Sex-and Region-Dependent Expression of the Autism-Linked *ADNP* Correlates with Social- and Speech-Related Genes in the Canary Brain

Gal Hacohe-Kleiman^{1,2} · Stan Moaraf^{2,3} · Oxana Kapitansky¹ · Illana Gozes¹

Accepted: 5 September 2020 / Published online: 14 September 2020
© Springer Science+Business Media, LLC, part of Springer Nature 2020

Abstract

The activity-dependent neuroprotective protein (*ADNP*) syndrome is an autistic-like disorder, instigated by mutations in *ADNP*. This syndrome is characterized by developmental delays, impairments in speech, motor function, abnormal hearing, and intellectual disabilities. In the *Adnp*-haploinsufficient mouse model, many of these impediments are evident, appearing in a sex-dependent manner. In zebra finch songbird (ZF; *Taeniopygia guttata*), an animal model used for song/language studies, *ADNP* mRNA most robust expression is observed in the cerebrum of young males, potentially corroborating with male ZF exclusive singing behavior and developed cerebral song system. Herein, we report a similar sex-dependent *ADNP* expression profile, with the highest expression in the cerebrum (qRT-PCR) in the brain of another songbird, the domesticated canary (*Serinus canaria domestica*). Additional analyses for the mRNA transcripts of the *ADNP* regulator, vasoactive intestinal peptide (*VIP*), sister gene *ADNP2*, and speech-related Forkhead box protein P2 (*FoxP2*) revealed multiple sex and brain region-dependent positive correlations between the genes (including *ADNP*). Parallel transcript expression patterns for *FoxP2* and *VIP* were observed alongside specific *FoxP2* increase in males compared with females as well as *VIP/ADNP2* correlations. In spatial view, a sexually independent extensive form of expression was found for *ADNP* in the canary cerebrum (RNA in situ hybridization). The songbird cerebral mesopallium area stood out as a potentially high-expressing *ADNP* tissue, further strengthening the association of *ADNP* with sense integration and auditory memory formation, previously implicated in mouse and human.

Keywords The *ADNP* syndrome · Activity-dependent neuroprotective protein (*ADNP*) · Transcript expression · Sexually dichotomous · Domesticated canary (*Serinus canaria domestica*) · RNA in situ hybridization

Introduction

Activity-dependent neuroprotective protein (*ADNP*) discovered by Gozes and colleagues is a well-conserved gene in Chordata (Bassan et al. 1999; Zamostiano et al. 2001; Gozes

et al. 2015). In humans, de novo mutations in *ADNP* result in the *ADNP* syndrome, characterized by intellectual (cognitive), auditory, and social impediments as well as motor and speech developmental delays/disabilities (Helsmoortel et al. 2014; Hacohe-Kleiman et al. 2015; Gozes et al. 2017a; Arnett et al. 2018; Van Dijck et al. 2019). In mice, *Adnp* haploinsufficiency, the *Adnp*^{+/-} model, developed by the Gozes laboratory (Pinhasov et al. 2003, Vulih-Shultzman et al. 2007), causes significant multi-system irregularities, bearing close resemblance to the phenotype of the *ADNP* syndrome (Malishkevich et al. 2015; Amram et al. 2016; Gozes et al. 2017b; Hacohe-Kleiman et al. 2018, 2019). More specifically, *Adnp*^{+/-} mice inhere a diverse set of deficiencies including hearing abnormalities and motor, social, learning, and memory deficits, all essentially underlied by significant synaptic and gene regulation abnormalities (Malishkevich et al. 2015; Amram et al. 2016; Hacohe-Kleiman et al. 2018, 2019). *ADNP* expression in mouse and human (hippocampus) is sexually dichotomous, also appearing in estrous cycle-synchronized fluctuations in the mouse arcuate

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12031-020-01700-x>) contains supplementary material, which is available to authorized users.

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

¹ 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 Natural and Life Sciences, The Open University of Israel, 43107 Ra'anana, Israel

³ School of Zoology, Tel Aviv University, 6997801 Tel Aviv, Israel

nucleus (hypothalamus) (Bassan et al. 1999; Furman et al. 2005; Malishkevich et al. 2015). With speech acquisition being a major impediment in *ADNP* syndrome patients (Gozes et al. 2017a; Van Dijck et al. 2019), *ADNP* potential effect on mouse vocal production was tested. A significant sex-dependent decline in the number of *Adnp*^{+/-} mouse pup calls was reported (Hacohen-Kleiman et al. 2019), whereas treatment with the *ADNP* snippet NAP corrected this abnormality (CP201) (Hacohen-Kleiman et al. 2018).

In contrast to rodents, songbirds are one of the few species able to learn vocalizations, alongside humans (Doupe and Kuhl 1999; Panaitof 2012). A previous study in the zebra finch songbird (ZF; *Taeniopygia guttata*) brain revealed greater *ADNP* transcript levels in the cerebrum of young males, compared with females and all other brain regions (Hacohen Kleiman et al. 2015). These findings were suggested to potentially correspond with male-exclusive singing ability and a fully developed cerebral song system (reviewed in Barnea and Pravosudov 2011).

Another highly conserved transcription factor is the *Forkhead box protein P2* (*FoxP2*), known to affect speech in human and song learning in songbirds (Lai et al. 2001; Haesler et al. 2004, 2007; Teramitsu et al. 2004; MacDermot et al. 2005; Feuk et al. 2006; Vernes et al. 2011). *FoxP2* has been associated with autism through interactions with known ASD-related genes, including *contactin-associated protein-like 2* (*CNTNAP2*) (Larsen et al. 2016; Adam et al. 2017; Li and Pozzo-Miller 2019). In mice, cortical *Foxp2* was suggested to be vital for social behavior, causing aberrations in autism-related gene expression and ultrasonic vocalization production (Medvedeva et al. 2019). In *Adnp*^{+/-} mice, *FoxP2* levels were previously shown to increase in the male hippocampus, compared with *Adnp*^{+/+} littermates (Hacohen-Kleiman et al. 2019), proposing *FoxP2* as a potential target for *ADNP* regulation.

To further our research, we strive to reveal the roles of *ADNP* in the songbird cerebrum of both sexes. Despite high resemblance in the *ADNP* sequence (predicted protein, 98.5% identical to ZF), canaries are characterized with subtler sex-dependent variations in song and volume of song nuclei (comparison presented in Table 1). Consequently, a study in canaries could subject sex-dependent roles in vocal production to scrutiny, thus allowing to potentially unravel *ADNP* underlying trajectory for vocal alteration.

Here, quantitative real-time PCR (qRT-PCR) and RNA in situ hybridization (ISH) spatial expression patterns of *ADNP* were tested in brains of male and female canaries. Corresponding with *ADNP* qRT-PCR data, transcript expression patterns for additional three genes, related to *ADNP* or autism/social behavior, were also examined: the *ADNP* regulator, vasoactive intestinal peptide (*VIP*) (Bodner et al. 1985; Gozes et al. 1989a; Giladi et al. 1990; Bassan et al. 1999), the *ADNP* sister gene (*ADNP2*) (Zamostiano et al. 2001; Kushnir et al. 2008; Dresner et al. 2011, 2012), and *FoxP2*. Correlation

Table 1 A comparison between canary and zebra finch

	Domesticated canary	Zebra finch (ZF)
Maximal lifespan reports in captivity	+ 10 years with reports of > 20-year-old individuals (Carey and Judge 2000; Pamplona et al. 1999; Holmes and Ottinger 2003; Nottebohm 2005; Austad 2011; Frankl-Vilches et al. 2015; Fishbein et al. 2019)	7–9 years (Austad 1997, Holmes and Ottinger 2003)
Male versus female song and song system	Females rarely produce songs (Ko et al. 2020). Female song control system nuclei appear smaller with a less-developed Area X (Nottebohm and Arnold 1976) The sex-dependent differences in nuclei volume were reported more striking in ZF compared with canary, coinciding with male-only song production in ZF (Nottebohm and Arnold 1976)	Females do not sing. Female song system nuclei appear smaller with missing or non-recognizable Area X (Nottebohm and Arnold 1976)
Seasonal effects	Seasonal breeders. Day-length increase triggers gonadal development and singing behavior (Ko et al. 2020). Song varies in stability, reported stable during breeding season. In non-breeding season, syllables are added, coupled with volume changes in song nuclei (Nottebohm et al. 1986).	Opportunistic breeders (Zann 1996)
<i>ADNP</i> conservation	(Predicted) <i>ADNP</i> protein is 98.51% identical (NCBI Blast, NCBI Reference Sequence: XM_018917433.2, XM_032751963.1 for canary and zebra finch, respectively)	
Experimental settings	Monitored daylight length, indoor cages	Outdoor cages (unmonitored)

analyses between these gene transcripts were performed for potential significant gene-gene correspondence.

Materials and Methods

Nomenclature For avian brain regions, we used the revised nomenclature proposed by the Avian Brain Nomenclature Forum (<http://avianbrain.org>) (Reiner et al. 2004b; Jarvis et al. 2013) as well as the brain atlas by Nixdorf-Bergweiler and Bischof (2007) and online atlas (<http://www.zebrafinchatlas.org>). For gene nomenclature, we followed

the convention proposed by the NCBI and the HUGO Gene Nomenclature Committee database (i.e., *ADNP*, *ADNP2*, *VIP*, and *FOXP2* in human and canary (excluding *FoxP2* in canary (Kaestner et al. 2000)) and *Adnp*, *Adnp2*, *Vip*, and *Foxp2* in mouse). Proteins are in roman type, and genes and RNA in italics (<https://www.genenames.org/>).

Animals Domesticated canary brains ($n = 13$, 2–6-year-old males; and $n = 12$, 2–5-year-old females) were obtained according to availability from a breeding colony in Professor Nottebohm laboratory at the Field Research Center in Rockefeller University, NY, USA. Canaries were previously kept in a co-sex aviary, housed in groups of two (breeding pair, $18 \times 10 \times 9$ in.) or 8–12 birds per cage ($32 \times 27 \times 22$ in.), subjected to 15.25 light hours/24 h a day. Birds were provided with seeds, water, and a high-protein egg diet available ad libitum. Prior to sacrifice, all canaries were in breeding mode. Preliminary analysis revealed that the selected gene expression levels did not change within the range of tested ages in both sexes (2–6 years old, Supplemental Fig. 1). Bird of ages ranging 2–6 years were therefore grouped together and analyzed accordingly. Two to 6 days prior to killing, birds were placed in groups of 3 birds per cage ($23.5 \times 20 \times 14$ in.; males and females together) in a 13-h lights-on/11-h lights-off cycle. For exclusion of song effect on gene expression, lights were kept “off” during morning hours of day of sacrifice, minimizing bird singing (up to 1 h prior to sacrifice). Canaries were decapitated following CO₂ inhalation overdose (Hacohen Kleiman et al. 2015).

Tissue Preparations for mRNA Analyses Brains were rapidly removed and snap-frozen in liquid nitrogen either separately cut into two hemispheres or dissected to cerebrum, cerebellum, and brain stem (for RNA ISH or qRT-PCR, respectively) (Hacohen Kleiman et al. 2015). Frozen samples were stored at -80°C until use. For qRT-PCR mRNA analysis, RNA extraction was done as previously described (Hacohen Kleiman et al. 2015). Cerebrum, cerebellum, and brain stem RNA transcripts were extracted using the TRI reagent (T9424, Sigma–Aldrich, St. Louis, MO). A total of 0.2 ml of chloroform was added for phase separation (pelleted at $12,000 \times g$ for 15 min, 4°C). A total of 0.5 ml of 2-propanol was added for total RNA pellet (pelleted at $12,000 \times g$ for 10 min, 4°C). The total RNA pellet was washed by adding 1 ml of 75% ethanol and then subjected to centrifugation at $7500 \times g$ for 5 min, 4°C . Total RNA was dissolved in distilled water (Tamar, Mevaseret Zion, Israel) (Hacohen Kleiman et al. 2015). Total RNA purity and concentration were determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All materials were added calculated in accordance with 1 ml of TRI Reagent.

Quantitative Real-time PCR qRT-PCR was performed as previously described (Hacohen Kleiman et al. 2015) (males ($n = 10$,

Table 2 Canary-specific primers utilized in qRT-PCR

Gene name	Primer	Primer sequence
<i>ADNP</i>	Forward	5'-GAAAATGAGCGAGAACTATG-3'
	Reverse	5'-TGTTCTTTACAGTACTCCAA ACC-3'
<i>VIP</i>	Forward	5'-AGAATGCCATTTGATGGA-3'
	Reverse	5'-GAGTGGCGTTTGACAGG-3'
<i>ADNP2</i>	Forward	5'-CAGTGCAGGGAATTGT-3'
	Reverse	5'-ATCTCTTCCTTTGCCCA-3'
<i>FoxP2</i>	Forward	5'-TGGCTGTGAAAGCGTTT-3'
	Reverse	5'-ATTTGCACCCGACACTG-3'
<i>RPL13</i>	Forward	5'-AACAAGTCCACCGAGTCC-3'
	Reverse	5'-TCCTCTGGAGAGCTGTCTC-3'

2–6 years old) and females ($n = 9$, 2–5 years old)). Equal amounts of total RNA (1 μg RNA/sample, obtained from each bird) were subjected to reverse transcription (RT) using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA). Reactions were all carried out in duplicates in 384-well plates. For sample variability exclusion, several samples were repeated between plates as controls. Real-time PCR was performed using PerfeCTa SYBR Green FastMix, Low ROX (Quanta Biosciences, Gaithersburg, MD, USA) and QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Messenger RNA expression levels were determined using specific canary primers presented in Table 2 (Mukai et al. 2009; Olias et al. 2014). The comparative Ct method was used for quantification of transcripts. As previously performed, data were normalized with those from *ribosomal protein L13* (*RPL-13*; control) (Hacohen Kleiman et al. 2015). Results are presented as mean ($\pm$ SEM) relative gene expression ($2^{-\Delta\text{CT}}$; Fig. 1). Analyses for potential significant correlations between the genes were performed (presented in Table 3).

ADNP cDNA Cloning from Canary Brain Desired *ADNP* cDNA sequence (260 bp) was amplified through PCR using *ADNP* primers (sense 5'-TCAGTGGCAACACAGCTG-3', antisense 5'-AGGACCTGTAGCAGCCAC-3') and a canary cDNA sample. The PCR product was examined on a 2% agarose gel, cleaned using Wizard® SV Gel and PCR Clean-Up System (Promega, WI, USA), and cloned into a pGEM T Easy vector (Promega, Madison, WI), as previously done (Haesler et al. 2004; Dresner et al. 2012). *ADNP* sense and antisense riboprobes were in vitro transcribed from T7 and SP6 promoter sides of the pGEM T easy cloning vector containing the *ADNP* cDNA clone, using digoxigenin (DIG) labeling (Roche, Germany).

RNA In Situ Hybridization ISH was performed as previously described (Mendoza et al. 2015). Canary brains (males ($n = 3$, 4–5 years old) and females ($n = 3$, 2–5 years old)) were cut frozen

Table 3 Gene correlations in the canary brain within three brain regions: cerebrum, cerebellum, brain stem. Male results are presented in blue; female results are presented in magenta. Data were normalized with those from *RPL-13* (control). Multiple statistically significant positive correlations were found between the genes within the different brain regions, in a sex-dependent manner. Correlation tests for all genes were

performed using either the Pearson correlation coefficient method or Spearman's rank correlation coefficient, if at least one of the data sets was not normally distributed. Table cells which stand for repeated comparisons between genes were disregarded and marked in gray color. Males ($n = 10$, 2–6 years old) and females ($n = 9$, 2–5 years old). Significant p values appear in asterisks

Cerebrum	<i>VIP</i>	<i>ADNP2</i>	<i>FoxP2</i>
<i>ADNP</i>	$r=0.74$, $*p=0.0174$	$r=0.92$, $***p=0.0004$	$r=0.73$, $*p=0.0159$ $r=0.76$, $*p=0.017$
<i>VIP</i>		$r=0.70$, $*p=0.0353$	$r=0.81$, $**p=0.0058$
<i>ADNP2</i>			$r=0.77$, $**p=0.0078$ $r=0.89$, $**p=0.0011$
<i>FoxP2</i>			

Cerebellum	<i>VIP</i>	<i>ADNP2</i>	<i>FoxP2</i>
<i>ADNP</i>		$r=0.77$, $**p=0.0085$	$r=0.81$, $**p=0.0038$ $r=0.71$, $*p=0.0298$
<i>VIP</i>			
<i>ADNP</i>			
<i>FoxP2</i>			

Brain stem	<i>VIP</i>	<i>ADNP2</i>	<i>FoxP2</i>
<i>ADNP</i>	$r=0.78$, $**p=0.007$	$r=0.88$, $***p=0.0007$ $r=0.81$, $**p=0.0071$	$r=0.94$, $***p<0.0001$
<i>VIP</i>		$r=0.82$, $**p=0.0034$	$r=0.87$, $***p=0.0008$ $r=0.72$, $*p=0.0262$
<i>ADNP2</i>			$r=0.88$, $***p=0.0006$
<i>FoxP2</i>			

on the sagittal plane using a rotary cryostat (14 μ m). Sections were then fixed in 4% paraformaldehyde for 5 min, washed in 0.025 $\times$ PBS, dehydrated in 70%, 95%, and 100% ethanol, and air-dried. Sections underwent acetylation for 10 min using triethanolamine (Carl-Roth, Germany) and acetic anhydride (Sigma–Aldrich, St. Louis, MO). Slides were washed twice with 2 $\times$ SSC (0.3 M NaCl, 0.03 M tri-Natriumcitrat Dihydrat, pH = 7), re-dehydrated with the same increasing concentrations of ethanol, and air-dried. Sections were pre-hybridized in hybridization buffer (5 $\times$ SSC, 2% blocking reagent, 50% formamide) for at

least 1 h at 60 °C. For hybridization, 7 μ l of probe/100 μ l hybridization solution was applied on each section and was left overnight at 55 °C in an oil bath (330779 light Mineral oil, Sigma–Aldrich, St. Louis, MO). The following day, sections were repeatedly rinsed using chloroform and 2 $\times$ SSC and washed for 20–30 min in the following solutions, preheated to 55 °C: 1 $\times$ SSC/50% formamide, 2 $\times$ SSC, and 0.2 $\times$ SSC (twice). Slides were then washed (RT) in MABT (100 mM malic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5), blocked with TNB (TSA Blocking Reagent, FP1020, PerkinElmer, USA) for 2 h,

Fig. 1 Gene expression and positive correlations in the canary brain. Results are presented as relative gene expression ($2^{-\Delta CT}$). Male results are presented in blue; female results are presented in magenta. Results were normalized to *RPL-13* (control). Two-way ANOVA with Tukey's post hoc test was performed for statistical analyses. **a** For *ADNP*, a significant interaction between brain regions and sex was found ($F_{(2,51)} = 4.366$, $p = 0.018$). Significantly higher *ADNP* expression was observed in the male cerebrum, compared with female and the other brain regions ($***p < 0.001$). In females, *ADNP* expression levels were significantly higher in the cerebrum, compared with the brain stem ($*p < 0.05$). **b** For *VIP*, main effect for brain region was found ($F_{(2,49)} = 36.842$, $p < 0.001$). *VIP* was significantly less expressed in the cerebellum compared with

other brain regions, in both sexes ($***p < 0.001$). **c** For *ADNP2*, main effect for brain region was found ($F_{(2,51)} = 15.194$, $p < 0.001$). *ADNP2* expression was significantly reduced in the brain stem of both sexes ($*p < 0.05$ in males, $***p < 0.001$ in females), with even lower expression in females ($*p < 0.05$). **d** For *FoxP2*, a statistically significant interaction between brain region and sex was found ($F_{(2,51)} = 4.174$, $p = 0.021$). In males, significantly higher expression was observed in both the cerebrum and brain stem, compared with females ($**p < 0.01$, $***p < 0.001$ respectively). For both sexes, *FoxP2* was less expressed in the cerebellum compared with other brain regions ($***p < 0.001$). Males ($n = 10$, 2–6 years old) and females ($n = 9$, 2–5 years old). Significant p values appear in asterisks

and incubated overnight with alkaline phosphatase-conjugated goat anti-DIG Fab' antibody (11093274910, Roche, Germany), 4 °C. Colorimetric detection was performed by an immunoalkaline phosphatase reaction with BM purple solution (11442074001, Roche, Germany) as the substrate. Antisense and sense probes were always run in parallel. Ten sections of each brain, in 280-μm intervals, were mapped along the sagittal axis of the brain for *ADNP*-expressed regions, with Olympus × 4/0.16 Objective (Fig. 2a, b). Images were taken using a light microscope (Olympus bx-65) equipped with a color camera (Micro-BrightField Ltd., USA), a motorized stage, and a computerized brain-mapping system (NeuroLucida; Stereo Investigator version 9; Micro-BrightField Ltd., USA). Settings for microscope lighting and camera exposure were kept constant for all images obtained.

ADNP mRNA Signal Quantification in ISH For all images, borders were drawn around desired brain region prior to

quantification as mean gray value, using the ImageJ software (Schneider et al. 2012). Significance of signal intensity is presented for mesopallium (M), compared with the *ADNP* sense probe in adjacent sections (Fig. 2b, c). Results are presented as 255-mean of signal intensity ± SEM (RGB; Fig. 2c) (males ($n = 1$, 5 years old) and females ($n = 3$, 2–5 years old)).

Statistics For qRT-PCR and ISH quantifications, results are presented as mean ± SEM. Data were tested for normal distribution using the Shapiro–Wilk test and outliers were excluded (<https://graphpad.com/quickcalcs/Grubbs1.cfm>). For two different categorically independent variables, a 2-way ANOVA followed by Tukey's post hoc test was performed. For correlation testing, Pearson's correlation analysis was performed if both plotted data sets were normally distributed. Spearman's rank correlation coefficient method was performed if at least one of the data sets was not normally distributed. Student's t test analysis was performed when needed. p values ≤ 0.05 were considered statistically

Fig. 2 ADNP expression shows a robust signal in the mesopallium (M). 14- μ m sagittal sections of male and female canary brains were tested for RNA in situ hybridization (ISH) of ADNP expression. In all images, arrows and dotted lines indicate the examined regions. Images of ADNP expression in mesopallium (M) are presented above with visible borders. All images were taken under the same illumination and camera settings (0.6 Lux), with $\times 4/0.16$ Objective. **a** Three representative images of male and female brains are presented similarly (upper images, males; lower images, females). Higher ADNP mRNA expression was implied by strong signal observed in M (marked on atlas, left side of the figure). Males ($n = 3$, 4–5 years old) and females ($n = 3$, 2–5 years old). **b** ADNP antisense probe specificity was verified by the lack of signal with the ADNP sense probe (representative pictures of ADNP antisense and sense probes appear respectively from left to right). **c** For signal

quantification, borders were drawn around each area using ImageJ. ADNP antisense probe was compared with signal from the ADNP sense probe in adjacent sections in each bird. In graph, data are presented as 255-mean of histogram gray value. Results for ADNP antisense probe signal are presented in blue; ADNP sense probe signal are presented in turquoise. ADNP antisense probe signal was significantly stronger compared with that of ADNP sense probe (** $p = 0.007$, Student's t test). Males ($n = 1$, 5 years old) and females ($n = 3$, 2–5 years old). The atlas image was reproduced from the ZEBra database with permission from Mello laboratory (Oregon Health & Science University, Portland, OR 97239; <http://www.zebrafinchatlas.org>) (Lovell et al. 2020). Directions are indicated by arrows: dorsal (D); lateral (L); caudal (C); rostral (R). Significant p values appear in asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Scale bar = 150 μ m

significant, and all tests were 2-tailed. All statistical analyses were conducted using the SigmaPlot software (version 11 Windows) or GraphPad Prism (version 6 Windows).

Results

ADNP Is Predominantly Expressed in the Canary Male Cerebrum, Similar to Zebra Finch

Given our interest to study ADNP relations to sex-dependent vocal production and circuits, we further examined ADNP transcript expression profile in a different songbird as compared with ZF, the domesticated canary songbird (*Serinus canaria domestica*) (presented in Table 1). Male and female canary gene expression

(qRT-PCR; males ($n = 10$, 2–6 years old) and females ($n = 9$, 2–5 years old)) data were analyzed for potential sex or brain region effects in the cerebrum, cerebellum, and brain stem (Fig. 1). Additional correlation measurements were applied for potential gene-gene associations in the canary brain (presented in Table 3). For ADNP, qRT-PCR results present a similar mRNA distribution to that seen in young ZF, in both sexes (Hacohen Kleiman et al. 2015) with highest expression in male cerebrum (2-fold, $p < 0.001$) and lowest in the female brain stem ($p < 0.05$) (Fig. 1a). For the ADNP regulator gene, *VIP*, expression was found to be similar for both sexes with lowest expression in the cerebellum ($p < 0.001$) (Fig. 1b). For the ADNP sister gene, *ADNP2*, a significantly lower expression was observed in the brain stem, with even lower expression in females ($p < 0.05$) (Fig. 1c).

FoxP2, a Known Speech Regulator, Is Grossly Distributed in a Similar Fashion to VIP, with Significant Sex-Dependent Differences in the Cerebrum, Like ADNP and the Brain Stem

The transcript levels of *FoxP2*, a known language acquisition regulator (Teramitsu et al. 2004; Scharff and Haesler 2005), were also tested (Fig. 1d). qRT-PCR results showcased an overall similar expression pattern to that of *VIP*, with lower expression in the cerebellum compared with the cerebrum and brain stem in males ($p < 0.001$). Similarly, lower *FoxP2* expression levels were observed in the female cerebellum, compared with the brain stem ($p < 0.001$). Like *ADNP*, a comparison between the sexes revealed significantly higher expression levels for *FoxP2* in the male cerebrum (Fig. 1a, d). Furthermore, a similar sex-dependent difference in *FoxP2* levels was found in the brain stem, comparing male expression levels with females ($p < 0.01$, $p < 0.001$, respectively) (Fig. 1d).

Multiple Positive Sex- and Brain Region-Dependent ADNP/ADNP2/VIP/FoxP2 Gene Transcript Correlations in the Canary Brain

Correlations between *ADNP*, *VIP*, *ADNP2*, and *FoxP2* transcripts were measured in the three tested brain regions, following qRT-PCR (above). Significant correlations ($p < 0.05$) are shown in Table 3. In short, high positive correlations were observed for *ADNP* and all tested transcripts in a sex/tissue-dependent manner as follows. For *VIP*, *ADNP* correlations were observed only in males in the cerebrum and brain stem. For *ADNP/ADNP2*, correlations were solely observed in the female cerebrum, the male cerebellum, and the brain stem of both sexes. The most extensive correlations were observed for *FoxP2* and *ADNP* appearing in all brain areas and in both sexes, except for the female brain stem. *VIP* also correlated with *ADNP2*, only in the female cerebrum (like with *ADNP*) and male brain stem. In the same brain areas, *VIP* correlated also with *FoxP2*, only in the male cerebrum and in the brain stem of both sexes.

Extensive ADNP mRNA Expression in the Brain with Robust Signal in Canary Mesopallium

As *ADNP* was significantly more expressed in the cerebrum (Fig. 1), we focused on this brain area for our ISH evaluations. An overall light and uniform signal was observed across the cerebrum with a robust signal detected in the mesopallium (M) region. Three male and three female panels are shown, indicating overall similar distribution (Fig. 2a). Figure 2b presents a sense and antisense *ADNP* probe comparison (in female) with a marked difference. Quantification of the *ADNP* signal in M is shown as 255-mean of histogram signal

intensity (RGB) with the *ADNP* sense probe signal found to be nearly negligible (control; Fig. 2c, including the brain coordinates). These results further indicate the significant labeling of *ADNP* in M.

Discussion

In the present study, sex/brain area-specific *ADNP* expression are shown in 2–6-year-old canary brains, with highest expression in the male cerebrum (Fig. 1a). Our previous findings in the ZF songbird demonstrated a similar sexual/brain area dichotomy in the young (6 months old) brain, which changed upon aging (Hacohen Kleiman et al. 2015).

Notably, this study holds potential limitation due to restricted selection of samples. As mentioned in the “Materials and Methods” section, canaries were obtained according to aviary availability, which constituted a great age repertoire (2–6 years of age) with a limited number of samples per age (1–3 or 2–5 samples per age in males and females, respectively). This predicament may have affected the variation of transcript data, possibly distorting result distribution. Importantly, despite given age range, preliminary analysis of qRT-PCR data disclosed constant gene expression for all tested genes, in both sexes (Supplemental Fig. 1). Therefore, while not ideal, analyzed data was treated regardless of age appearing in two groups: males and females. For *ADNP* transcript data (qRT-PCR), while significant sex-dependent differences were found, sample limitation may have consequently led to unexpected males’ and females’ partial overlap in the cerebrum. This impasse could possibly be settled by data presented in Table 1. Accordingly, canaries are reported with longer maximal lifespan, compared with ZF, potentially indicating a slower aging process. In this case, results in older canaries may stand in equal measure with the younger ZF (6 months old). Furthermore, unlike ZF studied before, the canaries here were kept in a monitored indoor environment (also presented in Table 1). Importantly, stress and environmental changes, including light/darkness conditions, may alter gene expression, encompassing, for example, *VIP* (Holtzman et al. 1989; Casal and Yanovsky 2005; Tung and Gilad 2013; Dawson 2015; Saunderson et al. 2016; Dominoni et al. 2018). In this respect, *ADNP* was also reported to increase in response to stress in humans, while *Adnp*^{+/-} mice were found more affected by light-related stress, compared with *Adnp*^{+/+} littermates (Sragovich et al. 2019). Ultimately, these results further amplify the intrigue of *ADNP* function in the songbird brain, suggesting potential sex-dependent roles. Such sex-dependent roles were previously described in mice with further association to behavioral regulation (Malishkevich et al. 2015; Amram et al. 2016; Hacohen-Kleiman et al. 2018).

Highly significant correlations were discovered among *ADNP*, *VIP*, *ADNP2*, and *FoxP2*. *VIP* is an *ADNP* regulator, important for embryogenesis (Gressens et al. 1993; Bassan

et al. 1999; Gozes et al. 1999; Giladi et al. 2007). VIP is highly conserved across species (Bodner et al. 1985; Giladi et al. 1990) and found identical in many mammals (Giladi et al. 1990) with minor differences observed between human and chicken (Nussdorfer and Malendowicz 1998; Nowak et al. 2003). In birds, VIP was previously associated with pair bonding and social and parental behaviors (Kingsbury et al. 2013; Kingsbury and Goodson 2014; Kingsbury et al. 2015; Vistoropsky et al. 2016). In our canaries, *VIP* expression of both sexes was increased in the cerebrum and brain stem with significantly less expression in the cerebellum (Fig. 1b). This corroborates with previous reports of very low density of VIP binding sites in the cerebellum of the pigeon *Columba livia* (Hof et al. 1991). *ADNP-VIP* positively correlated only in males, in both the cerebrum and brain stem (presented in Table 3), probably contingent to specific sex-dependent regulation on the *ADNP* gene (Malishkevich et al. 2015) and estrogen/steroid sex-dependent regulation of the *VIP* gene (Gozes et al. 1989b).

ADNP2 is an *ADNP* paralog (33% identical, 46% similar) and does not include the microtubule-binding neuroprotective NAP motif (Zamostiano et al. 2001) (Fig. 1c). The *ADNP* family (*ADNP*, *ADNP2*) was suggested to play a role in erythropoiesis in vertebrates (Dresner et al. 2012). To our knowledge, *ADNP2* has not been studied yet in birds. In mice, *VIP* reduction modulated *ADNP* and sister protein *ADNP2* expression patterns in the brain, presenting autistic like traits (Giladi et al. 2007). Here, *VIP* positively correlated with *ADNP2* expression in a sex-dependent manner, in the female cerebrum and the male brain stem. For *ADNP-ADNP2*, *ADNP2* was shown to correlate with *ADNP* mRNA as well as with premonitory intelligence in humans (Malishkevich et al. 2016). Resembling *ADNP* expression profile in the female canary, *ADNP2* transcripts were lowest in the canary brain stem, with significantly lower expression in females, compared with males. Accordingly, *ADNP-ADNP2* positively correlated in the brain stem of both sexes, as well as in the female cerebrum and male cerebellum. *ADNP2* correlation with *VIP* and *ADNP* in the female cerebrum may relate to past findings in mouse and human, associated with brain function importance, in a sex-dependent manner, with deregulated hippocampal *ADNP/ADNP2* linked to schizophrenia (Dresner et al. 2011). Furthermore, the *VIP* receptor *VPAC2* (*VIPR2*) gene copy number variations (CNVs) impact (cause) schizophrenia (Tian et al. 2019) and allow *VIP* regulation of *ADNP* (Zusev and Gozes 2004).

FoxP2 is a highly conserved transcription factor (Haesler et al. 2004) implicated in song and speech in both humans and songbirds (Teramitsu et al. 2004). Mutations in *FoxP2* were linked to a language comprehension and speech disorder in humans (Scharff and Haesler 2005). In the songbird brain, song production and learning pathways are both distributed in the cerebrum and brainstem (Kubikova et al. 2010). More specifically, *FoxP2* was previously reported to be expressed primarily in the striatum of avian and reptiles, as well as in the

thalamus, midbrain visual processing regions, the inferior olive of the medulla, Purkinje cells in the cerebellum, deep cerebellar nuclei, and sensory auditory midbrain structures (Takahashi et al. 2003; Haesler et al. 2004; Teramitsu et al. 2004; Scharff and Haesler 2005; Campbell et al. 2009; Kato et al. 2014; Wohlgemuth et al. 2014). Here, our results coincide with these previous reports, presenting higher *FoxP2* mRNA levels in both the cerebrum (including the striatum) and the brain stem (comprising the thalamus, midbrain, and hindbrain (Kubikova et al. 2010)), compared with much lower expression in the cerebellum, in both sexes (Fig. 1d).

FoxP2 male and female brain distribution patterns were grossly similar; nevertheless, significantly higher levels were reported in males, compared with females (Fig. 1d). The overall resemblance may be attributed to female canary ability to produce songs (Ko et al. 2020), whereas significant differences in song and song system anatomy may stand for the male-specific transcript increase. In this respect, reports of female spontaneous singing are relatively rare and still significantly differ from male or testosterone-induced female song (Ko et al. 2020). Anatomically, the female canary song control system nuclei appear smaller, compared with males (Nottebohm and Arnold 1976) (presented in Table 1). A previous study in black-capped chickadees (*Parus atricapillus*) may also correspond with our findings of subtler *FoxP2* sex-dependent differences, found in Area X (Phillmore et al. 2015). Likewise, this moderate discrepancy was potentially associated with female ability to produce learned chickadee call songs with lower output, compared with males (Phillmore et al. 2015).

For *ADNP-FoxP2*, a connection was previously implied with reports of a specific increase in *FoxP2* levels in the male *Adnp*^{+/-} mouse hippocampus, compared with *Adnp*^{+/+} littermates (Hacohen-Kleiman et al. 2019) as well as its normalization of expression in the presence of the *ADNP* snippet drug candidate NAP (NAPVSIPQ; CP201) (Bassan et al. 1999; Oz et al. 2014; Hacohen-Kleiman et al. 2018). Similarly, given the connection of *ADNP* and *VIP* to schizophrenia, previous studies have also assessed *FoxP2* expression in the disrupted-in-schizophrenia (*DISC1*) mouse model, with *FoxP2* regulating *DISC1*. Thus, previous results showed that *FoxP2* transcript levels were increased in the hippocampus of the *DISC1*-mutated mice and were significantly lowered after treatment with NAP (Vaisburd et al. 2015), as shown above for the *Adnp*^{+/-} mouse model.

In the current study, *ADNP* expression pattern resembled that of *FoxP2* with higher male expression in the cerebrum, coupled with significant positive correlations in both sexes. Additional *ADNP-FoxP2* positive correlations were observed in the cerebellum in both sexes and solely in the male brain stem. Like *FoxP2-ADNP*, *FoxP2-ADNP2* correlations were also present in the cerebrum of both sexes and in the brain stem of males only. These findings imply once again of sex-

dependent importance in the regulation and function for *ADNP*, *ADNP2*, and *FoxP2* in the brain.

For *FoxP2-VIP*, to the best of our knowledge, there is no previous description of a potential connection between the genes. Here, *FoxP2* and *VIP* gross expression patterns were found vastly similar, in both sexes with several *FoxP2-VIP* positive correlations, in the male cerebrum and in the brain stem of both sexes. We presume this could be referred to specific sexual dichotomous differences found in *FoxP2* (not in *VIP*), pointing to higher expression in males versus females in both the cerebrum and brain stem. In this regard, *FoxP2*, *ADNP*, and *VIP* may be connected through dendritic spine density regulation. *FoxP2* was found to regulate spine density in Area X in ZF (Haesler et al. 2007; Schulz et al. 2010), as well as *CNTNAP2* expression, suggesting a *CNTNAP2*-mediated *FoxP2* effect on spines (Adam et al. 2017; Mendoza and Scharff 2017). Importantly, *CNTNAP2* is an ASD-associated gene alongside *ADNP* (Helsmoortel et al. 2014; Larsen et al. 2016; Adam et al. 2017; Gozes et al. 2017a; Gozes et al. 2017b; Li and Pozzo-Miller 2019; Van Dijck et al. 2019; Grigg et al. 2020; Satterstrom et al. 2020). Like *FoxP2*, *ADNP* and *VIP* were also reported to regulate dendritic spines, possibly acting through *ADNP* (Hill et al. 1994; Hacohe-Kleiman et al. 2018). Treatment with NAP, in turn, normalized spine density in the *Adnp*^{+/-} mice (Hacohe-Kleiman et al. 2018).

ISH observations revealed *ADNP* expression in the mesopallium (M) of both sexes (Fig. 2). Notably, *VIP* mRNA was also similarly described in the bird M (Hof et al. 1991; Kuenzel et al. 1997). M, formerly known as hyperstriatum ventral (HV) (Reiner et al. 2004a), is a telencephalic region, part of the avian pallium (Reiner et al. 2004a). Previous studies associated M with color discrimination in pigeons (Chaves and Hodos 1997) while HV relative size was found to significantly correlate with the rate of feeding innovation in birds (Timmermans et al. 2000). In primates, a similar correlation was observed with the relative size of the neocortex (Timmermans et al. 2000; Michael et al. 2015). The intermediate medial HV was suggested to participate in the memory formation for avoidance learning in chicks (Patterson et al. 1990) and recognition following visual imprinting (Horn 1990). Additional past studies in the caudal medial mesopallium (CMM) and nidocaudal mesopallium (NCM), two important auditory areas, previously associated these brain areas to perceptual processing of song and the formation of auditory memories (Lynch et al. 2013; Haakenson et al. 2019). Respectively, this information could align with social and object recognition (visual-based) impairments and auditory pathway abnormalities following *Adnp* haploinsufficiency (Vulih-Shultzman et al. 2007; Malishkevich et al. 2015; Amram et al. 2016; Hacohe-Kleiman et al. 2018, 2019), as well as intellectual disability, autism-like traits, and atypical auditory brain stem response

(ABR) in the *ADNP* syndrome patients (Helsmoortel et al. 2014; Hacohe-Kleiman et al. 2019; Van Dijck et al. 2019). Furthering the association to song, functional connections between caudal medial mesopallium (CMM) and other important regions: HVC, and caudal medial nidopallium (CMN) were suggested to take part in song learning and production in males. In parallel, an experiment in female canaries indicates of distinct functions in female songbirds related to song recognition rather than song production (Lynch et al. 2013). For *Adnp*, haploinsufficiency was implicated in vocal production in mice, corresponding with reports of speech delays in children of the *ADNP* syndrome (Helsmoortel et al. 2014; Hacohe-Kleiman et al. 2018, 2019; Van Dijck et al. 2019). The notable decrease in *Adnp*^{+/-} number of calls was completely abolished following NAP treatment (Hacohe-Kleiman et al. 2018).

Finally, the intermediate medial mesopallium is known to partake in learning and memory and suggested to engage in rewarding effects (Csillag 1999; He et al. 2010). In this respect, *ADNP* was previously associated with the reward system in mice, affecting alcohol consumption in a sex-dependent manner (Ziv et al. 2019). NAP in turn normalized the phenotype presented in *Adnp*^{+/-} females (Ziv et al. 2019).

Taken all together, this study further strengthens previous findings in ZF, mouse, and human, validating a sexual dichotomous *ADNP* expression pattern in the brain, with higher levels found in males (Hacohe-Kleiman et al. 2015; Malishkevich et al. 2015). For *ADNP-VIP-FoxP2*, the role in dendritic spine density regulation, as well as *VIP* and *FoxP2* striking correlation patterns, captivates even further in the search of a potential non-direct connection between *ADNP* and *FoxP2*, possibly involving *VIP*. For *FoxP2*, a gross role in the cerebrum and brain stem is suggested for both sexes with a more significant/additional role in males, potentially attributed to the further-complex singing ability/song plasticity (Phillimore et al. 2015). In the current study, as canary song behavior was limited but not surely avoided prior to sacrifice, the potential existence of male singing towards females in the cage could only further strengthen our explanation for the differences in *FoxP2* expression between the sexes. For *ADNP* ISH, extensive mRNA distribution in the songbird brain may further corroborate with its essential roles as a transcription factor/chromatin remodeler and microtubule/autophagy regulator (Pinhasov et al. 2003; Mandel and Gozes 2007; Mandel et al. 2007; Merenlender-Wagner et al. 2015; Amram et al. 2016; Ivashko-Pachima et al. 2017, 2019a, b; Kaaij et al. 2019; Grigg et al. 2020). In turn, the palpable strong signal in canary M could indicate of a relation to songbird cognition, sense integration, and memory formation. This integrates well with *ADNP* association with similar functions, portrayed in the haploinsufficient mouse model, *ADNP* syndrome patients, and Alzheimer's disease patients who may suffer from *ADNP* somatic mutations (Ivashko-

Pachima et al. 2019a). Thus, additional investigation is necessary to unravel potential roles for *ADNP* in the establishment of auditory and visual-based memories and recognition in songbirds and humans. For singing behavior and perception, higher expression in M may be translated into different functions for each sex, playing a role in motor song production in males, while possibly implicating auditory song perception in females. Consequently, studies of *ADNP* in rodents and songbirds may be complimentary, paving the path for a better depiction of *ADNP* mutations and deficiencies in humans, potentially beneficial for treatment development.

Acknowledgments We would like to thank Professor Anat Barnea for her advice and student guidance, facilitating the connection established with the laboratories of Professor Fernando Nottebohm and Professor Constance Scharff. We would also like to thank Professor Fernando Nottebohm and his laboratory in Rockefeller University, NY, USA, as well as Mrs. Bhagwattie Haripal, Mrs. Sharon L. Sepe, and Professor Wan-chun Liu for assisting with the canary brains. Many thanks are given to Professor Constance Scharff and Doctor Ezequiel Mendoza for the demonstration of the RNA in situ hybridization technique. We also appreciate Mr. Yohan Benchimol, Mr. Michael Gruntfest, Mr. Gidon Karmon, and Doctor Eliezer Giladi for collaborative technical support.

Funding The Dr. Diana and Ziga (Zelman) Elton (Elbaum) Laboratory of Molecular Neuroendocrinology, Headed by Professor Illana Gozes (The Former, First Lily and Avraham Gildor Chair for the Investigation of Growth Factors), is supported by the following grants: European Research Area Networks (ERA-NET) neuron ADNPinMED, National Science Foundation (NSF) and US-Israel Binational Science Foundation (BSF) 2016746, and AMN Foundation as well as Drs. Ronith and Armand Stemmer (French Friends of Tel Aviv University). Professor Anat Barnea laboratory is supported by The Open University Research Fund. This study is in partial fulfillment of graduate studies requirements for Mrs. Gal Hacohen-Kleiman and Ms. Oxana Kapitansky. Gal Hacohen-Kleiman was supported by Eshkol fellowships, the Israel Ministry of Science and Technology, and by The Open University of Israel. Oxana Kapitansky is partially supported by the Israeli BioInnovators Fellowship and Mentors by Teva.

Compliance with Ethical Standards

Conflict of Interest Professor Illana Gozes is the Chief Scientific Officer of Coronis Neurosciences developing. NAP (CP201) for the *ADNP* syndrome. NAP (CP201) use is under patent protection (US patent nos. US7960334, US8618043, and USWO2017130190A1).

Ethics Approval All animal experiments and procedures were done in accordance with the permit granted by the Rockefeller University Institutional Animal Care and Use Committee (#13660-H).

References

- Adam I, Mendoza E, Kobalz U, Wohlgenuth S, Scharff C (2017) CNTNAP2 is a direct FoxP2 target in vitro and in vivo in zebra finches: complex regulation by age and activity. *Genes Brain Behav* 16:635–642
- 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 Psychiatry* 21:1467–1476
- Arnett AB, Rhoads CL, Hoekzema K, Turner TN, Gerdts J, Wallace AS, Bedrosian-Sermone S, Eichler EE, Bernier RA (2018) The autism spectrum phenotype in *ADNP* syndrome. *Autism Res* 11:1300–1310
- Austad SN (1997) Birds as models of aging in biomedical research. *ILAR J* 38:137–141
- Austad SN (2011) Candidate bird species for use in aging research. *ILAR J* 52:89–96
- Barnea A, Pravosudov V (2011) Birds as a model to study adult neurogenesis: bridging evolutionary, comparative and neuroethological approaches. *Eur J Neurosci* 34:884–907
- Bassan M, Zamostiano R, Davidson A, Pinhasov A, Giladi E, Perl O, Bassan H, Blat C, Gibney G, Glazner G, Brennehan DE, Gozes I (1999) Complete sequence of a novel protein containing a femtomolar-activity-dependent neuroprotective peptide. *J Neurochem* 72:1283–1293
- Bodner M, Fridkin M, Gozes I (1985) Coding sequences for vasoactive intestinal peptide and PHM-27 peptide are located on two adjacent exons in the human genome. *Proc Natl Acad Sci U S A* 82:3548–3551
- Campbell P, Reep RL, Stoll ML, Ophir AG, Phelps SM (2009) Conservation and diversity of Foxp2 expression in murine rodents: functional implications. *J Comp Neurol* 512:84–100
- Carey JR, Judge DS (2000) Longevity records: life spans of mammals, birds, amphibians, reptiles, and fish. University Press of Southern Denmark, Odense
- Casal JJ, Yanovsky MJ (2005) Regulation of gene expression by light. *Int J Dev Biol* 49:501–511
- Chaves LM, Hodos W (1997) Hyperstriatum ventrale in pigeons: effects of lesions on color-discrimination and color-reversal learning. *Vis Neurosci* 14:1029–1041
- Csillag A (1999) Striato-telencephalic and striato-tegmental circuits: relevance to learning in domestic chicks. *Behav Brain Res* 98:227–236
- Dawson A (2015) Annual gonadal cycles in birds: modeling the effects of photoperiod on seasonal changes in GnRH-1 secretion. *Front Neuroendocrinol* 37:52–64
- Dominoni DM, de Jong M, Bellingham M, O'Shaughnessy P, van Oers K, Robinson J, Smith B, Visser ME, Helm B (2018) Dose-response effects of light at night on the reproductive physiology of great tits (*Parus major*): integrating morphological analyses with candidate gene expression. *J Exp Zool Part A Ecol Integr Physiol* 329:473–487
- Doupe AJ, Kuhl PK (1999) Birdsong and human speech: common themes and mechanisms. *Annu Rev Neurosci* 22:567–631
- Dresner E, Agam G, Gozes I (2011) Activity-dependent neuroprotective protein (*ADNP*) expression level is correlated with the expression of the sister protein *ADNP2*: deregulation in schizophrenia. *Eur Neuropsychopharmacol* 21:355–361
- Dresner E, Malishkevich A, Arviv C, Leibman Barak S, Alon S, Ofir R, Gothilf Y, Gozes I (2012) Novel evolutionary-conserved role for the activity-dependent neuroprotective protein (*ADNP*) family that is important for erythropoiesis. *J Biol Chem* 287:40173–40185
- Feuk L, Kalervo A, Lipsanen-Nyman M, Skaug J, Nakabayashi K, Finucane B, Hartung D, Innes M, Kerem B, Nowaczyk MJ, Rivlin J, Roberts W, Senman L, Summers A, Szatmari P, Wong V, Vincent JB, Zeeman S, Osborne LR, Cardy JO, Kere J, Scherer SW, Hannula-Jouppi K (2006) Absence of a paternally inherited *FOXP2* gene in developmental verbal dyspraxia. *Am J Hum Genet* 79:965–972
- Fishbein AR, Lawson SL, Dooling RJ, Ball GF (2019) How canaries listen to their song: species-specific shape of auditory perception. *J Acoust Soc Am* 145:562–574

- Frankl-Vilches C, Kuhl H, Werber M, Klages S, Kerick M, Bakker A, de Oliveira EH, Reusch C, Capuano F, Vowinkel J, Leitner S, Ralsler M, Timmermann B, Gahr M (2015) Using the canary genome to decipher the evolution of hormone-sensitive gene regulation in seasonal singing birds. *Genome Biol* 16:19
- Furman S, Hill JM, Vulih I, Zaltzman R, Hauser JM, Brenneman DE, Gozes I (2005) Sexual dimorphism of activity-dependent neuroprotective protein in the mouse arcuate nucleus. *Neurosci Lett* 373:73–78
- Giladi E, Shani Y, Gozes I (1990) The complete structure of the rat VIP gene. *Brain Res Mol Brain Res* 7:261–267
- Giladi E, Hill JM, Dresner E, Stack CM, Gozes I (2007) Vasoactive intestinal peptide (VIP) regulates activity-dependent neuroprotective protein (ADNP) expression in vivo. *J Mol Neurosci* 33:278–283
- Gozes I, Meltzer E, Rubinrou S, Brenneman DE, Fridkin M (1989a) Vasoactive intestinal peptide potentiates sexual behavior: inhibition by novel antagonist. *Endocrinology* 125:2945–2949
- Gozes I, Werner H, Fawzi M, Abdelatty A, Shani Y, Fridkin M, Koch Y (1989b) Estrogen regulation of vasoactive intestinal peptide mRNA in rat hypothalamus. *J Mol Neurosci* 1:55–61
- Gozes I, Bassan M, Zamostiano R, Pinhasov A, Davidson A, Giladi E, Perl O, Glazner GW, Brenneman DE (1999) A novel signaling molecule for neuropeptide action: activity-dependent neuroprotective protein. *Ann N Y Acad Sci* 897:125–135
- Gozes I, Yeheskel A, Pasmanik-Chor M (2015) Activity-dependent neuroprotective protein (ADNP): a case study for highly conserved chordata-specific genes shaping the brain and mutated in cancer. *J Alzheimers Dis* 45:57–73
- Gozes I, Patterson MC, Van Dijk A, Kooy RF, Peeden JN, Eichenberger JA, Zawacki-Downing A, Bedrosian-Sermone S (2017a) 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
- Gozes I, Van Dijk 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 (2017b) Premature primary tooth eruption in cognitive/motor-delayed ADNP-mutated children. *Transl Psychiatry* 7:e1043
- Gressens P, Hill JM, Gozes I, Fridkin M, Brenneman DE (1993) Growth factor function of vasoactive intestinal peptide in whole cultured mouse embryos. *Nature* 362:155–158
- Grigg I, Ivashko-Pachima Y, Hait TA, Korenkova V, Touloumi O, Lagoudaki R, Van Dijk A, Marusic Z, Anicic M, Vukovic J, Kooy RF, Grigoriadis N, Gozes I (2020) Tauopathy in the young autistic brain: novel biomarker and therapeutic target. *Transl Psychiatry* 10:228
- Haakenson CM, Madison FN, Ball GF (2019) Effects of song experience and song quality on immediate early gene expression in female canaries (*Serinus canaria*). *Dev Neurobiol* 79:521–535
- Hacohen Kleiman G, Barnea A, Gozes I (2015) ADNP: a major autism mutated gene is differentially distributed (age and gender) in the songbird brain. *Peptides* 72:75–79
- 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 Invest* 128:4956–4969
- Hacohen-Kleiman G, Yizhar-Barnea O, Touloumi O, Lagoudaki R, Avraham KB, Grigoriadis N, Gozes I (2019) Atypical auditory brainstem response and protein expression aberrations related to ASD and hearing loss in the Adnp haploinsufficient mouse brain. *Neurochem Res* 44:1494–1507
- Haesler S, Wada K, Nshdejan A, Morrissey EE, Lints T, Jarvis ED, Scharff C (2004) FoxP2 expression in avian vocal learners and non-learners. *J Neurosci* 24:3164–3175
- Haesler S, Rochefort C, Georgi B, Licznarski 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:e321
- He X, Xiao L, Sui N (2010) Effects of SCH23390 and spiperone administered into medial striatum and intermediate medial mesopallium on rewarding effects of morphine in day-old chicks. *Eur J Pharmacol* 627:136–141
- 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* 46:380–384
- Hill JM, Mervis RF, Politi J, McCune SK, Gozes I, Fridkin M, Brenneman DE (1994) Blockade of VIP during neonatal development induces neuronal damage and increases VIP and VIP receptors in brain. *Ann N Y Acad Sci* 739:211–225
- Hof PR, Dietl MM, Charnay Y, Martin JL, Bouras C, Palacios JM, Magistretti PJ (1991) Vasoactive intestinal peptide binding sites and fibers in the brain of the pigeon *Columba livia*: an autoradiographic and immunohistochemical study. *J Comp Neurol* 305:393–411
- Holmes DJ, Ottinger MA (2003) Birds as long-lived animal models for the study of aging. *Exp Gerontol* 38:1365–1375
- Holtzman RL, Malach R, Gozes I (1989) Disruption of the optic pathway during development affects vasoactive intestinal peptide mRNA expression. *New Biol* 1(2):215–221
- Horn G (1990) Neural bases of recognition memory investigated through an analysis of imprinting. *Philos Trans R Soc Lond Ser B Biol Sci* 329:133–142 <https://www.genenames.org/>
- 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 Psychiatry* 22:1335–1344
- Ivashko-Pachima Y, Hadar A, Grigg I, Korenkova V, Kapitansky O, Karmon G, Gershovits M, Sayas CL, Kooy RF, Attems J, Gurwitz D, Gozes I (2019a) Discovery of autism/intellectual disability somatic mutations in Alzheimer's brains: mutated ADNP cytoskeletal impairments and repair as a case study. *Mol Psychiatry*. <https://doi.org/10.1038/s41380-019-0563-5>
- Ivashko-Pachima Y, Maor-Nof M, Gozes I (2019b) NAP (davunetide) preferential interaction with dynamic 3-repeat Tau explains differential protection in selected tauopathies. *PLoS One* 14:e0213666
- Jarvis ED, Yu J, Rivas MV, Horita H, Feenders G, Whitney O, Jarvis SC, Jarvis ER, Kubikova L, Puck AE, Siang-Bakshi C, Martin S, McElroy M, Hara E, Howard J, Pfenning A, Mouritsen H, Chen CC, Wada K (2013) Global view of the functional molecular organization of the avian cerebrum: mirror images and functional columns. *J Comp Neurol* 521:3614–3665
- Kaaij LJT, Mohn F, van der Weide RH, de Wit E, Buhler M (2019) The ChAHP complex counteracts chromatin looping at CTCF sites that emerged from SINE expansions in mouse. *Cell* 178:1437–1451 e1414
- Kaestner KH, Knochel W, Martinez DE (2000) Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev* 14:142–146
- Kato M, Okanoya K, Koike T, Sasaki E, Okano H, Watanabe S, Iriki A (2014) Human speech- and reading-related genes display partially overlapping expression patterns in the marmoset brain. *Brain Lang* 133:26–38
- Kingsbury MA, Goodson JL (2014) Pair bond formation is impaired by VPAC receptor antagonism in the socially monogamous zebra finch. *Behav Brain Res* 272:264–268
- Kingsbury MA, Miller KM, Goodson JL (2013) VPAC receptor signaling modulates grouping behavior and social responses to contextual

- novelty in a gregarious finch: a role for a putative prefrontal cortex homologue. *Horm Behav* 64:511–518
- Kingsbury MA, Jan N, Klatt JD, Goodson JL (2015) Nesting behavior is associated with VIP expression and VIP-Fos colocalization in a network-wide manner. *Horm Behav* 69:68–81
- Ko MC, Van Meir V, Vellena M, Gahr M (2020) Characteristics of song, brain-anatomy and blood androgen levels in spontaneously singing female canaries. *Horm Behav* 117:104614
- Kubikova L, Wada K, Jarvis ED (2010) Dopamine receptors in a songbird brain. *J Comp Neurol* 518:741–769
- Kuenzel WJ, McCune SK, Talbot RT, Sharp PJ, Hill JM (1997) Sites of gene expression for vasoactive intestinal polypeptide throughout the brain of the chick (*Gallus domesticus*). *J Comp Neurol* 381:101–118
- Kushnir M, Dresner E, Mandel S, Gozes I (2008) Silencing of the ADNP-family member, ADNP2, results in changes in cellular viability under oxidative stress. *J Neurochem* 105:537–545
- 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* 413:519–523
- Larsen E, Menashe I, Ziats MN, Pereanu W, Packer A, Banerjee-Basu S (2016) A systematic variant annotation approach for ranking genes associated with autism spectrum disorders. *Mol Autism* 7:44
- Li W, Pozzo-Miller L (2019) Dysfunction of the corticostriatal pathway in autism spectrum disorders. *J Neurosci Res*
- Lovell PV, Wirthlin M, Kaser T, Buckner AA, Carleton JB, Snider BR, McHugh AK, Tolpygo A, Mitra PP, Mello CV (2020) ZEBRA: Zebra finch Expression Brain Atlas—a resource for comparative molecular neuroanatomy and brain evolution studies. *J Comp Neurol* 528:2099–2131
- Lynch KS, Kleitz-Nelson HK, Ball GF (2013) HVC lesions modify immediate early gene expression in auditory forebrain regions of female songbirds. *Dev Neurobiol* 73:315–323
- MacDermot KD, Bonora E, Sykes N, Coupe AM, Lai CS, Vernes SC, Vargha-Khadem F, McKenzie F, Smith RL, Monaco AP, Fisher SE (2005) Identification of FOXP2 truncation as a novel cause of developmental speech and language deficits. *Am J Hum Genet* 76:1074–1080
- 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 Psychiatry* 5:e501
- Malishkevich A, Marshall GA, Schultz AP, Sperling RA, Aharon-Peretz J, Gozes I (2016) Blood-borne activity-dependent neuroprotective protein (ADNP) is correlated with premorbid intelligence, clinical stage, and Alzheimer's disease biomarkers. *J Alzheimers Dis* 50:249–260
- Mandel S, Gozes I (2007) Activity-dependent neuroprotective protein constitutes a novel element in the SWI/SNF chromatin remodeling complex. *J Biol Chem* 282:34448–34456
- Mandel S, Rechavi G, Gozes I (2007) Activity-dependent neuroprotective protein (ADNP) differentially interacts with chromatin to regulate genes essential for embryogenesis. *Dev Biol* 303:814–824
- Medvedeva VP, Rieger MA, Vieth B, Mombereau C, Ziegenhain C, Ghosh T, Cressant A, Enard W, Granon S, Dougherty JD, Groszer M (2019) Altered social behavior in mice carrying a cortical Foxp2 deletion. *Hum Mol Genet* 28:701–717
- Mendoza E, Scharff C (2017) Protein-protein interaction among the FoxP family members and their regulation of two target genes, VLDLR and CNTNAP2 in the zebra finch song system. *Front Mol Neurosci* 10:112
- Mendoza E, Tokarev K, During DN, Retamosa EC, Weiss M, Arpenik N, Scharff C (2015) Differential coexpression of FoxP1, FoxP2, and FoxP4 in the zebra finch (*Taeniopygia guttata*) song system. *J Comp Neurol* 523:1318–1340
- Merenlender-Wagner A, Malishkevich A, Shemer Z, Udawela M, Gibbons A, Scarr E, Dean B, Levine J, Agam G, Gozes I (2015) Autophagy has a key role in the pathophysiology of schizophrenia. *Mol Psychiatry* 20:126–132
- Michael N, Lowel S, Bischof HJ (2015) Features of the retinotopic representation in the visual wulst of a laterally eyed bird, the zebra finch (*Taeniopygia guttata*). *PLoS One* 10:e0124917
- Mukai M, Replogle K, Dmievich J, Wang G, Wacker D, Band M, Clayton DF, Wingfield JC (2009) Seasonal differences of gene expression profiles in song sparrow (*Melospiza melodia*) hypothalamus in relation to territorial aggression. *PLoS One* 4:e8182
- Nottebohm F (2005) The neural basis of birdsong. *PLoS Biol* 3:e164
- Nottebohm F, Arnold AP (1976) Sexual dimorphism in vocal control areas of the songbird brain. *Science* 194:211–213
- Nottebohm F, Nottebohm ME, Crane L (1986) Developmental and seasonal changes in canary song and their relation to changes in the anatomy of song-control nuclei. *Behav Neural Biol* 46:445–471
- Nowak JZ, Sedkowska P, Zawilska JB, Gozes I, Brenneman DE (2003) Antagonism of VIP-stimulated cyclic AMP formation in chick brain. *J Mol Neurosci* 20:163–172
- Nussdorfer GG, Malendowicz LK (1998) Role of VIP, PACAP, and related peptides in the regulation of the hypothalamo-pituitary-adrenal axis. *Peptides* 19:1443–1467
- Olias P, Adam I, Meyer A, Scharff C, Gruber AD (2014) Reference genes for quantitative gene expression studies in multiple avian species. *PLoS One* 9:e99678
- 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 Psychiatry* 19:1115–1124
- Pamplona R, Portero-Otin M, Riba D, Ledo F, Gredilla R, Herrero A, Barja G (1999) Heart fatty acid unsaturation and lipid peroxidation, and aging rate, are lower in the canary and the parakeet than in the mouse. *Aging* 11:44–49
- Panaitof SC (2012) A songbird animal model for dissecting the genetic bases of autism spectrum disorder. *Dis Markers* 33:241–249
- Patterson TA, Gilbert DB, Rose SP (1990) Pre- and post-training lesions of the intermediate medial hyperstriatum ventrale and passive avoidance learning in the chick. *Exp Brain Res* 80:189–195
- Phillmore LS, MacGillivray HL, Wilson KR, Martin S (2015) Effects of sex and seasonality on the song control system and FoxP2 protein expression in black-capped chickadees (*Parus atricapillus*). *Dev Neurobiol* 75:203–216
- 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* 144:83–90
- Reiner A, Perkel DJ, Bruce LL, Butler AB, Csillag A, Kuenzel W, Medina L, Paxinos G, Shimizu T, Striedter G, Wild M, Ball GF, Durand S, Gunturkun O, Lee DW, Mello CV, Powers A, White SA, Hough G, Kubikova L, Smulders TV, Wada K, Dugas-Ford J, Husband S, Yamamoto K, Yu J, Siang C, Jarvis ED, Avian Brain Nomenclature F (2004a) Revised nomenclature for avian telencephalon and some related brainstem nuclei. *J Comp Neurol* 473:377–414
- Reiner A, Perkel DJ, Mello CV, Jarvis ED (2004b) Songbirds and the revised avian brain nomenclature. *Ann N Y Acad Sci* 1016:77–108
- Satterstrom FK, Kosmicki JA, Wang J, Breen MS, De Rubeis S, An JY, Peng M, Collins R, Grove J, Klei L, Stevens C, Reichert J, Mulhern MS, Artomov M, Gerges S, Sheppard B, Xu X, Bhaduri A, Norman U, Brand H, Schwartz G, Nguyen R, Guerrero EE, Dias C, Autism Sequencing C, i P-BC, Betancur C, Cook EH, Gallagher L, Gill M, Sutcliffe JS, Thurm A, Zwick ME, Borglum AD, State MW, Cicek AE, Talkowski ME, Cutler DJ, Devlin B, Sanders SJ, Roeder K, Daly MJ, Buxbaum JD (2020) Large-scale exome sequencing study implicates both developmental and functional changes in the neurobiology of autism. *Cell* 180:568–584 e523

- Saunderson EA, Spiers H, Mifsud KR, Gutierrez-Mecinas M, Trollope AF, Shaikh A, Mill J, Reul JM (2016) Stress-induced gene expression and behavior are controlled by DNA methylation and methyl donor availability in the dentate gyrus. *Proc Natl Acad Sci U S A* 113:4830–4835
- Scharff C, Haesler S (2005) An evolutionary perspective on FoxP2: strictly for the birds? *Curr Opin Neurobiol* 15:694–703
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671–675
- Schulz SB, Haesler S, Scharff C, Rochefort C (2010) Knockdown of FoxP2 alters spine density in Area X of the zebra finch. *Genes Brain Behav* 9:732–740
- Sragovich S, Ziv Y, Vaisvaser S, Shomron N, Hendler T, Gozes I (2019) The autism-mutated ADNP plays a key role in stress response. *Transl Psychiatry* 9:235
- Takahashi K, Liu FC, Hirokawa K, Takahashi H (2003) Expression of Foxp2, a gene involved in speech and language, in the developing and adult striatum. *J Neurosci Res* 73:61–72
- 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* 24:3152–3163
- Tian X, Richard A, El-Saadi MW, Bhandari A, Latimer B, Van Savage I, Holmes K, Klein RL, Dwyer D, Goeders NE, Yang XW, Lu XH (2019) Dosage sensitivity intolerance of VIPR2 microduplication is disease causative to manifest schizophrenia-like phenotypes in a novel BAC transgenic mouse model. *Mol Psychiatry* 24:1884–1901
- Timmermans S, Lefebvre L, Boire D, Basu P (2000) Relative size of the hyperstriatum ventrale is the best predictor of feeding innovation rate in birds. *Brain Behav Evol* 56:196–203
- Tung J, Gilad Y (2013) Social environmental effects on gene regulation. *Cell Mol Life Sci* 70:4323–4339
- 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
- Van Dijk 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, BBA d V, Kury S, Rosenfeld JA, Meuwissen ME, Vandeweyer G, Kooy RF (2019) Clinical presentation of a complex neurodevelopmental disorder caused by mutations in ADNP. *Biol Psychiatry* 85:287–297
- Vernes SC, Oliver PL, Spiteri E, Lockstone HE, Puliyadi R, Taylor JM, Ho J, Mombereau C, Brewer A, Lowy E, Nicod J, Groszer M, Baban D, Sahgal N, Cazier JB, Ragoussis J, Davies KE, Geschwind DH, Fisher SE (2011) Foxp2 regulates gene networks implicated in neurite outgrowth in the developing brain. *PLoS Genet* 7:e1002145
- Vistorovsky Y, Heiblum R, Smorodinsky NI, Barnea A (2016) Active immunization against vasoactive intestinal polypeptide decreases neuronal recruitment and inhibits reproduction in zebra finches. *J Comp Neurol* 524:2516–2528
- 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* 323:438–449
- Wohlgemuth S, Adam I, Scharff C (2014) FoxP2 in songbirds. *Curr Opin Neurobiol* 28:86–93
- Zamostiano R, Pinhasov A, Gelber E, Steingart RA, Seroussi E, Giladi E, Bassan M, Wollman Y, Eyre HJ, Mulley JC, Brenneman DE, Gozes I (2001) Cloning and characterization of the human activity-dependent neuroprotective protein. *J Biol Chem* 276:708–714
- Zann RA (1996) The zebra finch: a synthesis of laboratory and field studies. Oxford University Press, USA
- Ziv Y, Rahamim N, Lezmy N, Even-Chen O, Shaham O, Malishkevich A, Giladi E, Elkon R, Gozes I, Barak S (2019) Activity-dependent neuroprotective protein (ADNP) is an alcohol-responsive gene and negative regulator of alcohol consumption in female mice. *Neuropsychopharmacology* 44:415–424
- Zusev M, Gozes I (2004) Differential regulation of activity-dependent neuroprotective protein in rat astrocytes by VIP and PACAP. *Regul Pept* 123:33–41

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.