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Cerebral Dopamine Neurotrophic Factor regulates multiple neuronal subtypes and behavior

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Abstract

Cerebral Dopamine Neurotrophic Factor (CDNF) protects dopaminergic neurons against toxic damage in the rodent brain and is in clinical trials to treat Parkinson's disease patients. Yet the underlying mechanism is poorly understood. To examine its significance for neural circuits and behavior, we examined the development of neurotransmitter systems from larval to male adult mutant zebrafish lacking *cdnf*. Although a lack of *cdnf* did not affect overall brain dopamine levels, dopaminergic neuronal clusters showed significant abnormalities. The number of histamine neurons that surround the dopaminergic neurons was significantly reduced. Expression of *tyrosine hydroxylase 2* in the brain was elevated in *cdnf* mutants throughout their lifespan. There were abnormally few GABA neurons in the hypothalamus in the mutant larvae, and expression of glutamate decarboxylase was reduced throughout the brain. *cdnf* mutant adults showed a range of behavioral phenotypes, including increased sensitivity to pentylenetetrazole-induced seizures. Shoaling behavior of mutant adults was abnormal, and they did not display social attraction to conspecifics. CDNF plays a profound role in shaping the neurotransmitter circuit structure, seizure susceptibility, and complex behaviors in zebrafish. These findings are informative for dissecting the diverse functions of this poorly understood factor in human conditions related to Parkinson's disease and complex behaviors.

Significance Statement

A zebrafish lacking cdnf grows normally and shows no overt morphologic phenotype throughout the life span. Remarkably, impaired social cohesion and increased seizure susceptibility was found in adult *cdnf* KO fish conceivably associated with significant changes of dopaminergic, GABAergic and histaminergic systems in selective brain areas. These findings suggest that cdnf has broad effects on regulating neurogenesis and

maturation of transmitter-specific neuronal types during development and throughout adulthood, rather than ones restricted to the dopaminergic systems.

Introduction

Neurotrophic factors (NTFs), such as neurotrophins, glial cell line-derived neurotrophic factor family of ligands, and neurokines are crucial regulators of neurogenesis and regeneration. These secretory proteins and their signaling receptors are responsible for the survival, maintenance, and synaptic plasticity of nervous systems (Chao, 2003). Several neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) are associated with dysregulation of trophic factors (Chao et al., 2006; Mitre et al., 2017).

An unconventional NTF family, which has a distinct two-domain protein structure and trophic effects on dopaminergic neurons, has recently been identified (Parkash et al., 2009; Latge et al., 2015). This evolutionarily conserved NTF family contains two proteins – mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF)(Lindahl et al., 2017). Their protein structure contains two main functional motifs. One is the N-terminus, which is similar to the saposin-like domain that has the capacity of lipid/cell membrane binding. The other is the C-terminus, composed of the unfolded Cys-X-X-Cys (CXXC) motif, the SAP domain of Ku70, and a putative endoplasmic reticulum (ER) retention signal (KDEL/RTDL) at the end of the C-terminal, which may protect cells from ER stress-induced apoptosis (Parkash et al., 2009; Latge et al., 2013; Latge et al., 2015). Both CDNF and MANF are detectable mainly in neurons in adult mouse and human brain, whereas the expression level of CDNF is generally lower than that of MANF (Lindholm et al., 2007; Lindahl et al., 2017). CDNF and MANF both protect

dopaminergic neurons against oxidative stress, neurotoxins, cerebral ischemia, and neuroinflammation-induced neuronal death (Liu et al., 2015; Lindahl et al., 2017; Sousa-Victor et al., 2018). As such, CDNF has become recognized as a promising candidate for clinical treatment of PD due to its potent neuroprotective and neurorestorative effects on midbrain dopamine neurons (Lindholm et al., 2016; Lindahl et al., 2017; Nasrolahi et al., 2018).

CDNF protects cultured mesencephalic neurons against alpha-synuclein-oligomer-induced toxicity (Latge et al., 2015). In 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonian animal models, the application of CDNF and MANF protects and rescues midbrain dopamine neurons (Lindholm et al., 2007; Voutilainen et al., 2011; Airavaara et al., 2012; Ren et al., 2013). In addition to its neuroprotective effects in PD animal models, CDNF improves long-term memory in an APP/PS1 mouse model of AD (Kemppainen et al., 2015), and reduces Aß25-35-induced ER stress and synaptotoxicity in cultured hippocampus neurons (Zhou et al., 2016). Growing evidence suggests that MANF and CDNF are ER stress response proteins involved in the unfolded protein response (UPR) through interactions with glucose-regulated protein 78 (BiP/GRP78) (Arancibia et al., 2018; Yan et al., 2019). Nevertheless, how CDNF responds to ER stress and its functions under healthy conditions remain largely unknown (Lindahl et al., 2017).

The role of CDNF in the development of dopaminergic or other neurons has not been addressed. The role and mechanisms of action of CDNF have remained elusive. To examine the biological function of cdnf and its role in neural systems and behavior, we first generated zebrafish null mutants by CRISPR/Cas9 genome editing. We investigated the role of cdnf on major neurotransmitter systems in the CNS, including dopaminergic, histaminergic, serotonergic, and GABAergic circuits using qPCR, *in situ* hybridization,

immunohistochemistry, and HPLC analysis, as well as locomotor behavioral analysis, from development throughout their life span. We found that adult *cdnf* mutant zebrafish show abnormal social behaviors and seizure susceptibility phenotypes that are conceivably associated with the multiple impairments of major neurotransmitter networks and deficient neural progenitors during embryonic neurogenesis. These results may provide new evidence that CDNF has a considerable impact on neurogenesis and formation of normal neurotransmitter systems in CNS, which may have important implications for human neurological disorders.

Materials and Methods

Zebrafish Maintenance

Zebrafish were obtained from our wild-type (Turku) line that has been maintained in the laboratory for more than a decade (Kaslin and Panula, 2001; Sundvik et al., 2011; Chen et al., 2016). Larvae were raised on 14:10 (light:dark, lights on at 8 a.m.) cycles at 28°C and fed daily once with flake food and two times with live artemia. Adult fish were raised in continuously cycling Aquatic Habitats™ systems (Apopka, FL, USA) with complete exchange of water in each tank every 6-10 min. Circulating water was UV sterilized, filtered with foam filters and activated charcoal. Water quality, including temperature (28±0.5°C), pH value (7.4±0.2) and conductivity (450±10µS) was monitored continuously. Embryos were obtained by natural spawning, collected from the breeding tanks and staged in hours post-fertilization (hpf), days post-fertilization (dpf), or months post-fertilization (mpf) as previously described (Kimmel et al., 1995). The permits for all experiments were obtained from the Office of the Regional Government of Southern Finland, in agreement with the ethical guidelines of the European convention.

RNA isolation and cDNA synthesis

Total RNA was extracted from ten pooled larval fish or one dissected adult brain per sample (RNeasy mini Kit; Qiagen, Valencia, CA, USA) for quantitative RT-PCR (qRT-PCR). To synthesize cDNA, 2µg of total RNA was reverse-transcribed using SuperScriptTM III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random hexamer primers (Roche Diagnostics, Germany) according to the manufacturer's instructions.

In Situ Hybridization

Whole-mount *in situ* hybridization (WISH) was performed on 4% paraformaldehyde (PFA)-fixed embryos and dissected brains as described earlier (Chen et al., 2009). Antisense and sense digoxigenin (DIG)-labelled RNA probes were generated using the DIG RNA labelling kit (Roche Diagnostics, Germany) following the manufacturer's instructions. The WISH procedure was followed according to the protocol described by Thisse & Thisse (Thisse and Thisse, 2008), with slight modifications. Briefly, the prehybridization and hybridization steps were conducted at 60°C for all riboprobes. The specificity of the anti-sense riboprobe *hdc* and *th2* have been described earlier (Chen et al., 2009; Chen et al., 2016). The cloning primers for the open-reading frames of *cdnf* and *vGAT* cDNA are listed in Table 1. *In situ* hybridization signals were detected with sheep anti-digoxigenin-AP Fab fragments (1:10,000; Roche Diagnostics, Germany). The color staining was carried out with chromogen substrates (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate) and incubated in the dark at room temperature. Stained samples were embedded in 80% glycerol and visualized under brightfield optics using a Leica DM IRB inverted microscope with a DFC 480 charge-coupled device camera. Z-stacks were processed with Leica Application Suit software, with the multifocal algorithm to identify the gene expression patterns (Chen et al., 2012).

Quantitative real-time PCR (qPCR)

qPCR was performed with a LightCycler® 480 instrument (Roche, Mannheim, Germany) using the Lightcycler®480 SYBR Green I master mix (Roche, Mannheim, Germany) according to the manufacturer's instructions. Primers for amplification were designed by Primer-BLAST (NCBI) and are listed in Table 1. Two housekeeping genes, β-*actin* and *ribosomal protein L13a* (*rpl13a*) were used as reference controls. All primer sets were confirmed to amplify only a single product of the correct size. Cycling parameters were as follows: 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. Fluorescence changes were monitored with SYBR Green after every cycle. Dissociation curve analysis was performed at the end of the cycles (0.1°C per s increase from 60°C to 95°C with continuous fluorescence

readings) to ensure that only a single amplicon (single melting peak) was obtained. All reactions were performed in duplicates, and at least three individual biological replicates were used (sample numbers indicated in figure legends). Duplicate quantification values were analyzed with the LightCycler 480 software. The data were calculated by the comparative method, using Ct values of β-*actin* and *rpl13a* as a reference control (Livak and Schmittgen, 2001). Since the changes of relative gene expression showed the same trend when normalized to the different housekeeping genes (data not shown), only the results from *rpl13a* are presented.

Establishing zebrafish mutants

CRISPR/Cas9-genome edited fish were generated in our Turku wild-type strain, based on the description of Hwang et al.(Hwang et al., 2013). To avoid off-target genomic mutagenesis effects, targeting sites were selected with a minimum of three mismatches in the genome as predicted by the CHOPCHOP software (http://chopchop.cbu.uib.no). The sequence-specific sgRNA template was generated in a pDR274 vector (Addgene Plasmid #42250; oligo sequence listed in Table 1). The sequences of the modified plasmids were verified by Sanger sequencing. sgRNAs were transcribed from linearized template plasmids (Ambion MEGAscritp), and Cas9 mRNA was transcribed *in vitro* from linearized template plasmid pMLM 3613 (Addgene Plasmid #42251). A mixture containing approximately 300ng/μl Cas9 mRNA and 20ng/μl sgRNA was injected into fertilized eggs at the one-cell stage. To verify the mutation efficiency of Cas9-sgRNA genome editing, the injected eggs were collected at 24 hpf. DNA was extracted from individual embryos and non-injected controls. PCR amplicons encompassing the targeted sites were amplified and analyzed via Sanger sequencing and high-resolution melting (HRM) analysis (Roche, Mannheim, Germany). Mutations were recognized as multiple sequencing peaks at the sgRNA target site. When the mutation efficiency was over 50%, the remaining Cas9/sgRNA-injected embryos were raised to adulthood and out-crossed to the wild-type fish to generate F1 progeny. To collect the tail biopsies, 3-dpf embryos were anesthetized with

0.02% Tricaine. The tip of the caudal fin within the pigment gap was removed using a microscalpel, and each larva was placed in an individual well of a 24-well plate with fresh embryonic medium until 5 dpf. We have consistently achieved 100% survival rate from the beginning of this procedure to adulthood. F1 genotyping was done by HRM assays and DNA sequencing. To identify mutated alleles from single embryos, each target locus was PCR amplified from individual genomic DNA with gene-specific primers (Table 1). PCR products were then cloned and sequenced. Mutated alleles were identified by comparison with the wild-type sequence. Heterozygous (HET) F1 siblings carrying the same mutations were pooled in one tank and raised to adulthood. Due to the sex imbalance in the F1 generation of *cdnf* HET fish, F1 male HET fish were outcrossed to Turku female wild-type fish; female mutants were obtained from the resulting F2 progeny. Genotyping of the F2s was done as described for the F1 generation.

Fin clipping and genomic DNA extraction of adult zebrafish and 3 dpf larvae

To lyse genomic DNA, individual tail clippings were incubated in 50µl lysis buffer (10mM Tris-HCl pH8.3, 50mM KCl, 0.3% Tween-20 and 0.3% NP-40) at 98°C for 10 min, followed by incubation on ice for 2 min. 1µl of Proteinase K (20mg/ml) was added to remove protein, and the mixture was incubated at 55°C for at least 4 h. To inactivate Proteinase K activity, samples were incubated at 98°C for 10 min and quenched on ice. To detect indel mutations, HRM curve acquisition and analysis was performed. Primers flanking the mutation site were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast; sequences are listed in Table 1). The HRM analysis was performed on a LightCycler® 480 instrument (Roche) using the following reaction mixtures: 1× LightCycler 480 HRM master mix (Roche, Mannheim, Germany), 2mM $MgCl_2$, and 0.15µM primer mixtures. The PCR cycling protocol was as follows: one cycle of 95°C for 10 min; 45 cycles of 95°C for 10 s, 60°C for 15 s, 72°C for 20 s, and melting curve acquisition; one cycle of 95°C for 60 s, and 40°C for 60 s. PCR products were denatured at 95°C for 60 s, renatured at 40°C for 60 s, and melted at 60°C to 95°C with 25 signal acquisitions per degree. Melting curves were generated

over a 65–95°C range. Curves were analyzed using the LightCycler® 480 gene-scanning software (version 1.5) according to the manufacturer's instructions (Roche Diagnostics Ltd., Switzerland). To identify deviations of the curves indicative of sequence mutations, a three-step analysis was performed using the Gene Scanning program (Roche) as follows: (1) Normalizing the raw melting-curve data by setting the initial fluorescence uniformly to a relative value of 100% and the final fluorescence to a relative value of 0%. (2) Determining the temperature threshold at which the entire double-stranded DNA was completely denatured. (3) Further analyzing the differences in melting-curve shapes (threshold setup 0) in order to cluster the melting curves with similar shapes into the same groups. Those with analogous melting curves were characterized as the same genotype.

Analysis of catecholamines and histamine by high performance liquid chromatography (HPLC)

For each sample, ten 8-dpf larvae were pooled into a group. The dissected brains of 8-mpf or 18- males were flash-frozen in liquid nitrogen and individually homogenized with sonication in 150μl of 2% perchloric acid. After centrifugation, 10μl of supernatant was assessed for monoamine concentration measurement. The detection details are described in Sallinen et al.(Sallinen et al., 2009). The results were normalized to the total protein concentration of each sample, which was measured using the Pierce© BCA Protein Assay Kit. The HPLC analysis was carried out as a blinded experiment.

Immunocytochemistry

Immunostaining was performed on zebrafish fixed in 2% PFA or 4% 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide (EDAC, Carbosynth, Berkshire, UK). For larvae older than 5 dpf, fixed brains were dissected to enhance antigen presentation and improve image quality. Antibody incubations were carried out with 4% normal goat serum and 1% dimethyl sulfoxide (DMSO) in 0.3% Triton X-100/ phosphate buffered saline

(PBS) for 16 h at 4°C with gentle agitation. Primary antibodies were mouse monoclonal anti-tubulin, acetylated antibody (1:1000; T6793, Sigma, St. Louis, MO, USA), rabbit anti-histamine 19C (1:5,000; (Panula et al., 1990; Sundvik et al., 2011), rabbit anti-TH2 antibody (1:2000; (Semenova et al., 2014), rabbit anti-serotonin antibody (1:1000; S5545, Sigma, St. Louis, MO, USA), anti-tyrosine hydroxylase (TH1) monoclonal mouse antibody (1:1000; Product No 22941, Immunostar, Husdon, WI, USA), anti-GABA 1H (1:1000; (Karhunen et al., 1993; Kukko-Lukjanov and Panula, 2003), rabbit anti-orexin A (1:1000; Millipore/Chemicon, Billerica, MA, USA) and mouse anti-zrf1 (Gfap; 1:1000, Zebrafish International Resource Center). The specificities of the anti-GABA (Karhunen et al. 1993) and anti-histamine, commercial anti- mouse monoclonal TH, anti-rabbit-TH2 and anti-serotonin antibodies have been verified previously (Kaslin and Panula, 2001). The following secondary antibodies were applied: Alexa Fluor® 488 and 568 anti-mouse or anti-rabbit IgG (1:1000; Invitrogen, Eugene, OR, USA).

Immunocytochemistry following EdU proliferation labeling

To detect the proliferating S-phase dividing cells, the Click-iT™EdU Alexa Fluor 488 imaging kit (Molecular Probes) was used following the manufacturer's instructions, with minor modifications. Briefly, 5-dpf larvae were incubated in 0.5mM EdU/E3 buffer (zebrafish embryonic medium; 5 mM NaCl, 0.44 mM $CaCl_2$, 0.33 mM $MgSO_4$, and 0.17 mM KCl) with 1% DMSO for 24 h at 28°C. Labelled samples were transferred back to E3 medium for 30 min and fixed in 4% EDAC/PB buffer pH 7.0 overnight at 4°C with gentle agitation. The skin and lower jaw of the fixed specimens were removed in order to enhance sample penetration. Dissected brains were incubated with rabbit anti-Histamine 19C antibody (1:5000) and mouse anti-HuC antibody (1:1000). The secondary antibody Alexa Fluor®568 anti-rabbit IgG and Alexa Fluor®633 anti-mouse IgG (1:1000; Invitrogen, Eugene, OR, USA) were applied. After immunostaining, labelled specimens were fixed in 4% PFA/PB for 20 min at room temperature, and then incubated in 1× Click-iT EdU cocktail with the green-fluorescent Alexa Fluor® 488 azide dye for one hour in the dark at room temperature. After removing

the reaction cocktail and rinsing in 1×PBST (phosphate-buffered saline and 0.25% Triton X-100) three times for 10 min, samples were mounted in 80% glycerol/PBS for confocal microscopy imaging.

Imaging

Brightfield images were taken with a Leica DM IRB inverted microscope with a DFC 480 charge-coupled device camera. Z-stacks were processed with Leica Application Suite software and Corel DRAW 2018 software (Chen et al., 2009). Immunofluorescence samples were examined using a Leica TCS SP2 AOBS confocal microscope. For excitation, an Argon laser (488 nm), green diode laser (561 nm), and red HeNe laser (633 nm) were used. Emission was detected at 500–550 nm, 560–620 nm, and 630–680 nm, respectively. Cross-talk between the channels and background noise were eliminated with sequential scanning and frame averaging as previously described (Sallinen et al., 2009). Stacks of images taken at 0.2–1.0 µm intervals were compiled, and the maximum intensity projection algorithm was used to produce final images with Leica Confocal software and Imaris imaging software (version 6.0; Bitplane AG, Zurich, Switzerland). Cell numbers were counted in each 1.0 µm optical slice using ImageJ 1.52b software (National Institutes of Health, Bethesda, USA). Fluorescence intensity was quantified in each 1.0 µm optical slice across entire z-slices with a standardized-selected region of interest (ROI) under the same parameters using ImageJ 1.52b software. All cell counts and fluorescence intensity measurements were performed by an investigator blinded to the sample type.

Estimation of the posterior swimbladder volume (V3)

The volume of the posterior chamber V3 region (geometrical figure like frustum of a con) was estimated according to the equation ($V = (1/3) * \pi * h * (r1^2 + r2^2 + (r1 * r2))$) as described in detail in Lindsey et al. (Lindsey et al., 2010). Measurements were done on 6-month-old male zebrafish (n=4 of both genotypes)

using Fiji (version 2.0) and were calculated using the lateral-aspect images of the 4% PFA-fixed posterior chamber.

Estimation of the vascular length density of the posterior swimbladder

The vascular length density was estimated using the vessel analysis plugin with Fiji (version 2.0) based on the user manual. The calculation of the vascular length density was presented as the ratio of skeletonized vasculature area to the total area (% Area)(Elfarnawany, 2015). Measurements were done on the lateral-aspect images of the 4% PFA-fixed posterior chamber of 6-month-old male zebrafish (n=4 of both genotypes).

Dark-light flash and sleep behavior test for larval zebrafish

Behavioral trials were done between 11:00 and 16:00. For larval locomotion tracking, 6-dpf zebrafish larvae were individually placed in a 24-well culture dish well containing approximately 1.5mL of E3 medium. The light level was set to approximately 330 lux based on the setting of Puttonen et al.(Puttonen et al., 2018). Before each trial, the larvae were habituated in the observation chamber for 10 min, followed by a 30 min locomotion tracking period with the lights on. A dark-light flash response was induced by switching off the lights for 2 min, then turning them back on for 2 min. One experiment consisted of three subsequent periods of white lights on and white lights off. Locomotor activity was monitored for one day with continuous illumination by infrared lights while white light remained on from 12:00 to 22:00 on the first day and from 8:00 to 12:00 on the next day. Locomotion response was monitored at room temperature using the Daniovision system (Noldus, Wageningen, The Netherlands). Video tracking was analyzed by EthoVision XT 8.5 locomotion tracking software (Noldus, Wageningen, The Netherlands).

Social interaction assay

The visually mediated social preference test was based on the setup of Baronio et al.(Baronio et al., 2018). Briefly, an acrylic apparatus (29 cm length × 19 cm height × 29 cm width) was divided into three arenas by two acrylic partitions. A rectangular compartment in the middle was the testing arena, referred to as the "distal" zone; to one side, the conspecific compartment housed a group of six fish, referred to as the "stimulus" zone; the other adjacent compartment was filled with stones and plant mockups, referred to as the "object" zone. A single 6-mpf male adult was placed in the testing arena to allow exploration and analysis of place preference. All experimental fish were raised in a social environment. All behavioral tests were performed between 11:00 and 16:00, and video-recorded from above the tank for 6 min. To quantify social preference, the videos were analyzed with the EthoVision XT 8.5 locomotion tracking software (Noldus, Wageningen, The Netherlands), and the amount of time each test fish spent in the proximity of each compartment was quantified.

Novel diving tank assay

The novel tank assay was performed based on Cachat et al.(Cachat et al., 2010). One day before the experiment, 6-mpf male adult fish with home tanks (19 cm x 34 cm x 21 cm) were habituated in the behavior testing room. In each trial, one fish was placed in a transparent tank (24 cm × 14.5 cm × 5 cm) with 1 L of fish system water. All behavioral tests were performed between 11:00 and 16:00, and video-recorded from the side of the tank for 6 min, using a Basler acA1300-60gm industrial CCD video camera. We performed a three-compartment novel tank test, with digitized divisions between top, bottom, and middle virtual zones. The time spent in each zone was quantified using EthoVision XT 8.5 software. Fish were returned to their home tanks immediately after the test.

Shoaling assay

Five 6-mpf or five 18-mpf male fish per cohort were placed in a round white polyethlene plastic flat-bottomed container (23 cm height, 23 cm diameter) with 2 L of fish system water (5.0 cm depth) based on the description of Green et al.(Green et al., 2012). Prior to testing, fish were habituated for 15 min followed by video recording for 10 min with a camera at a fixed height (60 cm) from the top of the container. All videos were analyzed with EthoVision XT 8.5 software, using the default setting (the center-point detection of the unmarked animals). The mean of the inter-fish distance (defined as distance between the body center of every member of the shoal) (Green et al., 2012) was quantified from the average data from all trials (n=4 trials for the 6-mpf fish, and n=3 trials for the 18-mpf fish). The proximity duration (in s) was defined as the average duration of time a fish stayed close to the shoal fish (i.e. within 2 cm for the 6-mpf fish or 2.5 cm for the 18-mpf fish). The misdetection rate of the video-tracking software was less than 1%. All behavioral trials were done between 11:00 and 16:00.

Seizures induced by pentylenetetrazole (PTZ) in adult zebrafish

Epileptic seizure stage scores (seizure behavior scores from 0 to 6) were assigned according to Mussulini et al.(Mussulini et al., 2013). 6-mpf male fish were individually exposed to 10 mM PTZ in a 1 L tank (24 cm length × 5 cm width × 14.5 cm height) for 5 min on three consecutive days, in order to induce experimental seizures based on the description of Duy et al.(Duy et al., 2017). After PTZ administration and seizure analysis, treated fish were transferred to a clean tank for one day until they recovered (i.e. seizure score = 0). They were then sacrificed by decapitation after cold-shock, and brains were dissected for RNA extraction. The PTZ concentration and the exposure period were selected and optimized based on our pilot study, which aimed to determine the shortest time of PTZ exposure that induces a seizure of score V

(including fish falling to the bottom of the tank and loss of the body posture for 1–2 s), but allowing full recovery after three daily exposures (Duy et al., 2017). Control fish were subjected to the same procedure but exposed to only clean system water.

Experimental design and Statistical analysis

The number of zebrafish for all experiments is indicated in the figure legends. Data analysis was performed by GraphPad Prism software (version 7; San Diego, CA, USA). Two-tailed p-values were generated by one-way analysis of variance (ANOVA) for multiple comparisons using Tukey's multiple comparison test, two-way ANOVA for multiple comparisons, and Student's unpaired *t*-test for comparison of two groups. Data were presented as mean ± SEM. Statistical significance was considered at p-value <0.05.

Results

Zebrafish cdnf 3D structure and mRNA distribution during embryogenesis and in adult tissues

Currently there is one human homologous *cdnf* (NM_001123281.1) documented in the latest zebrafish database (GRCz11). The zebrafish *cdnf* gene is located on chromosome 4 and contains four exons with the exon-intron splice sites conserved in mammalian CDNF and MANF. The open reading frame encodes a protein of 182 amino acid residues with a 25 amino-acid signal peptide and shares 67.4% and 65.1% of amino acid sequence similarity with zebrafish manf (NP_001070097) and human CDNF (NP_001025125), respectively. The predicted secondary structure analyzed by the protein homology/analogy recognition engine Phyre2 (Kelley et al., 2015) indicated that zebrafish cdnf contained seven α-helices (Figure 1A) and eight positioned-conserved cysteine residues. The N-terminus domain is highly similar to human saposin D, and the C-terminus is analogous to the SAP domain (Figure 1B), suggesting that zebrafish cdnf is structurally conserved with human CDNF (Latge et al., 2013). To determine the spatiotemporal expression of *cdnf*, WISH and qPCR was performed in embryos and adult tissues. The *cdnf* mRNA was widely expressed at 1 dpf in the brain, eyes, and muscles. In 2- and 3-dpf larvae, *cdnf* transcripts were detected in the midbrain-hindbrain boundary, hindbrain, otic vesicles, and heart (Figure 1C). qPCR detected *cdnf* transcripts as early as 2 hpf (Figure 1D), and expression gradually increased after 1 dpf. In adult organs, the highest expression level was found in the eyes compared with the brain, kidney, and liver; no sex differences were detected (Figure 1E).

Characterization of the CRISPR/Cas9-generated zebrafish *cdnf* mutant allele

To study the biological function of *cdnf*, we generated mutations using the CRISPR/Cas9 system. A sequence-specific guide RNA was designed to target exon2 of *cdnf* (Figure 2A). We identified two reading frame-shift mutant alleles: one with a 14 base-pair deletion (Figure 2A and 2B) and another with a 1 base-

pair insertion (data not shown). Both lesions are located in exon2, causing premature termination of the protein after amino acid 49 (NP_001116753.1, Figure 2B). We also cloned and sequenced the full-length reading frame in *cdnf* transcripts isolated from the mutant brain. The deletion sequence was the same as the genomic deletion sequence. In this study, the *cdnf* 14 base-pair deletion mutant allele was used for subsequent experiments. Heterozygous *cdnf* mutants (F3 and later generations) were mated to generate wild-type (WT), heterozygous (HET), and homozygous mutant embryos. The ratio of genotyped sibling offspring matched the normal Mendelian ratio (1:2:1). All progenies were tail clipped at 3 dpf and genotyped using HRM analysis, according to the distinct melting curves of each genotype. The embryos with the same HRM curve were grouped together before 5 dpf (Figure 2C). *In situ* hybridization results revealed that *cdnf* expression was mostly abolished in the caudal raphe, ventral part of the posterior tuberculum, tectum opticum and ventricular recess of the hypothalamus of *cdnf* mutant brains (Figure 2D, 2E). The faint signal in the *cdnf* mutant brain may be due to the remaining truncated mRNA. The *cdnf* mRNA quantification is shown in Figure 3A-3C. *cdnf* homozygous mutants were viable, swam normally, were fertile, and had no gross morphological phenotype (Figure 2F).

Upregulation of *tyrosine hydroxylase 2* (*th2*) expression in *cdnf* mutant fish

We addressed the question whether *cdnf* is required for development or maintenance of the dopaminergic neurons in the zebrafish brain. qPCR analysis was performed on 8-dpf larvae, 8-mpf brains, and 18-mpf brains to examine transcript levels of relevant marker genes of the dopaminergic and histaminergic systems (known to depend on dopaminergic system). *manf* was also analyzed to reveal if this closely related growth factor is upregulated as a consequence of genetic compensation in the *cdnf* mutant fish. In zebrafish, gene duplication has led to two non-allelic forms of human orthologous *tyrosine hydroxylase* (*th*) that are expressed in the brain in a largely complementary manner (Chen et al., 2009). We thus analyzed the mRNA levels of both tyrosine hydroxylases, *th1* and *th2*. Remarkably, a significant increase of *th2* was observed in

cdnf mutants compared with their WT siblings (Figure 3D-3F), whereas the expression level of *th1* mRNA showed no statistically significant difference (Figure 3G-3I). Additionally, a significant downregulation of *histidine decarboxylase* (*hdc*, a histaminergic marker) transcript was observed in 8-mpf mutant brains (Figure 3J-3L). Expression of *hdc* has been shown to be regulated by dopamine produced by *th2* (Chen et al., 2016). We also confirmed that a significant reduction of *cdnf* transcript remained in *cdnf* mutants throughout their lifespan (Figure 3A-3C), which agreed with the *in situ* hybridization results (Figure 2D, 2E) that most of the truncated *cdnf* mRNA went through nonsense-mediated mRNA decay pathway in *cdnf*-deficient mutant fish. The mRNA level of *manf*, a closely related trophic factor, was not significantly altered (data not shown). The qPCR results revealed that *cdnf* has an impact on the regulation of dopaminergic and histaminergic gene expression.

A dynamic change of dopaminergic neuron numbers in *cdnf* mutant fish

It is evident that dopaminergic signaling regulates the developing hypothalamic neurotransmitter identity (Chen et al., 2016). Due to the prominent upregulation of *th2* transcripts in *cdnf* mutant fish, we then quantified the number of cells of the dopaminergic populations in the prethalamus and caudal hypothalamus by immunohistochemistry to assess whether the TH1- and TH2-containing cell numbers were altered. 8-dpf dissected brains were co-stained with two antibodies that recognize dopaminergic neurons; one recognizes both TH1 and TH2 (Semenova et al., 2014), and one recognizes only TH1 in zebrafish. A significant increase in TH1- and TH2-positive cell numbers was observed in TH1/TH2 group 10/10b in the caudal hypothalamus area (Hc) of knock-out (KO) mutants compared with their WT siblings (Figure 4A-4E). However, the number of TH1-positive cells was unaffected in this region (Figure 4F), suggesting that the increase in TH1- and TH2-positive cell numbers in the Hc area of *cdnf* mutant brains is due to the increase in TH2-containing neurons, consistent with the qPCR results (Figure 3D-3F) and the *in situ* hybridization result (Figure 4J). Nevertheless, a significant decrease in TH1-positive cell number was

found in the prethalamus region (TH1 group 5,6,11) in *cdnf* mutants (Figure 4G). On the other hand, the serotonergic population in this location remained intact in KO mutants (Figure 4H and 4I). A dynamic change in dopaminergic neuron populations found in *cdnf* mutant fish suggests that *cdnf* may function distinctly on dopaminergic populations in different brain areas.

Decreased number of proliferating cells and histaminergic neurons in *cdnf* mutant fish

There are no reports about *cdnf* effects on embryonic neurogenesis. We previously reported that the dopaminergic populations in the caudal hypothalamus play a role in the regulation of histaminergic neuron development in zebrafish (Chen et al., 2016). To investigate whether the loss of functional *cdnf* affects neurogenesis and if the upregulated TH2 population in this area disturbs the development of histaminergic neurons in larval *cdnf* mutants, we studied 8-dpf dissected brains co-stained with antibodies recognizing 1) histamine (a histamine neuron marker), and 2) HuC (a panneuronal marker) following EdU staining (a proliferation marker) (Figure 5A and 5B). A significant reduction of EdU-positive cells (Figure 5C) as well as lower fluorescence intensity of HuC immunoreactivity (Figure 5D) was found in the caudal hypothalamus of *cdnf* mutant fish brains. Moreover, *sox2a* (neural progenitor marker) mRNA expression level was significantly downregulated in the *cdnf* mutant larvae (Figure 5H). Taken together, these findings suggest that lack of *cdnf* significantly affects embryonic neurogenesis in the caudal hypothalamus. Similarly, a significant decrease of histaminergic neurons was found in *cdnf* mutants (Figure 5E). Consistently, *in situ* hybridization of *hdc* (an enzyme converting L-histidine to histamine) showed a significant decrease in *hdc*-positive cell numbers in *cdnf* KO fish (Figure 5F). A significant decrease in the number of orexin-positive cells also found in *cdnf* KO fish (Figure 5G). This result was in agreement with reports that the histaminergic system regulates orexin neuron development in zebrafish (Sundvik et al., 2011).

Neurotransmitter profile by HPLC analysis

Due to the alterations of dopaminergic and histaminergic neurons in *cdnf* mutant fish, HPLC analysis was performed to measure the concentration of dopamine, norepinephrine, serotonin, histamine, and their metabolites throughout the zebrafish lifespan. No statistically significant difference was found in brain dopamine, norepinephrine, and serotonin levels, but a decrease in dopamine metabolites, DOPAC, and homovanillic acid and serotonin metabolite 5-hydroxyindoleaetic acid was found in adult *cdnf* mutant fish compared with the WT fish brains. (Table 2). A reduction in histamine level was found in 8-mpf *cdnf* mutants, consistent with a reduced expression of histaminergic marker *hdc* mRNA in 8-mpf *cdnf* KO brains (Figure 3K).

Impairments of GABAergic system

GABA acting through the GABA-A receptor in the adult brain is the major inhibitory neurotransmitter, and GABAergic neurons are widely distributed in the brain to modulate neural activity. It has been reported that MANF can potentiate presynaptic GABAergic inhibition (Zhou et al., 2006). Moreover, a dual dopaminergic and GABAergic phenotype is evident in the hypothalamic areas in zebrafish (Filippi et al., 2014). Dopamine signaling deficiency affects the development of GABAergic neurons in zebrafish (Souza et al., 2011). To assess whether the profound change of dopaminergic systems in *cdnf* mutant fish is associated with abnormalities in the GABAergic system, anti-GABA and anti-acetylated-alpha-tubulin antibodies (axonal alpha- tubulin marker) were used on 8-dpf dissected brains. Significantly fewer GABA-positive cells were observed in the ventral part of the posterior tuberculum (PTv) (Figure 6A-6C) and caudal hypothalamus area (Hc) (Figure 6D) of the mutant fish than in their WT siblings. The axonal tubulin pattern remained intact (Figure 6*A*, 6B and 6E) in *cdnf* mutants, suggesting that the loss of functional *cdnf* causes

specific abnormal neurotransmitter systems in the PTv and Hc areas rather than an overall change in neuronal organization.

GABA is converted from glutamic acid by glutamic acid decarboxylases (GADs: GAD65/gad2a and GAD67/gad1b), and vesicular GABA transporter (vGAT) is responsible for uptake and storage of GABA in the vesicles in the presynaptic terminals. To study whether the decreased number of GABA-containing cells in *cdnf* mutants stems from either dysfunction of GABA synthesis or neurotransmission, we performed qPCR to determine the expression level of GABAergic markers *slc32a1/vGAT*, *gad2a/gad65* and *gad1b/gda67* in 8-dpf larvae. A significant decrease in *slc32a1/vGAT* transcripts was detected in *cdnf* mutant larvae (Figure 7A), and the *in situ* hybridization results of *vGAT* in 8-dpf dissected brains showed lower expression in the ventral thalamus (VT), ventral part of posterior tuberculum (PTv) and hypothalamus (H) areas of *cdnf* KO mutants than in their WT siblings (Figure 7B). However, the expression levels of *gad1b*, *gad2a* and *vGlut2* in whole larvae were not statistically different (Figure 7C-7E). These findings suggest that the decreased number of GABA-positive cells is associated with downregulation of *vGAT* expression in the *cdnf* KO larvae.

Dark-flash and sleep-related behavior

Dopaminergic, histaminergic, and GABAergic circuitries play important roles in the regulation of startle response and sleep-wakefulness behavior (Jones, 2019). *cdnf* mutant larvae did not show impaired light/dark adaptions by the light-dark flash change (data not shown). Therefore, we examined the sleep-like locomotor behavior of 6-dpf larval fish under a 14:10 (light:dark) regime (Figure 8A, n=16 in each group). Interestingly, during the daylight period, the *cdnf* mutant larvae were more active than their WT siblings (Figure 8B) although locomotion during the second daylight period showed no statistically significant differences between groups (Figure 8C). In contrast, during the dark period, the *cdnf* mutant larvae moved

480 significantly less time than their WT siblings (Figure 8D, 8E). The abnormal locomotor activity during light
481 and dark conditions was thus associated with the dysfunctional neurotransmission in *cdnf* mutant larvae.

482

483 **Impaired social preference in adult *cdnf* mutant fish**

484 There is growing evidence to suggest that dysregulated neurotransmission causes neuropsychiatric
485 disorders, some of which alter social interactions (Laruelle, 2014; Kim and Yoon, 2017; Provensi et al.,
486 2018). Zebrafish are social animals and naturally tend to approach conspecifics by visual choice (Miller and
487 Gerlai, 2011). The social preference test conducted here measures this innate tendency. To investigate the
488 consequences of impaired dopaminergic, histaminergic, and GABAergic systems on adult fish behavior, we
489 performed social preference analysis/visually-mediated social preference on 6-mpf male fish by quantifying
490 the amount of time each fish spent in close proximity to conspecifics ("stimulus" arena) compared to the
491 empty "object" arena (Figure 9A). In comparison with their WT siblings, *cdnf* mutant fish spent significantly
492 less time in the stimulus/conspecific zone (Figure 9B and 9C), but spent more time in the "distal" testing
493 zone (Figure 9B, 9E). WT and KO fish spent similar amounts of time in the "object" zone (Figure 9B, 9D),
494 indicating that adult *cdnf* mutant fish show less social preference for conspecifics than the WT fish.

495

496 **Anxiolytic behavior appeared in adult *cdnf* mutant fish**

497 Zebrafish have a natural tendency to spend more time at the bottom of the tank when placed in a novel
498 environment, before gradually migrating to the surface (Kysil et al., 2017). We utilized a novel tank diving
499 assay to study anxiety-related risk-taking behavior. The novel tank diving area was digitally divided into
500 three zones, and representative swimming tracks are shown in Figure 10A. Compared with their WT
501 siblings, *cdnf* mutants spent significantly more time exploring the top zone (Figure 10B, 10C), and less time
502 in the bottom (Figure 10E); there was no difference in the time spent in the middle zone between WT and

KO siblings (Figure 10D). There was no significant difference for movement speed between WT and KO siblings, suggesting that impaired bottom-dwelling behavior was not caused by motor defects (Figure 10F). Moreover, buoyancy is regulated by the swim bladder and its innervation (Finney et al., 2006). We examined the size of the swim bladder (Figure 10G, 10H), vasculature (Figure 10G, 10I) and TH1 innervation (Figure 10G, 10J) of its posterior chamber, and no overt defect was found in the swim bladder of *cdnf* KO fish (n=4 in each group).

Decreased shoal cohesion in adult *cdnf* mutant fish

Zebrafish swim naturally in shoals (Miller and Gerlai, 2011). To test whether *cdnf* deficiency affects fish shoaling behavior, five 6-mpf fish (male young adult group) or five 18-mpf fish (male adult group) per trial were placed in a plastic cylindrical container (23 cm diameter, and monitored by video tracking for 10 min after a 15 min habituation period (n=4 trials for young adults, and n=3 trials for adults). The movement speed, average distance between the test fish and the other four shoal members, and duration of stays in proximity (the nearest inter-individual distance defined as less than 2 cm for young adults and 2.5 cm for adults) were analyzed. *cdnf* mutant fish showed higher swimming speed during 10 min locomotion activity in both age groups (Figure 11A, 11B 11E, 11F). In the young adult group, the inter-individual distance was significantly greater in the *cdnf* mutant group compared with their WT sibling group (Figure 11C). Furthermore, the time spent in proximity with shoal members was significantly shorter in *cdnf* mutant groups than in their WT siblings (Figure 11D). Similar results were obtained in the adult group. Collectively, the *cdnf* mutant fish were more hyperactive and kept at a greater distance to their neighbors (Figure 11G), although no significant difference in time spent in proximity was observed in the adult group (Figure 11H), suggesting that lack of functional *cdnf* causes social defect phenotypes in adult zebrafish.

Increased seizure susceptibility in adult *cdnf* mutant fish

We hypothesized that the impaired GABAergic phenotype found in the *cdnf* mutant fish may render the mutants more susceptible to drug-induced epileptic seizures. Pentylenetetazole (PTZ), a chemoconvulsant drug, is commonly used to induce seizures in rodents and zebrafish by inhibiting GABA-A receptor subunits (Huang et al., 2001; Mussulini et al., 2013). Six-month-old male fish were exposed to 10 mM PTZ (for 5 min periods, over three consecutive days) to allow analysis of the molecular consequences of PTZ-induced seizures (Figure 12A). Seizures were scored based on the description of Mussulini et al.(Mussulini et al., 2013). Briefly, score 3 was recorded when fish showed repetitive circular movements, score 4 included abnormal whole-body rhythmic muscular contractions, and score 5 was characterized by rigidity, loss of body posture, and sinking to the bottom of the tank. None of the tested fish died during the PTZ administration procedure. Throughout the 5 min PTZ administration, a significantly higher percentage of *cdnf* mutant fish reached score 5 compared to their WT siblings (Fig. 12B), revealing that *cdnf* mutant fish are more susceptible to PTZ-induced seizures. Moreover, the *cdnf* mutant fish showed a shorter onset latency to reach score 4 than their WT siblings, particularly on the second and third days of exposure (Figure 12C). The *cdnf* mutant fish demonstrated the longest periods of immobility (Figure 12D), but there were no significant differences between the genotypes in the total distance moved (Figure 12E).

Gene expression in PTZ-treated *cdnf* mutant and WT fish brains

To investigate the molecular alterations in the zebrafish brain caused by PTZ treatment, qPCR was used to quantify the gene expression of *manf* (a closely related growth factor), *glial fibrillary acidic protein* (*gfap*; an astrocyte marker), and GABAergic and glutamatergic markers. We first confirmed that 32% (Figure 13A) of the remaining *cdnf* truncated transcript was detected in untreated *cdnf* mutant fish brains compared with untreated adult *cdnf* WT fish brains, which agrees with the qPCR results on larvae (34%, Figure 3A). The

549 *manf* expression level did not alter significantly in untreated *cdnf* KO fish brains (Figure 13B). A significant
550 downregulation of *slc17a6a/vGlut2*, *slc32a1/vGAT* and *gad2a/gad65* mRNA expression was observed in
551 untreated adult *cdnf* KO fish brains (Figure 13C, 13D and 13E), but not the expression level of *gad1b/gad67*
552 (Figure 13F). PTZ administration did not significantly alter the mRNA expression of *cdnf*, *manf* , GABAergic
553 and glutamatergic markers in either WT or *cdnf* mutant fish (Figure 13A-13F), although there was a
554 tendency towards a higher level of mRNA expression of *manf* and GABAergic markers in PTZ treated *cdnf*
555 KO brains than in untreated *cdnf* KO fish group (Figure 13B-13F). Remarkably, mRNA expression of *gfap*
556 was significantly lower in untreated *cdnf* mutant fish brains than in WT fish brains (Figure 13G). After PTZ
557 treatment, a significant increase in *gfap* expression was seen in *cdnf* mutant fish brains, but not in their WT
558 siblings (Figure 13G). The immunostaining result of zrf-1 labeled *gfap* showed that *cdnf* mutant fish
559 revealed significantly less extensive radial glial fibers in the lateral region of hindbrain than their WT
560 siblings (Figure 13H and 13I).

Discussion

Despite the apparently typical general development and superficially normal behavior, zebrafish lacking CDNF displayed hyperactivity and impairments in anxiety-related behavior, social preference, and shoal cohesion. Reduction of proliferating cells, neural progenitor markers and radial glial cells in *cdnf* mutant larvae brain may cause abnormal neurogenesis consequently leading to brain dysfunction at later stages. Decreased sociability and increased seizure susceptibility were associated with deficiencies in several neurotransmitter systems, including dopaminergic, GABAergic, and histaminergic neurons. Notably, there was no overall difference in whole-brain dopamine levels, but a detailed examination of the two major dopaminergic systems showed significant abnormalities in *cdnf* KO fish**.** A recent study on cdnf-/- mice reports normal levels of brain dopamine and number of nigral dopamine neurons (Lindahl et al., 2020). Our findings lend support to the hypothesis that CDNF acts as a general modulator that regulates neurogenesis and maturation of transmitter-specific neuronal types during development and throughout adulthood, rather than a regulator of only dopaminergic systems. This concept is supported by the expression pattern of *cdnf* mRNA during development and in the mature brain: it is detected in the anteroposterior axis of the ventricular zones where neurogenesis actively happens from embryonal to adult zebrafish brain (Zupanc et al., 2005; Kizil and Brand, 2011; Schmidt et al., 2013).

There is evidence that the unfolded protein response (UPR), which is essential in the mechanisms of MANF and CDNF, is associated with the generation and maturation of CNS neurons and circuits. MANF/Armet is upregulated in various forms of ER stress (Apostolou et al., 2008). ATF6 is a transcription factor activated as one component of the ER stress cascade, and its conditional activation induces MANF/Armet in cardiomyocytes (Tadimalla et al., 2008). In addition to ATF6 activation, the UPR cascade consists of activation of inositol-requiring enzyme 6 (Ire-6) and protein kinase (PKR)-like ER kinase (Perk) and is essential in nervous system development. Additionally, it supports the generation, maturation, and

maintenance of CNS neurons (Godin et al., 2016; Hetz and Saxena, 2017). For example, lack of functional BiP/GRP78 – an essential component of the UPR – disturbs development of thalamocortical connections in mice (Favero et al., 2013). Moreover, downregulation of the UPR alters generation of progenitor cells and cell fate acquisition in the developing cerebral cortex (Laguesse et al., 2015). In agreement with the structural similarities of MANF and CDNF (Lindahl et al., 2017), CDNF is also activated in the UPR. Expression of CDNF in HEK293-T cells and hippocampal neurons activates the UPR during thapsigargin-induced ER stress in both cell types, and attenuates expression of ER stress activated by the apoptotic proteins CHOP and cleaved caspase 3 (Arancibia et al., 2018). There is clear evidence that exogenously administered CDNF is also neuroprotective (Lindholm et al., 2007; Voutilainen et al., 2015). Indeed, intrastriatally infused CDNF is taken up by neurons and transported retrogradely to, for example, the cortex and substantia nigra in rats, and localized in endosomes rather than the ER (Matlik et al., 2017). Although a cell surface receptor-mediated mechanism for secreted CDNF could be expected, no such mechanism has yet been identified. Moreover, all observed abnormalities are not necessarily direct consequences of a lack of CDNF, but may be secondary to the primary effects of the knockout, e.g. alterations in the GABAergic or dopaminergic systems, which are known to regulate neurogenesis and differentiation (Saito et al., 2010; Kim and Yoon, 2017).

Using the *cdnf* null mutant fish generated in this study, we first provide evidence that CDNF plays an important role in the regulation of developing neurotransmitter circuits, including dopaminergic, GABAergic, and histaminergic systems. Moreover, the increased seizure susceptibility revealed by PTZ administration in adult *cdnf* KO fish may be associated with the deficiency of the GABAergic and glutamatergic systems in *cdnf* KO fish. As a consequence of this lack of functional *cdnf*, the dysregulated homeostasis of the neurotransmitter connectivity leads to the impairment of social behaviors.

Loss of cdnf causes a dynamic alteration of dopaminergic systems in the brain

28

609 Due to the genome duplication in teleost fish (Postlethwait et al., 2004), two TH genes (Candy and Collet,
610 2005) complimentarily expressed in the brain (Chen et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010)
611 are found in the zebrafish. In our *cdnf* KO mutant, the number of TH1 neurons was reduced in the
612 prethalamus area, whereas an increased number of TH2-containing cells (teleost specific paralogous th)
613 appeared in the hypothalamic region. The zebrafish TH1 cell population in the prethalamus is homologous
614 to the mammalian DA population A13 in the zona incerta of the thalamus (Tay et al., 2011), which is more
615 susceptible to neurotoxic MPTP and MPP+ injury in the zebrafish brain (Sallinen et al., 2009). The
616 hypothalamic TH1 groups correspond to A12 and A14 DA groups in the arcuate and periventricular nucleus
617 of the hypothalamus (Tay et al., 2011), respectively. In the L1CAM (neural cell adhesion molecule L1) null
618 mice, abnormal distribution of dopaminergic neurons was evident in A12, A13 and A14 DA groups – but not
619 the A9 group – in the substantia nigra (Demyanenko et al., 2001). It has remained unclear whether cdnf
620 binds to potential signaling receptors to trigger downstream signal pathways. Alternatively, it is possible
621 that in the zebrafish brain, cdnf serves as a survival-promoting factor affecting the expression of essential
622 regulators that could regulate the number of TH1 dopaminergic neurons but negatively control the TH2-
623 containing cell numbers in a regional-specific fashion. Finally, we cannot exclude the possibility that loss of
624 cdnf *per se* may induce unpredicted endoplasmic reticulum stress, oxidative stress or chronic inflammation,
625 which could result in neurodegeneration in other more vulnerable dopaminergic populations (Sprenkle et
626 al., 2017).

627

628 ***Impaired GABAergic and histaminergic system***

629 It is evident that most of the dopaminergic neurons contain GABA as a co-transmitter in the preoptic area,
630 prethalamus, and hypothalamus regions (Filippi et al., 2014). Histamine neurons also contain GABA in all
631 vertebrates studied thus far (Airaksinen et al., 1992), including zebrafish (Sundvik and Panula, 2012).
632 Moreover, we have reported that dopaminergic signaling plays a crucial role in the specification of

633 hypothalamic neurotransmitter identity (Chen et al., 2016). The number of histaminergic neurons is
634 determined at least in part by dopamine produced by *th2*-expressing dopaminergic neurons in zebrafish
635 (Chen et al., 2016). The number of histaminergic neurons shows life-long plasticity in adult zebrafish
636 through a Notch1 pathway regulated by presenilin 1 in the gamma-secretase complex (Sundvik et al.,
637 2013). Dopamine activation also has a direct impact on GABAergic neuron development in zebrafish larvae
638 (Souza et al., 2011). Consequently, a decreased number of GABA-containing cells and histaminergic
639 neurons found in the hypothalamic area may also be caused by the dynamic alteration of dopaminergic
640 systems, specifically the increased expression of *th2* in the hypothalamus, caused by a lack of cdnf.
641 Nevertheless, the wide distribution of abnormal cell populations in cdnf-deficient zebrafish is more likely to
642 derive from a direct effect on early neuronal proliferation, maturation, and transmitter specification.

643

644 ***Neurotransmitter systems associated with abnormal social behaviors***

645 The neurotransmitter phenotype in the developing and mature nervous system is regulated by genetic and
646 environmental cues, in order to compensate for the changing homeostatic requirements and to maintain
647 the appropriate neuronal circuits during development in nervous system function (Dulcis et al., 2013; Dulcis
648 et al., 2017). Proper social responses require coordinated neurotransmitter circuits in the CNS. Dysfunction
649 of any main transmitter system is known to cause mental disorders and neurodegenerative diseases (Ng et
650 al., 2015). The behavioral phenotype of the *cdnf* KO zebrafish is reminiscent of many neurological and
651 psychiatric conditions, such as attention deficit disorder, autism spectrum disorder, schizophrenia, or
652 epilepsy. The observed sensitivity to pentylenetetrazole-induced seizures may depend on an abnormal
653 GABAergic system and low expression of vGAT, since PTZ acts through the GABA-A receptors (Saito et al.,
654 2010; Kim and Yoon, 2017). The abnormal histaminergic system may be responsible for the low level of
655 anxiety-related behavior observed in our novel tank test, since reducing histamine levels in the adult
656 zebrafish brain by prohibiting the *hdc* inhibitor alpha-fluoromethylhistidine has the same effect (Peitsaro et

al., 2003). The decrease in *hdc* expression and number of histamine-containing posterior hypothalamic neurons found in this study may be a direct result of lack of CDNF, or a secondary effect of the increased production of dopamine by the *th2*-expressing neurons in the same cluster of cells (Chen et al., 2016).

Taken together, this study highlights the novel and broad role of CDNF in shaping the neurotransmitter circuits in the zebrafish brain, and provides evidence that cdnf has an impact on regulation of neural progenitors and maintenance of neurotransmitter properties. Although the *cdnf* KO fish are superficially normal, the altered transmitter networks produce a range of abnormal behaviors that resemble some human neuropsychiatric conditions, including schizophrenia. Indeed, one study has already shown an association between one SNP/haplotype in the human cdnf gene and schizophrenia characterized by negative symptoms in the Han Chinese population (Yang et al., 2018). Interestingly, ER quality control of protein processing is known to be associated with schizophrenia (Kim et al., 2019).

Author contributions

YCC designed the study, conducted and performed experiments, interpreted data and wrote the manuscript. DB and SS performed experiments, acquired data and assisted in preparation of the manuscript. SA provided vGAT materials. PP conceived and designed the study and wrote the manuscript.

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References

Airaksinen MS, Alanen S, Szabat E, Visser TJ, Panula P (1992) Multiple neurotransmitters in the tuberomammillary nucleus: comparison of rat, mouse, and guinea pig. J Comp Neurol 323:103-116.

Airavaara M, Harvey BK, Voutilainen MH, Shen H, Chou J, Lindholm P, Lindahl M, Tuominen RK, Saarma M, Hoffer B, Wang Y (2012) CDNF protects the nigrostriatal dopamine system and promotes recovery after MPTP treatment in mice. Cell Transplant 21:1213-1223.

Apostolou A, Shen Y, Liang Y, Luo J, Fang S (2008) Armet, a UPR-upregulated protein, inhibits cell proliferation and ER stress-induced cell death. Exp Cell Res 314:2454-2467.

Arancibia D, Zamorano P, Andres ME (2018) CDNF induces the adaptive unfolded protein response and attenuates endoplasmic reticulum stress-induced cell death. Biochim Biophys Acta Mol Cell Res 1865:1579-1589.

Baronio D, Puttonen HAJ, Sundvik M, Semenova S, Lehtonen E, Panula P (2018) Embryonic exposure to valproic acid affects the histaminergic system and the social behaviour of adult zebrafish (Danio rerio). Br J Pharmacol 175:797-809.

Benjamini Y, Krieger, A. M. & Yekutieli, D. (2006) Adaptive linear step-up procedures that control the false discovery rate. Biometrika 93:17.

Cachat J et al. (2010) Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat Protoc 5:1786-1799.

Candy J, Collet C (2005) Two tyrosine hydroxylase genes in teleosts. Biochim Biophys Acta 1727:35-44.

Chao MV (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 4:299-309.

Chao MV, Rajagopal R, Lee FS (2006) Neurotrophin signalling in health and disease. Clin Sci (Lond) 110:167-173.

Chen YC, Priyadarshini M, Panula P (2009) Complementary developmental expression of the two tyrosine hydroxylase transcripts in zebrafish. Histochem Cell Biol 132:375-381.

Chen YC, Sundvik M, Rozov S, Priyadarshini M, Panula P (2012) MANF regulates dopaminergic neuron development in larval zebrafish. Dev Biol 370:237-249.

Chen YC, Semenova S, Rozov S, Sundvik M, Bonkowsky JL, Panula P (2016) A Novel Developmental Role for Dopaminergic Signaling to Specify Hypothalamic Neurotransmitter Identity. J Biol Chem 291:21880-21892.

Demyanenko GP, Shibata Y, Maness PF (2001) Altered distribution of dopaminergic neurons in the brain of L1 null mice. Brain Res Dev Brain Res 126:21-30.

714 Dulcis D, Jamshidi P, Leutgeb S, Spitzer NC (2013) Neurotransmitter switching in the adult brain regulates
715 behavior. Science 340:449-453.

716 Dulcis D, Lippi G, Stark CJ, Do LH, Berg DK, Spitzer NC (2017) Neurotransmitter Switching Regulated by
717 miRNAs Controls Changes in Social Preference. Neuron 95:1319-1333 e1315.

718 Duy PQ, Berberoglu MA, Beattie CE, Hall CW (2017) Cellular responses to recurrent pentylenetetrazole-
719 induced seizures in the adult zebrafish brain. Neuroscience 349:118-127.

720 Elfarnawany MH (2015) Signal Processing Methods for Quantitative Power Doppler Microvascular
721 Angiography. In. Electronic Thesis and Dissertation Repository. 3106.

722 Favero CB, Henshaw RN, Grimsley-Myers CM, Shrestha A, Beier DR, Dwyer ND (2013) Mutation of the
723 BiP/GRP78 gene causes axon outgrowth and fasciculation defects in the thalamocortical
724 connections of the mammalian forebrain. J Comp Neurol 521:677-696.

725 Filippi A, Mueller T, Driever W (2014) vglut2 and gad expression reveal distinct patterns of dual GABAergic
726 versus glutamatergic cotransmitter phenotypes of dopaminergic and noradrenergic neurons in the
727 zebrafish brain. J Comp Neurol 522:2019-2037.

728 Filippi A, Mahler J, Schweitzer J, Driever W (2010) Expression of the paralogous tyrosine hydroxylase
729 encoding genes th1 and th2 reveals the full complement of dopaminergic and noradrenergic
730 neurons in zebrafish larval and juvenile brain. J Comp Neurol 518:423-438.

731 Finney JL, Robertson GN, McGee CA, Smith FM, Croll RP (2006) Structure and autonomic innervation of the
732 swim bladder in the zebrafish (Danio rerio). J Comp Neurol 495:587-606.

733 Godin JD, Creppe C, Laguesse S, Nguyen L (2016) Emerging Roles for the Unfolded Protein Response in the
734 Developing Nervous System. Trends Neurosci 39:394-404.

735 Green J, Collins C, Kyzar EJ, Pham M, Roth A, Gaikwad S, Cachat J, Stewart AM, Landsman S, Grieco F,
736 Tegelenbosch R, Noldus LP, Kalueff AV (2012) Automated high-throughput neurophenotyping of
737 zebrafish social behavior. J Neurosci Methods 210:266-271.

738 Hetz C, Saxena S (2017) ER stress and the unfolded protein response in neurodegeneration. Nat Rev Neurol
739 13:477-491.

740 Huang RQ, Bell-Horner CL, Dibas MI, Covey DF, Drewe JA, Dillon GH (2001) Pentylenetetrazole-induced
741 inhibition of recombinant gamma-aminobutyric acid type A (GABA(A)) receptors: mechanism and
742 site of action. J Pharmacol Exp Ther 298:986-995.

743 Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK (2013) Efficient
744 genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31:227-229.

745 Jones BE (2019) Arousal and sleep circuits. Neuropsychopharmacology.

746 Karhunen T, Airaksinen MS, Tuomisto L, Panula P (1993) Neurotransmitters in the nervous system of
747 Macoma balthica (Bivalvia). J Comp Neurol 334:477-488.

748 Kaslin J, Panula P (2001) Comparative anatomy of the histaminergic and other aminergic systems in
749 zebrafish (Danio rerio). J Comp Neurol 440:342-377.

750 Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ (2015) The Phyre2 web portal for protein modeling,
751 prediction and analysis. Nat Protoc 10:845-858.

752 Kemppainen S, Lindholm P, Galli E, Lahtinen HM, Koivisto H, Hamalainen E, Saarma M, Tanila H (2015)
753 Cerebral dopamine neurotrophic factor improves long-term memory in APP/PS1 transgenic mice
754 modeling Alzheimer's disease as well as in wild-type mice. Behav Brain Res 291:1-11.

755 Kim P, Scott MR, Meador-Woodruff JH (2019) Abnormal ER quality control of neural GPI-anchored proteins
756 via dysfunction in ER export processing in the frontal cortex of elderly subjects with schizophrenia.
757 Transl Psychiatry 9:6.

758 Kim YS, Yoon BE (2017) Altered GABAergic Signaling in Brain Disease at Various Stages of Life. Exp
759 Neurobiol 26:122-131.

760 Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the
761 zebrafish. Dev Dyn 203:253-310.

762 Kizil C, Brand M (2011) Cerebroventricular microinjection (CVMI) into adult zebrafish brain is an efficient
763 misexpression method for forebrain ventricular cells. PLoS One 6:e27395.

764 Kukko-Lukjanov TK, Panula P (2003) Subcellular distribution of histamine, GABA and galanin in
765 tuberomamillary neurons in vitro. J Chem Neuroanat 25:279-292.

766 Kysil EV, Meshalkina DA, Frick EE, Echevarria DJ, Rosemberg DB, Maximino C, Lima MG, Abreu MS,
767 Giacomini AC, Barcellos LJG, Song C, Kalueff AV (2017) Comparative Analyses of Zebrafish Anxiety-
768 Like Behavior Using Conflict-Based Novelty Tests. Zebrafish 14:197-208.

769 Laguesse S, Creppe C, Nedialkova DD, Prevot PP, Borgs L, Huysseune S, Franco B, Duysens G, Krusy N, Lee G,
770 Thelen N, Thiry M, Close P, Chariot A, Malgrange B, Leidel SA, Godin JD, Nguyen L (2015) A Dynamic
771 Unfolded Protein Response Contributes to the Control of Cortical Neurogenesis. Dev Cell 35:553-
772 567.

773 Laruelle M (2014) Schizophrenia: from dopaminergic to glutamatergic interventions. Curr Opin Pharmacol
774 14:97-102.

775 Latge C, Cabral KM, Almeida MS, Foguel D (2013) (1)H-, (13)C- and (15)N-NMR assignment of the N-
776 terminal domain of human cerebral dopamine neurotrophic factor (CDNF). Biomol NMR Assign
777 7:101-103.

778 Latge C, Cabral KM, de Oliveira GA, Raymundo DP, Freitas JA, Johanson L, Romao LF, Palhano FL, Herrmann
779 T, Almeida MS, Foguel D (2015) The Solution Structure and Dynamics of Full-length Human Cerebral
780 Dopamine Neurotrophic Factor and Its Neuroprotective Role against alpha-Synuclein Oligomers. J
781 Biol Chem 290:20527-20540.

782 Lindahl M, Saarma M, Lindholm P (2017) Unconventional neurotrophic factors CDNF and MANF: Structure,
783 physiological functions and therapeutic potential. Neurobiol Dis 97:90-102.

784 Lindahl M, Chalazonitis A, Palm E, Pakarinen E, Danilova T, Pham TD, Setlik W, Rao M, Voikar V, Huotari J,
785 Kopra J, Andressoo JO, Piepponen PT, Airavaara M, Panhelainen A, Gershon MD, Saarma M (2020)
786 Cerebral dopamine neurotrophic factor-deficiency leads to degeneration of enteric neurons and
787 altered brain dopamine neuronal function in mice. Neurobiol Dis 134:104696.

788 Lindholm D, Makela J, Di Liberto V, Mudo G, Belluardo N, Eriksson O, Saarma M (2016) Current disease
789 modifying approaches to treat Parkinson's disease. Cell Mol Life Sci 73:1365-1379.

790 Lindholm P, Voutilainen MH, Lauren J, Peranen J, Leppanen VM, Andressoo JO, Lindahl M, Janhunen S,
791 Kalkkinen N, Timmusk T, Tuominen RK, Saarma M (2007) Novel neurotrophic factor CDNF protects
792 and rescues midbrain dopamine neurons in vivo. Nature 448:73-77.

793 Lindsey BW, Smith FM, Croll RP (2010) From inflation to flotation: contribution of the swimbladder to
794 whole-body density and swimming depth during development of the zebrafish (Danio rerio).
795 Zebrafish 7:85-96.

796 Liu H, Tang X, Gong L (2015) Mesencephalic astrocyte-derived neurotrophic factor and cerebral dopamine
797 neurotrophic factor: New endoplasmic reticulum stress response proteins. Eur J Pharmacol
798 750:118-122.

799 Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR
800 and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

801 Matlik K, Vihinen H, Bienemann A, Palgi J, Voutilainen MH, Booms S, Lindahl M, Jokitalo E, Saarma M,
802 Huttunen HJ, Airavaara M, Arumae U (2017) Intrastriatally Infused Exogenous CDNF Is Endocytosed
803 and Retrogradely Transported to Substantia Nigra. eNeuro 4.

804 Miller NY, Gerlai R (2011) Shoaling in zebrafish: what we don't know. Rev Neurosci 22:17-25.

805 Mitre M, Mariga A, Chao MV (2017) Neurotrophin signalling: novel insights into mechanisms and
806 pathophysiology. Clin Sci (Lond) 131:13-23.

807 Mussulini BH, Leite CE, Zenki KC, Moro L, Baggio S, Rico EP, Rosemberg DB, Dias RD, Souza TM, Calcagnotto
808 ME, Campos MM, Battastini AM, de Oliveira DL (2013) Seizures induced by pentylenetetrazole in
809 the adult zebrafish: a detailed behavioral characterization. PLoS One 8:e54515.

810 Nasrolahi A, Mahmoudi J, Akbarzadeh A, Karimipour M, Sadigh-Eteghad S, Salehi R, Farhoudi M (2018)
811 Neurotrophic factors hold promise for the future of Parkinson's disease treatment: is there a light
812 at the end of the tunnel? Rev Neurosci 29:475-489.

813 Ng J, Papandreou A, Heales SJ, Kurian MA (2015) Monoamine neurotransmitter disorders--clinical advances
814 and future perspectives. Nat Rev Neurol 11:567-584.

815 Panula P, Airaksinen MS, Pirvola U, Kotilainen E (1990) A histamine-containing neuronal system in human
816 brain. Neuroscience 34:127-132.

817 Parkash V, Lindholm P, Peranen J, Kalkkinen N, Oksanen E, Saarma M, Leppanen VM, Goldman A (2009) The
818 structure of the conserved neurotrophic factors MANF and CDNF explains why they are
819 bifunctional. Protein Eng Des Sel 22:233-241.

820 Peitsaro N, Kaslin J, Anichtchik OV, Panula P (2003) Modulation of the histaminergic system and behaviour
821 by alpha-fluoromethylhistidine in zebrafish. J Neurochem 86:432-441.

822 Postlethwait J, Amores A, Cresko W, Singer A, Yan YL (2004) Subfunction partitioning, the teleost radiation
823 and the annotation of the human genome. Trends Genet 20:481-490.

824 Provensi G, Costa A, Izquierdo I, Blandina P, Passani MB (2018) Brain histamine modulates recognition
825 memory: possible implications in major cognitive disorders. Br J Pharmacol.

826 Puttonen HAJ, Sundvik M, Semenova S, Shirai Y, Chen YC, Panula P (2018) Knockout of histamine receptor
827 H3 alters adaptation to sudden darkness and monoamine levels in the zebrafish. Acta Physiol (Oxf)
828 222.

829 Ren X, Zhang T, Gong X, Hu G, Ding W, Wang X (2013) AAV2-mediated striatum delivery of human CDNF
830 prevents the deterioration of midbrain dopamine neurons in a 6-hydroxydopamine induced
831 parkinsonian rat model. Exp Neurol 248:148-156.

832 Saito K, Kakizaki T, Hayashi R, Nishimaru H, Furukawa T, Nakazato Y, Takamori S, Ebihara S, Uematsu M,
833 Mishina M, Miyazaki J, Yokoyama M, Konishi S, Inoue K, Fukuda A, Fukumoto M, Nakamura K,
834 Obata K, Yanagawa Y (2010) The physiological roles of vesicular GABA transporter during
835 embryonic development: a study using knockout mice. Mol Brain 3:40.

836 Sallinen V, Torkko V, Sundvik M, Reenila I, Khrustalyov D, Kaslin J, Panula P (2009) MPTP and MPP+ target
837 specific aminergic cell populations in larval zebrafish. J Neurochem 108:719-731.

838 Schmidt R, Strahle U, Scholpp S (2013) Neurogenesis in zebrafish - from embryo to adult. Neural Dev 8:3.

839 Semenova SA, Chen YC, Zhao X, Rauvala H, Panula P (2014) The tyrosine hydroxylase 2 (TH2) system in
840 zebrafish brain and stress activation of hypothalamic cells. Histochem Cell Biol 142:619-633.

841 Sousa-Victor P, Jasper H, Neves J (2018) Trophic Factors in Inflammation and Regeneration: The Role of
842 MANF and CDNF. Front Physiol 9:1629.

843 Souza BR, Romano-Silva MA, Tropepe V (2011) Dopamine D2 receptor activity modulates Akt signaling and
844 alters GABAergic neuron development and motor behavior in zebrafish larvae. J Neurosci 31:5512-
845 5525.

846 Sprenkle NT, Sims SG, Sanchez CL, Meares GP (2017) Endoplasmic reticulum stress and inflammation in the
847 central nervous system. Mol Neurodegener 12:42.

848 Sundvik M, Panula P (2012) Organization of the histaminergic system in adult zebrafish (Danio rerio) brain:
849 neuron number, location, and cotransmitters. J Comp Neurol 520:3827-3845.

850 Sundvik M, Chen YC, Panula P (2013) Presenilin1 regulates histamine neuron development and behavior in
851 zebrafish, danio rerio. J Neurosci 33:1589-1597.

852 Sundvik M, Kudo H, Toivonen P, Rozov S, Chen YC, Panula P (2011) The histaminergic system regulates
853 wakefulness and orexin/hypocretin neuron development via histamine receptor H1 in zebrafish.
854 FASEB J 25:4338-4347.

855 Tadimalla A, Belmont PJ, Thuerauf DJ, Glassy MS, Martindale JJ, Gude N, Sussman MA, Glembotski CC
856 (2008) Mesencephalic astrocyte-derived neurotrophic factor is an ischemia-inducible secreted
857 endoplasmic reticulum stress response protein in the heart. Circ Res 103:1249-1258.

858 Tay TL, Ronneberger O, Ryu S, Nitschke R, Driever W (2011) Comprehensive catecholaminergic projectome
859 analysis reveals single-neuron integration of zebrafish ascending and descending dopaminergic
860 systems. Nat Commun 2:171.

861 Thisse C, Thisse B (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat
862 Protoc 3:59-69.

863 Voutilainen MH, Arumae U, Airavaara M, Saarma M (2015) Therapeutic potential of the endoplasmic
864 reticulum located and secreted CDNF/MANF family of neurotrophic factors in Parkinson's disease.
865 FEBS Lett 589:3739-3748.

866 Voutilainen MH, Back S, Peranen J, Lindholm P, Raasmaja A, Mannisto PT, Saarma M, Tuominen RK (2011)
867 Chronic infusion of CDNF prevents 6-OHDA-induced deficits in a rat model of Parkinson's disease.
868 Exp Neurol 228:99-108.

869 Yamamoto K, Ruuskanen JO, Wullimann MF, Vernier P (2010) Two tyrosine hydroxylase genes in
870 vertebrates New dopaminergic territories revealed in the zebrafish brain. Mol Cell Neurosci 43:394-
871 402.

872 Yan Y, Rato C, Rohland L, Preissler S, Ron D (2019) MANF antagonizes nucleotide exchange by the
873 endoplasmic reticulum chaperone BiP. Nat Commun 10:541.

874 Yang Y, Yu H, Li W, Liu B, Zhang H, Ding S, Lu Y, Jiang T, Lv L (2018) Association between cerebral dopamine
875 neurotrophic factor (CDNF) 2 polymorphisms and schizophrenia susceptibility and symptoms in the
876 Han Chinese population. Behav Brain Funct 14:1.

877 Zhou C, Xiao C, Commissiong JW, Krnjevic K, Ye JH (2006) Mesencephalic astrocyte-derived neurotrophic
878 factor enhances nigral gamma-aminobutyric acid release. Neuroreport 17:293-297.

879 Zhou W, Chang L, Fang Y, Du Z, Li Y, Song Y, Hao F, Lv L, Wu Y (2016) Cerebral dopamine neurotrophic factor
880 alleviates Abeta25-35-induced endoplasmic reticulum stress and early synaptotoxicity in rat
881 hippocampal cells. Neurosci Lett 633:40-46.

882 Zupanc GK, Hinsch K, Gage FH (2005) Proliferation, migration, neuronal differentiation, and long-term
883 survival of new cells in the adult zebrafish brain. J Comp Neurol 488:290-319.

Figure legends

Figure 1. Predicted domain structures and spatiotemporal expression of zebrafish cdnf. (**A**) Secondary structure, and (**B**) predictive 3D model of zebrafish cdnf by Phyre2 modeling with human CDNF. (**C**) whole-mount *in situ* hybridization highlighting *cdnf* expression in the head, eyes, heart, muscles, and optic vesicles during development. (**D**) Quantitative RT-PCR showing the zygotic *cdnf* mRNA expression at various developmental stages (one-way ANOVA, $F(6, 14) = 40.31$, $p<0.0001$ n=3), and (**E**) in various male and female adult organs (two-way ANOVA, $F(1, 32) = 3.885$,$p=0.0574$, n=4/group). Green helices indicate alpha-helices, blue arrows indicate beta-strands. Grey lines indicate the conserved amino acid residues. G indicates the 3-trun helix. T indicates hydrogen bonded turn. S indicates the bend. H, heart. HB, hindbrain. ov, optic vesicle. Data are mean ± SEM; one-way or two-way ANOVA was used for statistics.

Figure 2. CRISPR/Cas9-generated *cdnf* mutant zebrafish. (**A**) Scheme of CRISPR/Cas9-generated 14-base pair deletion in exon2 of *cdnf*. (**B**) Sequence chromatogram showing a premature stop codon of a homozygous *cdnf* mutant zebrafish. (**C**) Results of the HRM analysis showing the distinctive melting curves of each genotype. (**D**) Reduction in *cdnf* mRNA expression shown in the brains of 8-dpf and (**E**) 1-mpf *cdnf* mutant fish. (**F**) No obvious gross phenotype in larvae and adult *cdnf* mutant fish. PPa, anterior parvocellular preoptic nucleus. PTv, ventral part of posterior tuberculum. T, midbrain tegmentum. TeO, tectum opticum. Scale bar is 200μm in (D), 1mm (10 dpf) and 1cm (10 mpf) in (F).

Figure 3. qPCR analysis of *cdnf* and neurotransmitter synthesis enzymes at larval, adulthood, and aging stages. (**A-C**) Significant reduction of *cdnf* mRNA in *cdnf*-deficient (knock-out) fish (Unpaired t test, $t=4.675$, $df=22$, two-tailed $p=0.0001$; $t=4.245$, $df=8$, $p=0.0028$; $t=3.280$, $df=8$, $p=0.0112$). (**D-F**) Significant increase of *th2* transcripts in *cdnf*-deficient fish (Unpaired t test, $t=4.043$, $df=22$, $p= 0.0005$; $t=2.901$, $df=8$, $p= 0.0198$;

t=5.450, df=8, p= 0.0006). (**G-I**) Non-significant differences in *th1* mRNA expression between groups (Unpaired t test, t=0.6318, df=22, p= 0.5340; t=1.024, df=8, p= 0.3358; t=0.7477, df=8, p= 0.4760). (**J-L**) Significant reduction of *hdc* mRNA expression in adult brains (Unpaired t test, t=0.052, df=22, p=0.9590; t=2.473, df=8, p=0.0385; t=1.042, df=8, p=0.3278). qPCR analysis relative to expression of the housekeeping gene *rpl13a*; values are mean ± SEM. n=12/ group for 8-dpf fish, n=5/ group for 8-mpf brains, n=5/ group for 18-mpf brains in each *cdnf* knock-out and wild-type group. Data are mean ± SEM. Student's *t*-test was used for statistical analysis *p<0.05, **p<0.01, and ***p<0.001.

Figure 4. Selective alteration in dopaminergic systems in *cdnf* mutant fish. (**A**) Co-labeling of zebrafish tyrosine hydroxylase 1 (th1) and tyrosine hydroxylase 2 (th2) of 8-dpf *cdnf* wild-type (WT) brains. (**B**) Co-labeling of zebrafish tyrosine hydroxylase 1 (th1) and tyrosine hydroxylase 2 (th2) of 8-dpf *cdnf* knock-out (KO) brains. (**C**) Higher magnification images of TH2 10/10b (Hc) group, TH1 10 group, and TH1 5,6,11 group of *cdnf* WT brains. (**D**) Higher magnification images of TH2 10/10b (Hc) group, TH1 10 group, and TH1 5,6,11 group of *cdnf* KO brains. (**E**) Significant increase in TH1/TH2 immunoreactive cell number in the caudal hypothalamus area (Unpaired t test, t=3.398, df=14, p= 0.0043, n=8/group; Hc, 10/10b th population) in the *cdnf* KO group. (**F**) No significant change in TH1 immunoreactive cell number in the caudal hypothalamus area (Unpaired t test, t=0.9245, df=14, p= 0.3709, n=8/ group; Hc, 10 th population) in the *cdnf* KO group. (**G**) Significant decrease in TH1-positive cell number in the prethalamus (Unpaired t test, t=4.472, df=16, p=0.0004; n=8/ group, th1 group 5,6,11) in the *cdnf* KO group. (**H**) 5-HT immunoreactivity in 8-dpf WT larval brains and *cdnf* KO larval brains (n=7). (**I**) No significant difference in 5HT immunoreactive cell numbers in the caudal hypothalamus area (Unpaired t test, t=0.7817, df=12, p= 0.4495, n=7/ group) (**J**) *in situ* hybridization results showing a higher intensity of *th2* signals in *th2* 10b group (Hc, caudal hypothalamus) in 8-dpf *cdnf* KO fish (n=8). In particular, TH1+TH2 (rabbit anti-th1 and th2 antibody) recognized both zebrafish th1 and th2 dopaminergic neurons. TH1 (mouse anti-tyrosine hydroxylase antibody) specifically

933 recognized zebrafish th1. 5-HT, rabbit anti-serotonin antibody. Data are mean ± SEM. Student's *t*-test was
934 used for statistical analysis. **p<0.01, ***p<0.001. Scale bar is 100μm.

935

936 **Figure 5. Deficient proliferating cells and histaminergic neurons in *cdnf* mutant fish.** (**A**) Co-labeling of
937 proliferation marker (EdU), anti-histamine immunoreactivity, anti-HuC immunoreactivity, and a merged
938 image of triple labeled stacks of an 8-dpf *cdnf* WT brain. (**B**) Triple labeling of an 8-dpf *cdnf* KO brain. (**C**)
939 Quantification of EdU-positive cell numbers, and higher magnification images of EdU-labeled cells in the
940 caudal hypothalamic area (Hc) (Unpaired t test, t=2.193, df=14, p=0.0457, n=8 *cdnf* WT, and n=8 *cdnf* KO
941 fish). (**D**) Quantification of fluorescence intensity of HuC immunoreactive cells, and higher magnification
942 images of the caudal hypothalamus (Unpaired test, t=2.362, df=14, p=0.0332, n=8 *cdnf* WT, and n=8 *cdnf*
943 KO fish). (**E**) Quantification of histamine-positive cell numbers, and higher magnification images of
944 histamine immunostaining images (Unpaired t test, t=6.584, df=12, p<0.0001, n=7 *cdnf* WT, and n=7 *cdnf*
945 KO fish). (**F**) Quantification of *histidine decarboxylase* (*hdc*) mRNA-containing cell numbers (unpaired t test,
946 t=4.124, df=12, p=0.0014, n=6 *cdnf* wild-type, and n=8 *cdnf* KO fish). (**G**) Quantification of orexin-positive
947 cell numbers, and higher magnification images of orexin immunostaining images (Unpaired t test, t=2.683,
948 df=16, p= 0.0163, n=9 *cdnf* WT, and n=9 *cdnf* KO fish). (**H**) Significant decrease of *sox2a* transcripts in *cdnf*-
949 deficient fish (Unpaired t test, t=2.614, df=12, p= 0.0226). Marked areas were used a regions of interest for
950 measurement of fluorescence intensity. Data are mean ± SEM. Student's *t*-test was used for statistical
951 analysis. **p<0.01, ***p<0.001. Scale bar is 100μm.

952

953 **Figure 6. Selective impairment of GABAergic system in *cdnf* KO fish.** (**A**) Co-labeling of anti-acetylated
954 tubulin (showing axons) and GABA with antibody (showing GABAergic cells) of 8-dpf *cdnf* WT brains. (**B**)
955 Double immunostaining of 8-dpf *cdnf* KO brains. (**C**) Quantification of GABA immunoreactive cell numbers

in the ventral part of the posterior tuberculum (PTv), and higher magnification images of GABA-labelled cells (Unpaired t test, t=9.989, df=14, p <0.0001, n=8/group). (**D**) Quantification of GABA immunoreactive cell numbers in the caudal hypothalamus (Hc), and higher magnification images of GABA-labelled cells (Unpaired test, t=5.503, df=14, p <0.0001, n=8/group). (**E**) Quantification of fluorescence intensity of acetylated tubulin immunoreactive projections, and higher magnification images of acetylated tubulin immunoreactive projections (Unpaired t test, t=0.7879, df=14, p=0.4439, n=8/group). White rectangles and arrows indicate a noteworthy reduction of GABA-staining cells in the PTv and Hc area, respectively. The marked area was used as a region of interest for measurement of fluorescence intensity. Data are mean ± SEM. Student's *t*-test was used for statistical analysis. ***p<0.001. Scale bar is 100μm.

Figure 7. *slc32a/vGAT* downregulation in *cdnf* KO fish. (**A**) Quantification of *slc32a/vGAT* mRNA expression relative to the housekeeping gene *rpl13a* in 8-dpf fish (Unpaired t test, t=2.508, df=14, p= 0.0251, n=8/group). (**B**) *In situ* hybridization of *vGAT* mRNA expression of 8-dpf brains (n=5). (**C**) Quantification of *gad1b* mRNA expression relative to *rpl13a* in 8-dpf fish (Unpaired t test, t=2.077, df=14, p= 0.0567). (**D**) Quantification of *gad2a* mRNA expression relative to the *rpl13a* in 8-dpf fish (Unpaired t test, t=2.508, df=14, p= 0.0251). (**E**) Quantification of *vGlut2* mRNA expression relative to *rpl13a* in 8-dpf fish (Unpaired t test, t=2.508, df=14, p= 0.0251). Arrows and red rectangles indicate downregulated expression of *vGAT* in the hypothalamus (H), preoptic region (Po), ventral posterior tuberculum (PTv) and ventral thalamus (VT). Data are mean ± SEM. Student's *t*-test was used for statistical analysis. *p<0.05. Scale bar is 100μm.

Figure 8. 8-dpf larval sleep-related behavior

(**A**) Locomotor activity under 14:10 (light:dark) illumination conditions (Multiple t tests using Two-stage step-up method of Benjamini, Krieger and Yekutieli)(Benjamini, 2006). (**B**) Average cumulative duration (in s) in 15 min time bins of movement during light conditions (Unpaired t test, t=2.304, df=62, p= 0.0246). (**C**) Average cumulative duration of movement during the second treatment of light conditions in 15 min time bins (Unpaired t test, t=2.151, df=12, p= 0.0525). (**D**) Duration of rest (not moving) during dark conditions in 15 min time bins (Unpaired t test, t=9.159, df=76, p <0.0001). (**E**) Average cumulative duration of movement during dark conditions in 15 min time bins (Unpaired t test, t=9.159, df=76, p <0.0001). n=16/ group. Data are mean ± SEM. Student's *t*-test and one-way ANOVA analysis with multiple comparisons was used for statistical analysis *p<0.05 and ***p<0.001.

Figure 9. Social preference deficiency in *cdnf* KO adult fish. (**A**) Schemes of visually mediated social preference behavior setup and representative movement traces of *cdnf* WT and KO fish during 10 min recording intervals. (**B**) Ratio of cumulative duration in the distal, object and stimulus/conspecific zone. (**C**) Cumulative time spent in the stimulus (conspecific) zone. (Unpaired t test, t=2.357, df=28, p= 0.0257). (**D**) Cumulative time spent in the object zone (Unpaired t test, t=1.156, df=28, p= 0.2575). (**E**) Cumulative time spent in the distal zone (Unpaired t test, t=2.217, df=28, p= 0.0349). n=15/ group. Data are mean ± SEM. Student's *t*-test was used for statistical analysis. *p<0.05.

Figure 10. Impaired bottom-dwelling behavior in *cdnf* KO adult fish. (**A**) Schemes of the novel tank test with three digitized zones and representative movement traces of *cdnf* WT and KO siblings during a 6 min recording period. (**B**) Ratio of cumulative duration in the top, middle and bottom zone. (**C**) Amount of time spent in the top zone (Unpaired t test, t=2.942, df=18, p= 0.0087). (**D**) Amount of time spent in the middle zone (Unpaired t test, t=0.01053, df=18, p= 0.9917). (**E**) Amount of time spent in the bottom zone (unpaired

t test, t=3.029, df=18, p= 0.0072). (**F**) Average velocity during the 6 min video tracking period (Unpaired t test, t=1.253, df=18, p= 0.2261). (**G**) Vasculature by autofluorescence and TH1 immunostaining of the posterior chamber of the swim bladder in *cdnf* WT and *cdnf* KO fish. Inserts show images of dissected swim bladders. (**H**) Quantification of V3 volume of the posterior chamber (Unpaired t test, t=0.3488, df=6, p= 0.7392). (**I**) Quantification of vascular length density of the posterior chamber (Unpaired t test, t=0.0646, df=6, p= 0.9506). (J) Quantification of TH fluorescence intensity of the posterior chamber (Unpaired t test, t=1.138, df=6, p= 0.2983). n=10 for WT and *cdnf* KO fish in the Novel tank analysis. n=4 for WT and *cdnf* KO in the swim bladder analysis. Data are mean ± SEM. Student's *t*-test was used for statistical analysis. **p<0.01. Scale bar is 100 μm.

Figure 11. Decreased shoal cohesion in *cdnf* KO adult fish. The upper panel shows the shoaling behavior test on 6-mpf male *cdnf* WT and KO fish (n=5 in each trial) during a 10 min video tracking period; four trials were analyzed. (**A**) Average velocity, in 1 min time bins (Multiple t tests, 1min, t=2.794, df=38.0 p= 0.0477; 2min, t=3.576, df=38, p=0.0087; 3min, t=4.344, df=38, p= 0.001; 7min, t= 2.991, df=38, p=0.033; 10min, t=3.361, df=38.00, p=0.014). (**B**) Average velocity during the 10 min video tracking period (Unpaired t test, t=3.300, df=38, p= 0.0021). (**C**) Average inter-individual distance of total trials (Unpaired t test, t=4.964, df=78, p <0.0001). (**D**) Average time spent in the proximity (i.e. less than 2 cm) of the nearest neighbor. The lower panel shows the shoaling behavior test on 18-mpf male *cdnf* WT and KO fish (n=5 in each trial); three trials were analyzed (Unpaired t test, t=2.973, df=38, two-tailed p= 0.0051). (**E**) Average velocity, in 1 min time bins (Multiple t tests, 1min, t= 4.351, df=28.0 p= 0.0016; 2min, t= 3.898, df=28, p= 0.0050; 3min, t= 3.726, df=28, p= 0.0070; 8min, t= 3.392, df=28, p= 0.0145). (**F**) Average velocity during the 10 min video tracking period (Unpaired t test, t=4.122, df=28, p= 0.0003). (**G**) Average inter-individual distance of total trials (Unpaired t test, t=7.264, df=58, p <0.0001). (**H**) Average time spent in the proximity of closest neighbor (Unpaired t test, t=1.649, df=28, p= 0.1103). Data are mean ± SEM. Student's *t*-test and one-way

ANOVA analysis with multiple comparisons was used for statistical analysis. *p<0.05, **p<0.01, and ***p<0.001.

Figure 12. Increased seizure susceptibility in *cdnf* KO adult fish. (**A**) Scheme of the PTZ administration procedure. (**B**) Seizure scores after 5 min of PTZ exposure (Two-way ANOVA, F (2, 15) = 0.8333, P=0.4538; Multiple t test; Day1, t= 2.712, df=10, p= 0.0433; Day2, t= 2.712, df=10, p= 0.0433; Day3, t= 4.000, df=10, p= 0.0075). (**C**) Seizure onset latency to score of 4 (Two-way ANOVA, F (2, 30) = 2.973, P=0.0664; Multiple t tests; Day1, t= 1.265, df=10, p= 0.2344; Day2, t= 6.078, df=10, p= 0.0004; Day 3, t= 4.065, df=10, p= 0.0045). (**D**) Duration of immobility during 5 min of PTZ exposure (Two-way ANOVA, F (2, 30) = 0.3478, P=0.7091; Multiple t tests; Day1, t= 0.4509, df=10, p= 0.6616; Day2, t= 1.853, df=10, p= 0.2554; Day 3, t= 1.674, df=10, p= 0.2554). (**E**) Total distance travelled during 5 min of PTZ exposure (Two-way ANOVA, F (2, 30) = 0.4349, P=0.6513; Multiple t tests; Day1, t= 1.053, df=10, p= 0.6168; Day2, t=1.158, df=10, p= 0.6168; Day 3, t= 0.0195, df=10, p=0.9848). n=6 in each group. Data are mean ± SEM. Two-way ANOVA analysis with multiple comparisons was used for statistical analysis. *p<0.05, **p<0.01, and ***p<0.001.

Figure 13. Results of qPCR analysis in PTZ-treated *cdnf* knock-out fish. Quantification of relative expression of (**A**) *cdnf* (Two-way ANOVA, F (1, 16) = 40.14, P<0.0001), (**B**) *manf* (Two-way ANOVA, F (1, 16) = 0.02213, P=0.8836), (**C**) *slc17a6a/vGlut2* (Two-way ANOVA, F (1, 16) = 16.16, P=0.0010), (**D**) *slc32a1/vGAT* (Two-way ANOVA, F (1, 16) = 40.14, P<0.0001), (**E**) *gad2a/gad65* (Two-way ANOVA, F (1, 16) = 10.68, P=0.0048), (**F**) *gad1b/gad67* (Two-way ANOVA, F (1, 16) = 4.052, P=0.0613), and (**G**) *gfap* (Two-way ANOVA, F (1, 16) = 8.813, P=0.0091) in 6-mpf brains with or without PTZ treatment. n=5 in each group. (**H**) Quantification of fluorescence intensity of zrf-1 immunostaining signals of 10-dpf *cdnf* wild-type and knock-out fish brains (Two-way ANOVA , F (1,24)= 33.8 P<0.0001). (**I**) Quantification of fluorescence intensity of

gfap-positive radial glial cells (shown in panel **H**) was done in the regions of interest shown in white boxes. RV, rhombencephalic ventricle; LR, lateral region of raphe. Data are mean ± SEM. Two-way ANOVA analysis with Tukey's multiple comparisons test was used for statistical analysis. *p<0.05, **p<0.01, and ***p<0.001. Scale bar is 100μm.

Table 1

List of primers used in this study

Table 2

HPLC analysis results of monoamine concentrations of larvae, adult brains and aging brains

Table 1 List of primers used in this study

gene	Forward primer	Reverse primer	note	ACCESSION
actb1	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	qPCR	NM_131031.1
cdnf	TGAAGTTCCCTTGGAAGTGCG	TGTGCAGATATTGCACATGGC	RT-PCR cloning	NM_001123281.1
cdnf	TTCTGCAGCCAAAGTGACCG	TCTGTGCTCCAGTCAAGAACC	qPCR	NM_001123281.1
cdnf	CACGAAATCAGCCCTCCAGT	GCTGAAGCCTCTGGCAGATT	crispr_cloning	BX901962.8
cdnf	TGTGTGTGGGGTTTTTGGGA	GCCGATTCTCTTTCCCAGTAGTCTC	HRM	BX901962.8
cdnf	TAGGCCCTCCTCCACCAGCTCT	AAACAGAGCTGGTGGAGGAGGG	sgRNA cloning	NM_001123281.1
gad1b/67	GGCCAAGGGCACGATTGGGT	GCATGCCACGCAGACTCGGT	qPCR	NM_194419.1
gad2/65	GCGGAGGCATCGGCTCCAAA	GCCGCAGCTCTCGGCTGTAG	qPCR	NM_001017708.2
gfap	GAAGCAGGAGGCCAATGACTATC	GGACTCATTAGACCCACGGAGAG	qPCR	NM_131373.2
hdc	TTCATGCGTCCTCTCCTGC	CCCCAGGCATGATGATGTTC	qPCR	NM_001102593.1
manf	ACCATGTGCCAGTGGAAAAGA	TCGACGGAGCTCAAGTCAAC	qPCR	NM_001076629.1
rpl13a	AGAGAAAGCGCATGGTTGTCC	GCCTGGTACTTCCAGCCAACTT	qPCR	NM_212784.1
sox2a	CCTATTCGCAGCAAAGCACG	GGAATGAGACGACGACGTGA	qPCR	NM_213118.1
slc17a6a/vGlut2	CATCCTGTCTACAACTACGGTT	CCAACACCAGAAATGAAATAGCCA	qPCR	NM_001009982.1
slc32a1/vGAT	AATACGCGTCACCACGAGAG	GAGCTCGATGATCTGTGCCA	cloning	NM_001080701.1
slc32a1/vGAT	CGGACAAGCCCAGAATCACT	CGACGGCGGCGAATATAATG	qPCR	NM_001080701.1
th	GACGGAAGATGATCGGAGACA	CCGCCATGTTCCGATTTCT	qPCR	NM_131149.1
th2	CTCCAGAAGAGAATGCCACATG	ACGTTCACTCTCCAGCTGAGTG	qPCR	NM_001001829.1

Table 2 HPLC analysis of monoamine concentrations of larvae, adult brains and aging brains

	19-momth-old brain			8-momth-old brain			8-dpf (8 larvae per tube)		
pmole/mg protein	WT (n=5)	KO (n=5)	*p value*	WT (n=9)	KO (n=9)	*p value*	WT (n=3)	KO (n=3)	*p value*
Dopamine	22.39 ± 0.3935	21.14 ± 0.6298	0.13	16.52 ± 0.542	15.97 ± 0.433	0.4451	2.834 ± 0.1438	3.21 ± 0.2845	0.3041
Norepinephrine	58.79 ± 3.076	56.32 ± 2.85	0.5707	46.29 ± 1.956	43.3 ± 1.243	0.216	7.2 ± 0.1838	8.239 ± 0.56.	0.1528
DOPAC	4.699 ± 0.3663	3.506 ± 0.2053	0.0218	2.764 ± 0.1298	2.452 ± 0.2412	0.2713	1.721 ± 0.1544	1.605 ± 0.08641	0.549
Homovanillic acid	12.48 ± 1.131	8.847 ± 0.1623	0.013	17.54 ± 1.768	8.494 ± 1.607	0.0016	2.235 ± 0.007714	4.195 ± 1.205	0.1794
3-Methoxytyramine	1.539 ± 0.1343	1.343 ± 0.2165	0.4639	5.42 ± 0.6011	5.795 ± 0.438	0.6205	2.854 ± 0.1544	3.351 ± 0.4868	0.3855
Serotonin	23 ± 1.162	24.63 ± 0.6439	0.2543	16.83 ± 0.6696	19.43 ± 0.6398	0.0124	3.693 ± 0.157	3.902 ± 0.1672	0.4142
5-Hydroxyindoleacetic acid	19.81 ± 0.9596	13.7 ± 0.4645	0.0004	15.99 ± 0.8659	15.08 ± 1.173	0.5423	3.769 ± 0.03767	5.02 ± 0.6834	0.1416
Histamine	9.065 ± 0.4468	11.33 ± 1.745	0.244	5.958 ± 0.516	4.285 ± 0.3379	0.0154	7.805 ± 0.854	5.899 ± 1.09	0.2406

(A significant reduction in KO is indicated in red; a significant increase in KO is indicated in green. Data are mean ± SEM. Student's *t*-test was used for statistical analysis)