay or speech-generation deficits in children and adults, accordingly. Remarkably, children with *ADNP*- mutations were reported to suffer from severe delays in language acquisition while $Adnp^{+/-}$ mice were portrayed with significant sex-dependent vocal impediments, exhibiting less distressed calls in response to maternal separation [14, 19, 21]. Also, $Adnp^{+/-}$ mice were also described with impaired gait, showcasing sex-dependent abnormalities in stand, step cycle and stride length [19]. These impediments were significantly affected by daily treatment with NAP (NAPVSIPQ; CP201), a short ADNP-derived snippet, drug candidate [19, 28, 38, 77].

The cerebellum was found as part of an important network, activated during auditory and verbal tasks [43, 78, 79]. More importantly, is was named as the second highest activated region during auditory tasks, after auditory cortex, even in the absence of motor activation [80]. The cerebellum has been further connected to the auditory system, specifically to the tinnitus phenomenon, suggested as a potential tinnitus generator, alongside the auditory cortex [43, 81, 82]. Similarly, it has been identified as active during tinnitus in humans (imaging studies) [43, 83, 84]. Although much is still unknown, cerebellar connectivity and activation also indicate linkage to the auditory circuit, more specifically to the auditory cortex and cochlea [43, 85].

In conclusion, we find significant new similarities between the $Adnp^{+/-}$ mouse model and the *ADNP* syndrome characteristics. *ADNP* dysfunction resulted in ABR aberrations and hearing loss in mice and humans. Immunostaining further validated the important connection between autism and *ADNP* research as it revealed important changes in proteins associated with ASD, hearing loss and brain inflammation. We continuously show that the trajectory underlying *ADNP* deficiency pathophysiology is through its important role as a transcription factor throughout life as well as through its essential role in synapse formation and function. We believe that the continuation of this study will be of great interest, providing important data regarding the $Adnp^{+/-}$ mouse model relevance to ABR in infants with the *ADNP* syndrome in particular and ASD in general [86]. Furthermore, the $Adnp^{+/-}$ mouse model may become a relevant platform to test therapeutic applications such as NAP (CP201), for effects on hearing loss and other ASD-related predicaments.

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Compliance with Ethical Standards

Conflict of interest Professor Illana Gozes is the Chief Scientific Officer of Coronis Neurosciences (http://www.coronisns.com/) developing NAP (CP201) for the ADNP syndrome.

Human and Animal Rights Statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Care and Use Committee of Tel Aviv University and the Israeli Ministry of Health (M-15-059).

References

1. Autism and Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators (2014) Prevalence of autism spectrum disorder among children aged 8 years—autism and developmental disabilities monitoring network, 11 sites, United States, 2010. MMWR Surveill Summ 63 (2):1–21
2. Campisi L, Imran N, Nazeer A, Skokauskas N, Azeem MW (2018) Autism spectrum disorder. Br Med Bull 127(1):91–100. https://doi.org/10.1093/bmb/ldy026
3. Gillberg C, Wing L (1999) Autism: not an extremely rare disorder. Acta Psychiatr Scand 99(6):399–406
4. Pinto D, Pagnamenta AT, Klei L, Anney R, Merico D, Regan R, Conroy J, Magalhaes TR,Correia C, Abrahams BS, Almeida J, Bacchelli E, Bader GD, Bailey AJ, Baird G, Battaglia A, Berney T, Bolshakova N, Bolte S, Bolton PF, Bourgeron T, Brennan S, Brian J, Bryson SE, Carson AR, Casallo G, Casey J, Chung BH, Cochrane L, Corsello C, Crawford EL,Crossett A, Cytrynbaum C, Dawson G, de Jonge M, Delorme R, Drmic I, Duketis E, Duque F, Estes A, Farrar P, Fernandez BA, Folstein SE, Fombonne E, Freitag CM, Gilbert J,Gillberg C, Glessner JT, Goldberg J, Green A, Green J, Guter SJ, Hakonarson H, Heron EA, Hill M, Holt R, Howe JL, Hughes G, Hus V, Igliozzi R, Kim C, Klauck SM, Kolevzon A, Korvatska O, Kustanovich V, Lajonchere CM, Lamb JA, Laskawiec M, Leboyer M, Le Couteur A, Leventhal BL, Lionel AC, Liu XQ, Lord C, Lotspeich L, Lund SC, Maestrini E, Mahoney W, Mantoulan C, Marshall CR, McConachie H, McDougle CJ, McGrath J, McMahon WM, Merikangas A, Migita O, Minshew NJ, Mirza GK, Munson J, Nelson SF, Noakes C, Noor A, Nygren G, Oliveira G, Papanikolaou K, Parr JR, Parrini B, Paton T, Pickles A, Pilorge M, Piven J, Ponting CP, Posey DJ, Poustka A, Poustka F, Prasad A, Ragoussis J, Renshaw K, Rickaby J, Roberts W, Roeder K, Roge B, Rutter ML, Bierut LJ, Rice JP, Salt J,Sansom K, Sato D, Segurado R, Sequeira AF, Senman L, Shah N, Sheffield VC, Soorya L, Sousa I, Stein O, Sykes N, Stoppioni V, Strawbridge C, Tancredi R, Tansey K, Thiruvahindrapduram B, Thompson AP, Thomson S, Tryfon A, Tsiantis J, Van Engeland H, Vincent JB, Volkmar F, Wallace S, Wang K, Wang Z, Wassink TH, Webber C, Weksberg R, Wing K, Wittemeyer K, Wood S, Wu J, Yaspan BL, Zurawiecki D, Zwaigenbaum L, Buxbaum JD, Cantor RM, Cook EH, Coon H, Cuccaro ML, Devlin B, Ennis S, Gallagher L, Geschwind DH, Gill M, Haines JL, Hallmayer J, Miller J, Monaco AP, Nurnberger JI Jr., Paterson AD, Pericak-Vance MA, Schellenberg GD, Szatmari P, Vicente AM, Vieland VJ, Wijsman EM, Scherer SW, Sutcliffe JS, Betancur C (2010) Functional impact of global rare copy number variation in autism spectrum disorders. Nature 466 (7304):368–372. doi:10.1038/nature09146
5. Gillberg C, Billstedt E (2000) Autism and asperger syndrome: coexistence with other clinical disorders. Acta Psychiatr Scand 102(5):321–330
6. Talkowski ME, Rosenfeld JA, Blumenthal I, Pillalamarri V, Chiang C, Heilbut A, Ernst C, Hanscom C, Rossin E, Lindgren AM, Pereira S, Ruderfer D, Kirby A, Ripke S, Harris DJ, Lee JH, Ha K, Kim HG, Solomon BD, Gropman AL, Lucente D, Sims K, Ohsumi TK, Borowsky ML, Loranger S, Quade B, Lage K, Miles J, Wu BL, Shen Y, Neale B, Shaffer LG, Daly MJ, Morton CC, Gusella JF (2012) Sequencing chromosomal abnormalities reveals neurodevelopmental loci that confer risk across diagnostic boundaries. Cell 149(3):525–537. https://doi.org/10.1016/j.cell.2012.03.028
7. Panaitof SC (2012) A songbird animal model for dissecting the genetic bases of autism spectrum disorder. Dis Mark 33(5):241–249. https://doi.org/10.3233/DMA-2012-0918
8. Abrahams BS, Geschwind DH (2008) Advances in autism genetics: on the threshold of a new neurobiology. Nat Rev Genet 9(5):341–355. https://doi.org/10.1038/nrg2346
9. Bassan M, Zamostiano R, Davidson A, Pinhasov A, Giladi E, Perl O, Bassan H, Blat C, Gibney G, Glazner G, Brenneman DE, Gozes I (1999) Complete sequence of a novel protein containing a femtomolar-activity-dependent neuroprotective peptide. J Neurochem 72(3):1283–1293
10. Pinhasov A, Mandel S, Torchinsky A, Giladi E, Pittel Z, Goldsweig AM, Servoss SJ, Brenneman DE, Gozes I (2003) Activity-dependent neuroprotective protein: a novel gene essential for brain formation. Brain Res Dev Brain Res 144(1):83–90
11. Borozdin W, Graham JM Jr, Bohm D, Bamshad MJ, Spranger S, Burke L, Leipoldt M, Kohlhase J (2007) Multigene deletions on chromosome 20q13.13-q13.2 including SALL4 result in an expanded phenotype of Okihiro syndrome plus developmental delay. Hum Mutat 28(8):830
12. Mandel S, Gozes I (2007) Activity-dependent neuroprotective protein constitutes a novel element in the SWI/SNF chromatin remodeling complex. J Biol Chem 282(47):34448–34456
13. Gozes I, Yeheskel A, Pasmanik-Chor M (2014) Activity-dependent neuroprotective protein (ADNP): a case study for highly conserved chordata-specific genes shaping the brain and mutated in cancer. J Alzheimer's Dis. https://doi.org/10.3233/JAD-142490
14. Helsmoortel C, Vulto-van Silfhout AT, Coe BP, Vandeweyer G, Rooms L, van den Ende J, Schuurs-Hoeijmakers JH, Marcelis CL, Willemsen MH, Vissers LE, Yntema HG, Bakshi M, Wilson M, Witherspoon KT, Malmgren H, Nordgren A, Anneren G, Fichera M, Bosco P, Romano C, de Vries BB, Kleefstra T, Kooy RF, Eichler EE, Van der Aa N (2014) A SWI/SNF-related autism syndrome caused by de novo mutations in ADNP. Nat Genet 46(4):380–384. https://doi.org/10.1038/ng.2899
15. Gozes I, Helsmoortel C, Vandeweyer G, Van der Aa N, Kooy F, Sermone SB (2015) The compassionate side of neuroscience: Tony Sermone's undiagnosed genetic journey–ADNP mutation. J Mol Neurosci 56(4):751–757. https://doi.org/10.1007/s12031-015-0586-6
16. Gozes I, Patterson MC, Van Dijck A, Kooy RF, Peeden JN, Eichenberger JA, Zawacki-Downing A, Bedrosian-Sermone S (2017) The eight and a half year journey of undiagnosed AD: gene sequencing and funding of advanced genetic testing has led to hope and new beginnings. Front Endocrinol 8:107. https://doi.org/10.3389/fendo.2017.00107
17. Malishkevich A, Amram N, Hacohen-Kleiman G, Magen I, Giladi E, Gozes I (2015) Activity-dependent neuroprotective protein (ADNP) exhibits striking sexual dichotomy impacting

on autistic and Alzheimer's pathologies. Transl Psychiatr 5:e501. https://doi.org/10.1038/tp.2014.138
18. Amram N, Hacohen-Kleiman G, Sragovich S, Malishkevich A, Katz J, Touloumi O, Lagoudaki R, Grigoriadis NC, Giladi E, Yeheskel A, Pasmanik-Chor M, Jouroukhin Y, Gozes I (2016) Sexual divergence in microtubule function: the novel intranasal microtubule targeting SKIP normalizes axonal transport and enhances memory. Mol Psychiatr. https://doi.org/10.1038/mp.2015.208
19. Hacohen-Kleiman G, Sragovich S, Karmon G, Gao AYL, Grigg I, Pasmanik-Chor M, Le A, Korenkova V, McKinney RA, Gozes I (2018) Activity-dependent neuroprotective protein deficiency models synaptic and developmental phenotypes of autism-like syndrome. J Clin Investig 128(11):4956–4969. https://doi.org/10.1172/JCI98199
20. Gkogkas CG, Khoutorsky A, Ran I, Rampakakis E, Nevarko T, Weatherill DB, Vasuta C, Yee S, Truitt M, Dallaire P, Major F, Lasko P, Ruggero D, Nader K, Lacaille JC, Sonenberg N (2013) Autism-related deficits via dysregulated eIF4E-dependent translational control. Nature 493(7432):371–377. https://doi.org/10.1038/nature11628
21. Van Dijck A, Vulto-van Silfhout AT, Cappuyns E, van der Werf IM, Mancini GM, Tzschach A, Bernier R, Gozes I, Eichler EE, Romano C, Lindstrand A, Nordgren A, Consortium A, Kvarnung M, Kleefstra T, de Vries BBA, Kury S, Rosenfeld JA, Meuwissen ME, Vandeweyer G, Kooy RF (2018) Clinical presentation of a complex neurodevelopmental disorder caused by mutations in ADNP. Biol Psychiatr. https://doi.org/10.1016/j.biopsych.2018.02.1173
22. Miron O, Roth DAE, Gabis LV, Henkin Y, Shefer S, Dinstein I, Geva R (2016) Prolonged auditory brainstem responses in infants with autism. Autism Res 9(6):689–695. https://doi.org/10.1002/aur.1561
23. World-Health-Organization (2010) Newborn and infant hearing screening—current issues and guiding principels for action outcome of a WHO informal consultation. WHO, Geneva
24. Fujikawa-Brooks S, Isenberg AL, Osann K, Spence MA, Gage NM (2010) The effect of rate stress on the auditory brainstem response in autism: a preliminary report. Int J Audiol 49(2):129–140. https://doi.org/10.3109/14992020903289790
25. Kwon S, Kim J, Choe BH, Ko C, Park S (2007) Electrophysiologic assessment of central auditory processing by auditory brainstem responses in children with autism spectrum disorders. J Korean Med Sci 22(4):656–659. https://doi.org/10.3346/jkms.2007.22.4.656
26. Roth DA, Muchnik C, Shabtai E, Hildesheimer M, Henkin Y (2012) Evidence for atypical auditory brainstem responses in young children with suspected autism spectrum disorders. Dev Med Child Neurol 54(1):23–29. https://doi.org/10.1111/j.1469-8749.2011.04149.x
27. Miron O, Beam AL, Kohane IS (2018) Auditory brainstem response in infants and children with autism spectrum disorder: a meta-analysis of wave V. Autism Res 11(2):355–363. https://doi.org/10.1002/aur.1886
28. Vulih-Shultzman I, Pinhasov A, Mandel S, Grigoriadis N, Touloumi O, Pittel Z, Gozes I (2007) Activity-dependent neuroprotective protein snippet NAP reduces tau hyperphosphorylation and enhances learning in a novel transgenic mouse model. J Pharmacol Exp Ther 323(2):438–449
29. Walsh VL, Raviv D, Dror AA, Shahin H, Walsh T, Kanaan MN, Avraham KB, King MC (2011) A mouse model for human hearing loss DFNB30 due to loss of function of myosin IIIA. Mamm Genome 22(3–4):170–177. https://doi.org/10.1007/s00335-010-9310-6
30. Muller U, Barr-Gillespie PG (2015) New treatment options for hearing loss. Nat Rev Drug Discov 14(5):346–384. https://doi.org/10.1038/nrd4533
31. McCullough BJ, BL T (2004) Haplo-insufficiency revealed in deafwaddler mice when tested for hearing loss and ataxia. Hear Res 195(1–2):90–102. https://doi.org/10.1016/j.heares.2004.05.003
32. Shu W, Cho JY, Jiang Y, Zhang M, Weisz D, Elder GA, Schmeidler J, De Gasperi R, Sosa MA, Rabidou D, Santucci AC, Perl D, Morrisey E, Buxbaum JD (2005) Altered ultrasonic vocalization in mice with a disruption in the Foxp2 gene. Proc Natl Acad Sci USA 102(27):9643–9648. https://doi.org/10.1073/pnas.0503739102
33. Hertzano R, Montcouquiol M, Rashi-Elkeles S, Elkon R, Yucel R, Frankel WN, Rechavi G, Moroy T, Friedman TB, Kelley MW, Avraham KB (2004) Transcription profiling of inner ears from Pou4f3(ddl/ddl) identifies Gfi1 as a target of the Pou4f3 deafness gene. Hum Mol Genet 13(18):2143–2153. https://doi.org/10.1093/hmg/ddh218
34. Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, Santos A, Doncheva NT, Roth A, Bork P, Jensen LJ, von Mering C (2017) The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Res 45(D1):D362–D368. https://doi.org/10.1093/nar/gkw937
35. Zhou X, Jen PH, Seburn KL, Frankel WN, Zheng QY (2006) Auditory brainstem responses in 10 inbred strains of mice. Brain Res 1091(1):16–26. https://doi.org/10.1016/j.brainres.2006.01.107
36. Burkard RF, Eggermont JJ, Manuel D (eds) (2007) Auditory evoked potentials: basic principles and clinical application. Lippincott Williams & Wilkins, Philadelphia
37. Hodges SL, Nolan SO, Reynolds CD, Lugo JN (2017) Spectral and temporal properties of calls reveal deficits in ultrasonic vocalizations of adult Fmr1 knockout mice. Behav Brain Res 332:50–58. https://doi.org/10.1016/j.bbr.2017.05.052
38. Oz S, Kapitansky O, Ivashco-Pachima Y, Malishkevich A, Giladi E, Skalka N, Rosin-Arbesfeld R, Mittelman L, Segev O, Hirsch JA, Gozes I (2014) The NAP motif of activity-dependent neuroprotective protein (ADNP) regulates dendritic spines through microtubule end binding proteins. Mol Psychiatr 19(10):1115–1124. https://doi.org/10.1038/mp.2014.97
39. Ivashko-Pachima Y, Sayas CL, Malishkevich A, Gozes I (2017) ADNP/NAP dramatically increase microtubule end-binding protein-Tau interaction: a novel avenue for protection against tauopathy. Mol Psychiatr 22(9):1335–1344. https://doi.org/10.1038/mp.2016.255
40. Dong S, Mulders WHAM, Rodger J, Robertson D (2009) Changes in neuronal activity and gene expression in guinea-pig auditory brainstem after unilateral partial hearing loss. Neuroscience 159(3):1164–1174. https://doi.org/10.1016/j.neuroscience.2009.01.043
41. Sarro EC, Kotak VC, Sanes DH, Aoki C (2008) Hearing loss alters the subcellular distribution of presynaptic GAD and postsynaptic GABAA receptors in the auditory cortex. Cerebral Cortex 18(12):2855–2867. https://doi.org/10.1093/cercor/bhn044
42. Godfrey DA, Kaltenbach JA, Chen K, Ilyas O (2013) Choline acetyltransferase activity in the hamster central auditory system and long-term effects of intense tone exposure. J Neurosci Res 91(7):987–996. https://doi.org/10.1002/jnr.23227
43. Bauer CA, Kurt W, Sybert LT, Brozoski TJ (2013) The cerebellum as a novel tinnitus generator. Hear Res 295:130–139
44. Wang SS, Kloth AD, Badura A (2014) The cerebellum, sensitive periods, and autism. Neuron 83(3):518–532. https://doi.org/10.1016/j.neuron.2014.07.016

45. Miron O, Ari-Even Roth D, Gabis LV, Henkin Y, Shefer S, Dinstein I, Geva R (2016) Prolonged auditory brainstem responses in infants with autism. Autism Res 9(6):689–695. https://doi.org/10.1002/aur.1561
46. Gozes I, Van Dijck A, Hacohen-Kleiman G, Grigg I, Karmon G, Giladi E, Eger M, Gabet Y, Pasmanik-Chor M, Cappuyns E, Elpeleg O, Kooy RF, Bedrosian-Sermone S (2017) Premature primary tooth eruption in cognitive/motor-delayed ADNP-mutated children. Transl Psychiatr 7(2):e1043. https://doi.org/10.1038/tp.2017.27
47. Shulz DE, Cohen S, Haidarliu S, Ahissar E (1997) Differential effects of acetylcholine on neuronal activity and interactions in the auditory cortex of the guinea-pig. Eur J Neurosci 9(2):396–409
48. Chavez C, Zaborszky L (2017) Basal forebrain cholinergic-auditory cortical network: primary versus nonprimary auditory cortical areas. Cerebral cortex 27(3):2335–2347. https://doi.org/10.1093/cercor/bhw091
49. Chauhan PS, Misra UK, Kalita J, Chandravanshi LP, Khanna VK (2016) Memory and learning seems to be related to cholinergic dysfunction in the JE rat model. Physiol Behav 156:148–155. https://doi.org/10.1016/j.physbeh.2016.01.006
50. Nordberg A, Alafuzoff I, Winblad B (1986) Muscarinic receptor subtypes in hippocampus in Alzheimer's disease and mixed dementia type. Neurosci Lett 70(1):160–164
51. Vreugdenhil M, Jefferys JG, Celio MR, Schwaller B (2003) Parvalbumin-deficiency facilitates repetitive IPSCs and gamma oscillations in the hippocampus. J Neurophysiol 89(3):1414–1422. https://doi.org/10.1152/jn.00576.2002
52. Wohr M, Orduz D, Gregory P, Moreno H, Khan U, Vorckel KJ, Wolfer DP, Welzl H, Gall D, Schiffmann SN, Schwaller B (2015) Lack of parvalbumin in mice leads to behavioral deficits relevant to all human autism core symptoms and related neural morphofunctional abnormalities. Transl Psychiatr 5:e525. https://doi.org/10.1038/tp.2015.19
53. Martin del Campo HN, Measor KR, Razak KA (2012) Parvalbumin immunoreactivity in the auditory cortex of a mouse model of presbycusis. Hear Res 294(1–2):31–39. https://doi.org/10.1016/j.heares.2012.08.017
54. Penttonen M, Kamondi A, Acsady L, Buzsaki G (1998) Gamma frequency oscillation in the hippocampus of the rat: intracellular analysis in vivo. Eur J Neurosci 10(2):718–728
55. Nguyen MV, Du F, Felice CA, Shan X, Nigam A, Mandel G, Robinson JK, Ballas N (2012) MeCP2 is critical for maintaining mature neuronal networks and global brain anatomy during late stages of postnatal brain development and in the mature adult brain. J Neurosci 32(29):10021–10034. https://doi.org/10.1523/JNEUROSCI.1316-12.2012
56. Zhang FX, Ge SN, Dong YL, Shi J, Feng YP, Li Y, Li YQ, Li JL (2018) Vesicular glutamate transporter isoforms: the essential players in the somatosensory systems. Progr Neurobiol. https://doi.org/10.1016/j.pneurobio.2018.09.006
57. Caporali P, Bruno F, Palladino G, Dragotto J, Petrosini L, Mangia F, Erickson RP, Canterini S, Fiorenza MT (2016) Developmental delay in motor skill acquisition in Niemann-Pick C1 mice reveals abnormal cerebellar morphogenesis. Acta Neuropathol Commun 4(1):94. https://doi.org/10.1186/s40478-016-0370-z
58. Nualart-Marti A, Solsona C, Fields RD (2013) Gap junction communication in myelinating glia. Biochim Biophys acta 1828(1):69–78. https://doi.org/10.1016/j.bbamem.2012.01.024
59. Yamasaki R (2018) Connexins in health and disease. Clin Exp Neuroimmunol 9:30–36
60. Maezawa I, Calafiore M, Wulff H, Jin LW (2011) Does microglial dysfunction play a role in autism and Rett syndrome? Neuron glia Biol 7(1):85–97. https://doi.org/10.1017/S1740925X1200004X
61. Abbasian M, Sayyah M, Babapour V, Mahdian R (2013) Intracerebroventricular injection of lipopolysaccharide increases gene expression of connexin32 gap junction in rat hippocampus. Basic Clin Neurosci 4(4):334–340
62. Braitch M, Kawabe K, Nyirenda M, Gilles LJ, Robins RA, Gran B, Murphy S, Showe L, Constantinescu CS (2010) Expression of activity-dependent neuroprotective protein in the immune system: possible functions and relevance to multiple sclerosis. Neuroimmunomodulation 17(2):120–125. https://doi.org/10.1159/000258695
63. Wasseff SK, Scherer SS (2015) Activated immune response in an inherited leukodystrophy disease caused by the loss of oligodendrocyte gap junctions. Neurobiol Dis 82:86–98. https://doi.org/10.1016/j.nbd.2015.05.018
64. Maturana CJ, Aguirre A, Saez JC (2017) High glucocorticoid levels during gestation activate the inflammasome in hippocampal oligodendrocytes of the offspring. Dev Neurobiol 77(5):625–642. https://doi.org/10.1002/dneu.22409
65. Hou Q, Wang Y, Li Y, Chen D, Yang F, Wang S (2018) A developmental study of abnormal behaviors and altered GABAergic signaling in the VPA-treated rat model of autism. Front Behav Neurosci 12:182. https://doi.org/10.3389/fnbeh.2018.00182
66. Teramitsu I, Kudo LC, London SE, Geschwind DH, White SA (2004) Parallel FoxP1 and FoxP2 expression in songbird and human brain predicts functional interaction. J Neurosci 24(13):3152–3163. https://doi.org/10.1523/JNEUROSCI.5589-03.2004
67. Haesler S, Rochefort C, Georgi B, Licznerski P, Osten P, Scharff C (2007) Incomplete and inaccurate vocal imitation after knockdown of FoxP2 in songbird basal ganglia nucleus Area X. PLoS Biol 5(12):e321. https://doi.org/10.1371/journal.pbio.0050321
68. Lai CS, Fisher SE, Hurst JA, Vargha-Khadem F, Monaco AP (2001) A forkhead-domain gene is mutated in a severe speech and language disorder. Nature 413(6855):519–523. https://doi.org/10.1038/35097076
69. Bowers JM, Konopka G (2012) ASD-relevant animal models of the Foxp family of transcription factors. Autism-Open Access Suppl. https://doi.org/10.4172/2165-7890.S1-010
70. Coutinho P, Pavlou S, Bhatia S, Chalmers KJ, Kleinjan DA, van Heyningen V (2011) Discovery and assessment of conserved Pax6 target genes and enhancers. Genome Res 21(8):1349–1359. https://doi.org/10.1101/gr.124115.111
71. Vaisburd S, Shemer Z, Yeheskel A, Giladi E, Gozes I (2015) Risperidone and NAP protect cognition and normalize gene expression in a schizophrenia mouse model. Sci Rep 5:16300. https://doi.org/10.1038/srep16300
72. Sia GM, Clem RL, Huganir RL (2013) The human language-associated gene SRPX2 regulates synapse formation and vocalization in mice. Science 342(6161):987–991. https://doi.org/10.1126/science.1245079
73. Sutor B, Hagerty T (2005) Involvement of gap junctions in the development of the neocortex. Biochim biophys acta 1719(1–2):59–68. https://doi.org/10.1016/j.bbamem.2005.09.005
74. Caillard O, Moreno H, Schwaller B, Llano I, Celio MR, Marty A (2000) Role of the calcium-binding protein parvalbumin in short-term synaptic plasticity. Proc Natl Acad Sci USA 97(24):13372–13377. https://doi.org/10.1073/pnas.230362997
75. Becker EB, Stoodley CJ (2013) Autism spectrum disorder and the cerebellum. Int Rev Neurobiol 113:1–34. https://doi.org/10.1016/B978-0-12-418700-9.00001-0
76. Parikshak NN, Luo R, Zhang A, Won H, Lowe JK, Chandran V, Horvath S, Geschwind DH (2013) Integrative functional genomic analyses implicate specific molecular pathways and circuits in autism. Cell 155(5):1008–1021. https://doi.org/10.1016/j.cell.2013.10.031
77. Rotstein M, Bassan H, Kariv N, Speiser Z, Harel S, Gozes I (2006) NAP enhances neurodevelopment of newborn

apolipoprotein E-deficient mice subjected to hypoxia. J Pharmacol Exp Ther 319(1):332–339. https://doi.org/10.1124/jpet.106.106898
78. Fiez JA, Raichle ME, Balota DA, Tallal P, Petersen SE (1996) PET activation of posterior temporal regions during auditory word presentation and verb generation. Cerebral Cortex 6(1):1–10
79. Schlosser R, Hutchinson M, Joseffer S, Rusinek H, Saarimaki A, Stevenson J, Dewey SL, Brodie JD (1998) Functional magnetic resonance imaging of human brain activity in a verbal fluency task. J Neurol Neurosurg Psychiatr 64(4):492–498
80. Petacchi A, Laird AR, Fox PT, Bower JM (2005) Cerebellum and auditory function: an ALE meta-analysis of functional neuroimaging studies. Hum Brain Mapp 25(1):118–128. https://doi.org/10.1002/hbm.20137
81. Yang G, Lobarinas E, Zhang L, Turner J, Stolzberg D, Salvi R, Sun W (2007) Salicylate induced tinnitus: behavioral measures and neural activity in auditory cortex of awake rats. Hear Res 226(1–2):244–253. https://doi.org/10.1016/j.heares.2006.06.013
82. Yang S, Weiner BD, Zhang LS, Cho SJ, Bao S (2011) Homeostatic plasticity drives tinnitus perception in an animal model. Proc Natl Acad Sci USA 108(36):14974–14979. https://doi.org/10.1073/pnas.1107998108
83. Shulman A, Strashun A (1999) Descending auditory system/cerebellum/tinnitus. Int Tinnitus J 5(2):92–106
84. Mirz F, Pedersen B, Ishizu K, Johannsen P, Ovesen T, Stodkilde-Jorgensen H, Gjedde A (1999) Positron emission tomography of cortical centers of tinnitus. Hear Res 134(1–2):133–144
85. Azizi SA, Burne RA, Woodward DJ (1985) The auditory corticopontocerebellar projection in the rat: inputs to the paraflocculus and midvermis. An anatomical and physiological study. Exp Brain Res 59(1):36–49
86. Chang A, Chen P, Guo S, Xu N, Pan W, Zhang H, Li C, Tang J (2018) Specific influences of early acoustic environments on cochlear hair cells in postnatal mice. Neural Plast 2018:5616930. https://doi.org/10.1155/2018/5616930