Development/Plasticity/Repair

Presenilin1 Regulates Histamine Neuron Development and Behavior in Zebrafish, *Danio rerio*

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Modulatory neurotransmitters, including the histaminergic system, are essential in mediating cognitive functions affected in Alzheimer's disease (AD). The roles of disease genes associated with AD, most importantly the *presenilin1* gene (*psen1*), are poorly understood. We studied the role of *psen1* in plasticity of the brain histaminergic system using a novel *psen1* mutant zebrafish, *Danio rerio*. We found that in *psen1*$^{-/-}$ zebrafish, the histaminergic system is altered throughout life. At 7 d postfertilization (dpf) the histamine neuron number was reduced in *psen1*$^{-/-}$ compared with wild-type (WT) fish; at 2 months of age the histamine neuron number was at the same level as that in WT fish. In 1-year-old zebrafish, the histamine neuron number was significantly increased in *psen1*$^{-/-}$ fish compared with WT fish. These changes in histamine neuron number were accompanied by changes in histamine-driven behaviors. Treatment with DAPT, a γ-secretase inhibitor, similarly interfered with the development of the histaminergic neurons. We also assessed the expression of γ-secretase-regulated *Notch1a* mRNA and β-catenin at different time points. *Notch1a* mRNA level was reduced in *psen1*$^{-/-}$ compared with WT fish, whereas β-catenin was slightly upregulated in the hypothalamus of *psen1*$^{-/-}$ compared with WT fish at 7 dpf. The results reveal a life-long brain plasticity in both the structure of the histaminergic system and its functions induced by altered *Notch1a* activity as a consequence of *psen1* mutation. The new histaminergic neurons in aging zebrafish brain may arise as a result of phenotypic plasticity or represent newly differentiated stem cells.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by cognitive decline and dementia (Bishop et al., 2010). The cognitive decline in AD is associated with significant reduction in subcortical modulatory inputs, including the cholinergic (Whitehouse et al., 1982) and the histaminergic (Saper and German, 1987; Airaksinen et al., 1991; Panula et al., 1998) ones. Histamine is a biogenic amine discovered a century ago (Dale and Laidlaw, 1910). In the CNS, histamine is confined to neurons of the tuberomammillary nucleus (Panula et al., 1984; Ekström et al., 1995; Eriksson et al., 1998). The histaminergic neurotransmitter system is involved in different functions from cognition to hormonal regulation (Haas and Panula, 2003; Haas et al., 2008). Postmortem studies of the his-

taminergic neurotransmitter system in AD brains have shed light on the role of histamine in brain and found that histamine is reduced in AD patients (Panula et al., 1998). Histamine improves cognitive performance (Medhurst et al., 2007), and histamine H3 receptor ligands are attractive drug candidates (Schwartz, 2011; Tiligada et al., 2011). Patients with inherited early onset AD [familial AD (FAD)] carry mutations mainly within the genes for presenilin1 (PSEN1), presenilin2 (PSEN2), and amyloid precursor protein (APP) (Tanzi and Bertram, 2005). PSEN1 is associated with the γ-secretase complex (Kimberly et al., 2003) within the neuronal membranes (Kovacs et al., 1996; Xu et al., 2002; Hansson et al., 2004). PSEN1 in the γ-secretase complex has many functions, including cleavage of APP (Borchelt et al., 1996), regulation of Ca^{2+} homeostasis (Guo et al., 1996; Stutzmann et al., 2004), cleavage of the Notch receptor (Levitan and Greenwald, 1995; De Strooper et al., 1999; Jack et al., 2001), and involvement in Wnt/β-catenin signaling (Serban et al., 2005). PSEN1 affects the proliferation of cells in the CNS both in the hippocampus and subventricular zone of two different transgenic mouse models of AD (Wen et al., 2002; Rodríguez et al., 2009) *via* Notch signaling (Veeraraghavalu et al., 2010). PSEN1 also functions outside the γ-secretase complex, and the ability to degrade β-catenin is one example (De Strooper and Annaert, 2010). Histamine has been implicated as a neurogenesis-promoting factor; an *in vitro* study (Molina-Hernández and Velasco, 2008) showed that histamine induces neuronal stem cell proliferation through histamine H2 receptor and promotes neuronal fate through histamine H1 receptor. Zebrafish has established itself as a model organism within neurosciences (Panula et al., 2010). In zebrafish, the histaminergic neurotransmitter sys-

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>gi|56790305|ref|NP_571099.1| presenilin 1 [Danio rerio]

MADLVQNAANNVLNDGMDTSRHTSSTAAPPSRNEVELNGQPPTAPPPQVVTDSEEDEDEELTLKYGAKHV
IMLFIPVTLCMVVVVATIKSVSFYTQKDGQQLIYTPFREDTETVGQRALHSMLNAIIMISVIVVMTLVLV
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B WT   *psen1-/-*

C   Proteolytic site

N   Hydrophilic loop domain
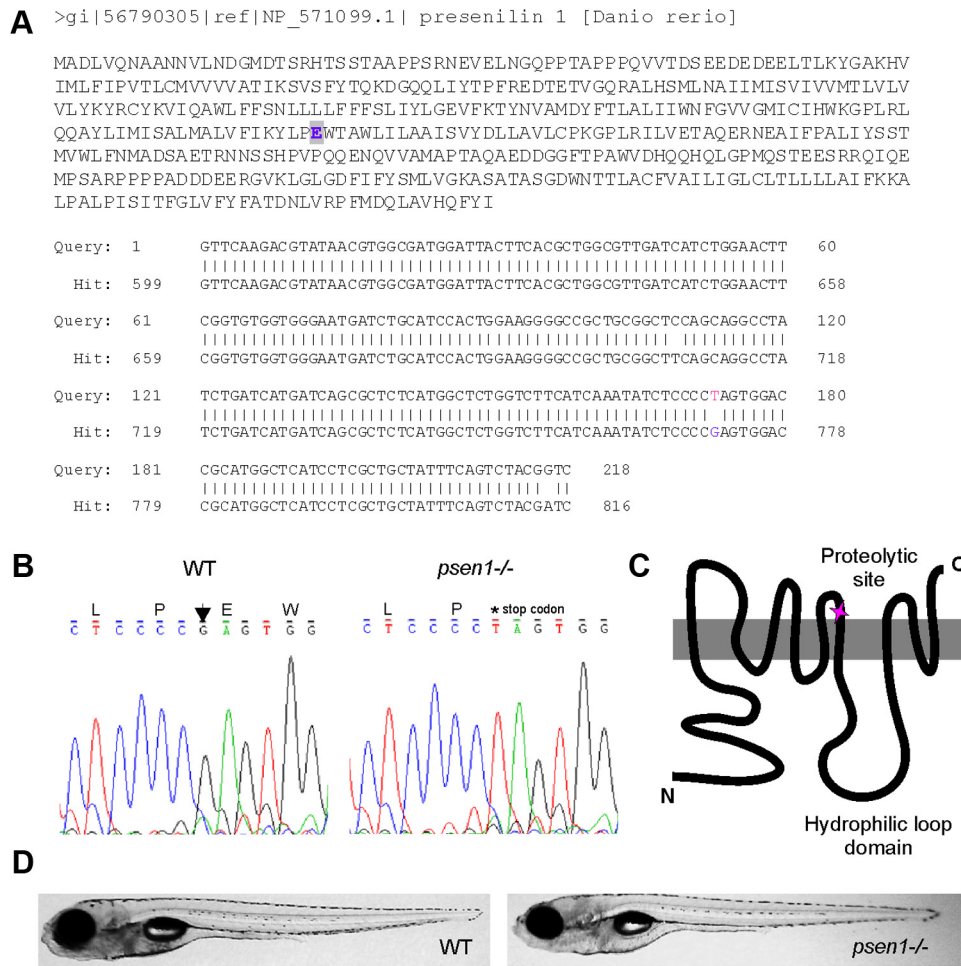
D   WT   *psen1-/-*

Figure 1. Identification of mutation leading to production of truncated PSEN1. ***A***, ***B***, A point mutation in exon 5 induces a premature stop codon. ***C***, Structure of the transmembrane regions of PSEN1. The mutation in *psen1*$^{-/-}$ fish is indicated with a star on amino acid E within the third extracellular loop. The image is modified (Dillen and Annaert, 2006). ***D***, Gross phenotype of *psen1*$^{-/-}$ and WT fish at 7 dpf.

tem, including its receptors, is highly similar to that of other vertebrates, and histamine receptor ligands affect behavior in both adult and larval zebrafish (Peitsaro et al., 2003; Peitsaro et al., 2007). PSEN1 is conserved in many species, including zebrafish (Leimer et al., 1999). We hypothesized that the histaminergic system might be affected during the development of zebrafish carrying a mutation in a FAD-associated disease gene, *psen1*. We identified psen1 as a regulator of the development of histaminergic neurons in a complex manner and found behavioral alterations that match the changes observed in histaminergic neurotransmission in *psen1*$^{-/-}$ fish.

Materials and Methods

Animals. The permits for the experiments were obtained from the Office of the Regional Government of Southern Finland in agreement with the ethical guidelines of the European convention. The wild-type (WT) animals used for this study were of the TL strain, and *psen1*$^{-/-}$ fish (PS1 hu2547) were made in a TL background by TILLING at the Hubrecht Laboratory (Utrecht, The Netherlands). The mutation was identified by RT-PCR and sequencing as described below. This fish strain was a kind gift from Dr. Dana Zivkovic (Hubrecht Institute, Utrecht, The Netherlands). The experiments were done on either sex. The gender effect was not taken into account when assessing the histaminergic system, as no significant difference between sexes has been found (Sundvik and Panula, 2012).

Behavioral assay. To assess the effect of histamine on behavior, two behavioral assays were used. First the basic locomotor activity and place

preference was observed during 10 min in both larvae (6-well plate) and adult zebrafish. This method has been described earlier (Peitsaro et al., 2003; Panula et al., 2006; Peitsaro et al., 2007). In the second behavioral test we studied whether light *per se* affects the activity of the mutant fish as has been observed in histidine decarboxylase (*hdc*) morphant fish (Sundvik et al., 2011). The response to light of *psen1* mutants was assessed by observing 6 d postfertilization (dpf) fish during 2 min periods of lights on and off for a period of 30 min. All data were further analyzed with the EthoVision software (Noldus Information Technologies) and GraphPad Prism (GraphPad Software).

Genotyping. DNA was isolated from fin clips of adult zebrafish with SZL buffer and stored at −80°C until further processing. Standard touchdown RT-PCR was used for verifying the mutation in *psen1* hu2547 fish. The following primers were used: GAAACCCATTTGGGAAGTG and AATACAAGCCTACACACAACC for the first RT-PCR and primers AGGAAACAGCTATGACCAT GAACGCAGAAGAATGAACG and TGTAAAACGACGGCCAGT CAAGTTTGACATTTGCATGG for the second RT-PCR.

Immersion with DAPT. DAPT (*N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester) in solution (Tocris Bioscience) was diluted in DMSO to reach a final concentration of 2.5 mM. At 1 dpf, 30 fish per treatment group were dechorionated and moved to 6-well plates and kept in 3 ml of embryo medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$, 0.33 mM MgSO$_4$; all reagents were from Sigma-Aldrich) containing either 1% DMSO or DAPT.

RNA isolation and quantitative RT-PCR. RNA was isolated from 10 or 30 pooled 7 dpf larvae for a single sample. The RNA was isolated by

RNeasy Mini Kit (Qiagen). RNA quality and amount were analyzed spectrophotometrically and cDNA was prepared by SuperScript III (Invitrogen). Primers used for quantitative RT-PCR were galanin (forward, GA CCAACTGATACTCAGGATGCA; reverse, ATCCCGAGTGTTTCTGT CAGAA) and β-actin (forward, CGAGCAGGAGATGGGAACC; reverse, CAACGGAAACGCTCATTGC) (Keegan et al., 2002).

In situ *hybridization*. In situ hybridization was preformed according to the protocol of Thisse and Thisse (Thisse and Thisse, 2008) with several modifications as described previously (Chen et al., 2009; Kudo et al., 2009). *hdc* probe was produced as described (Sundvik et al., 2011). The Notch1a plasmid was a kind gift from Dr. Michael Brand (Biotec, Biotechnology Center TU Dresden, Dresden, Germany). *Notch1a* RNA digoxigenin-labeled probes were produced according to the abovementioned protocols using XbaI (BioLabs Inc.), and *in situ* hybridization was preformed following standard *in situ* hybridization protocols (Thisse and Thisse, 2008).

Immunohistochemistry. Immunohistochemistry on both larvae and adults was done following the protocol outlined in (Kaslin and Panula, 2001). The following antibodies were used: rabbit anti-histamine 19C, 1:10000 (Panula et al., 1990); rabbit anti-active caspase-3, 1:500 (clone C92-605, catalog no. 559565, BD Biosciences); mouse anti-tyrosine hydroxylase 1, 1:1000 (LOT22941, DiaSorin); rabbit anti-galanin, 1:1000–1:5000 (catalog no. AB1985/AB5909, Millipore); rabbit anti-β-catenin, 1:1000 (clone 2206, LOT056K4763, Sigma-Aldrich); rat anti-serotonin, 1:250 (MAB352, Millipore); the secondary antibodies were the highly cross-purified Alexa Fluor-conjugated antibodies (488 or 568 fluorophores, Invitrogen). The use of these primary antibodies in zebrafish has been reported elsewhere: rabbit anti-histamine 19C (Eriksson et al., 1998); rabbit anti-active caspase-3 (Kratz et al., 2006); mouse anti-tyrosine hydroxylase 1 and rat anti-serotonin (Kaslin and Panula, 2001); anti-galanin (Sundvik and Panula, 2012); rabbit anti-β-catenin (Matsuda and Chitnis, 2009; Tallafuss et al., 2009). As all of the histamine neurons are located extremely ventrally in the hypothalamus, we carried out whole mount immunostaining of whole adult brains to visualize the entire three-dimensional structure and to obtain true numbers at histamine-immunoreactive neurons as described in detail (Sundvik and Panula, 2012).

Microscopy and image analysis. An inverted light microscope, Leica DM IRB, with Leica Application Suite, MultiFocus option (Leica Microsystems GmbH) was used to acquire pictures of the samples where the mRNA expression was detected by in situ hybridization. A confocal microscope, Leica TCS SP2 AOBS, was used to visualize and quantify the histaminergic system detected by immunohistochemistry in both larval and adult zebrafish brains, as well as the immunoreactivity for caspase-3, tyrosine hydroxylase 1, galanin, serotonin, and β-catenin. Excitation wavelength for samples with Alexa Fluor 488 fluorophore was 488 nm, and the emission was collected from 500 to 550 nm. The excitation wavelength used for samples stained with Alexa Fluor 568 fluorophore was 561 nm, and the emission was collected at 600–700 nm. Maximum projection images of the acquired stacks were produced with Leica software to render the 3D material 2D. Quantification of the neuron numbers in the 3D material was done in Fiji software. All pictures were collected into panels in CorelDraw (Corel).

Statistical methods. Comparisons between two groups were done with Student's *t* test. Comparisons between several groups were done with one-way ANOVA, two-way ANOVA, and repeated measures two-way ANOVA with appropriate *post hoc* tests. The data in graphs were presented as mean ± SEM.

Results

Genotyping

The zebrafish *psen1-null* mutant was identified with TILLING (Wienholds et al., 2003) by Dr. Dana Zivkovic's Laboratory at Hubrecht Institute, Developmental Biology and Stem Cell Research, Royal Netherlands Academy of Arts and Sciences and University Medical Center Utrecht, The Netherlands. The mutation was localized to exon 5 where it induced a point mutation resulting in a premature stop codon by exchanging a
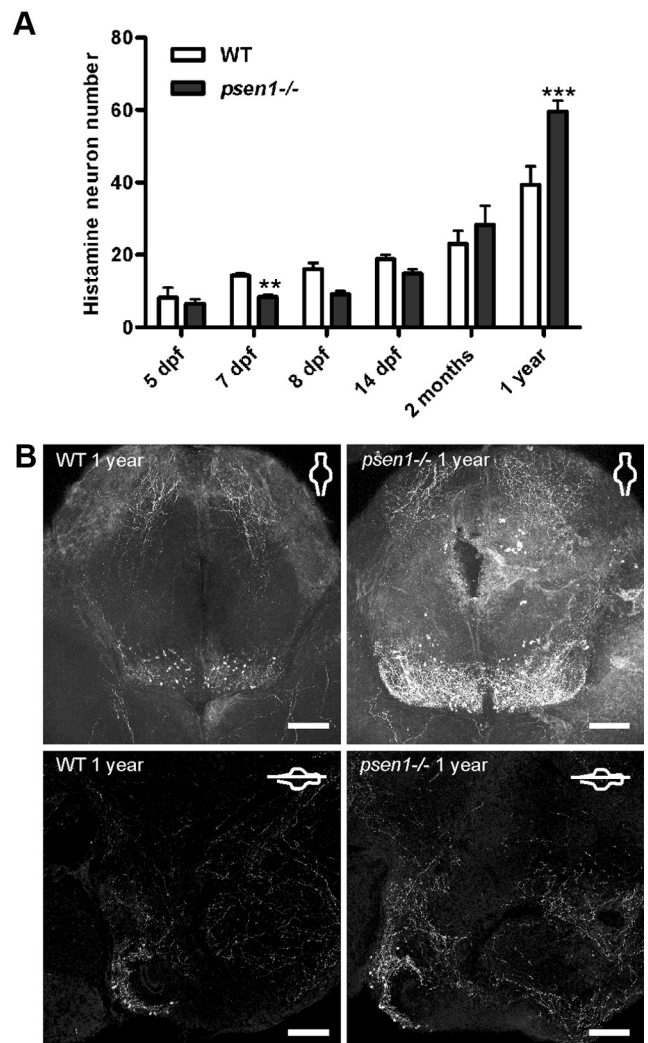

Figure 2. The histaminergic neurons at different ages in *psen1* $^{-/-}$ and WT zebrafish. ***A***, Histamine neuron numbers at different ages. The graph shows mean values ± SEM (5 dpf, $n = 4$–6; 7 dpf, $n = 31$–32; 8 dpf, $n = 5$; 14 dpf, $n = 11$–12; 2 months old, $n = 4$–5; 1 year old, $n = 11$; significance between genotypes is only shown at specific time points; **$p \leq 0.01$, ***$p \leq 0.001$, two-way ANOVA, Bonferroni *post hoc* test). ***B***, The top panels are from whole mount brains of 1-year-old *psen1* $^{-/-}$ and WT fish, and the bottom panels are sagittal sections of fish of the same age. Whole mounts were used to enable counting of the total neuron number, and sections were used to visualize the extremely ventral and superficial location of all the histaminergic neurons. Scale bar, 100 μm.

guanine to thymine in the codon that in normal cases codes for glutamic acid (Fig. 1*A*,*B*). The truncated protein in the mutants lacks the proteolytic site of PSEN1 and the C terminus (Fig. 1*C*). The gross phenotype of the *psen1* $^{-/-}$ fish was normal (Fig. 1*D*) at 7 dpf. As these individuals were viable and developed normally to adulthood in contrast to the mouse *psen1* knockout animals (Shen et al., 1997), the *psen1* mutant fish offers an excellent tool to understand the role of *psen1* in development and neural functions.

Development of the histaminergic system from larvae to adulthood in *psen1* $^{-/-}$

The histamine-immunoreactive neurons were counted at different ages of larval and adult fish (Fig. 2*A*). A significant interaction between time and genotype was found (two-way ANOVA, $p < 0.0001$, $F = 13.40$, DF$n = 5$, DF$d = 125$, where n is numerator and d is denominator of the degrees of freedom, DF). No signif-
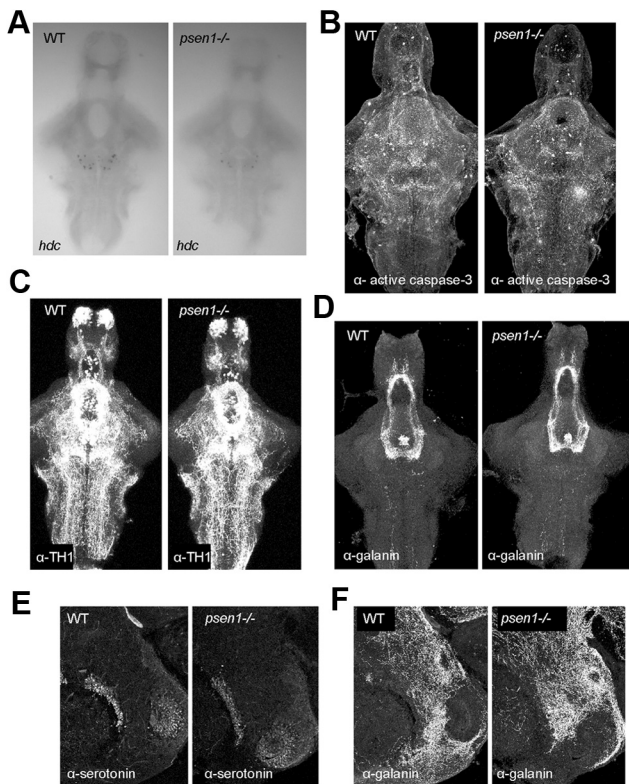
Figure 3. Neurotransmitter systems in *psen1*$^{-/-}$ and WT zebrafish. *A*, Representative images of *hdc* mRNA expression in 7 dpf fish brains. *B*, Active caspase-3 immunoreactivity in 7 dpf fish brain, $n = 10$. *C*, Tyrosine hydroxylase 1 (TH1) immunoreactivity in 7 dpf fish brain, $n = 6 – 8$. *D*, Galanin immunoreactivity in 7 dpf fish brain, $n = 14$. *E*, *F*, Serotonin immunoreactivity (*E*) and galanin (*F*) immunoreactivity were unaltered in the posterior hypothalamus in both genotypes at adult stage (~2.5 years), $n = 3$.

Figure 4. Behavior of larval zebrafish. *A*, Basic locomotor activity is not changed in *psen1*$^{-/-}$ fish compared with WT fish at 5 dpf. Mean values ± SEM ($n = 17–21$, $p > 0.05$, Student's t test). *B*, At 7 dpf the *psen1*$^{-/-}$ fish move significantly less than WT fish. Mean values ± SEM (WT $n = 62$, *psen1*$^{-/-}$ $n = 128$, ***$p \le 0.001$, Student's t test). *C*, Dark-induced flash response in WT larvae at 6 dpf. Mean values without standard errors are indicated in the graphs ($n = 11$). *D*, *psen1*$^{-/-}$ larvae fail to respond consistently to darkness and lack a dark-induced flash response at 6 dpf. Mean values without standard errors are indicated in the graphs ($n = 9$).

icant difference was observed in the number of histamine neurons between the WT and *psen1*$^{-/-}$ fish at 5 dpf, but at 7 dpf the histamine neuron number in *psen1*$^{-/-}$ fish was significantly lower compared with that of WT fish (two-way ANOVA, Bonferroni post test, **$p \le 0.01$). At 2 months of age, just before the fish reach sexual maturity, no significant difference was observed between the *psen1*$^{-/-}$ and WT fish. In a sagittal view of the adult brain (Fig. 2*B*, bottom) it was possible to observe the location and morphology of the histaminergic neuron population. Imaging all neuron populations throughout the entire whole mount adult brains is not possible at the moment, but imaging the entire histaminergic neuron population was possible because the depth (ventral to dorsal) of the system is about 200 μm. This approach allowed us to quantify the histamine neuron number in the brain without sectioning, and thus reveal the complete three-dimensional structure of the nucleus. When histamine neuron number was counted in the brains of 1-year-old zebrafish, a significantly higher (about 30%) number of histamine-immunoreactive neurons was observed in *psen1*$^{-/-}$ fish compared with WT fish (two-way ANOVA, Bonferroni *post hoc* test, ***$p \le 0.001$, Fig. 2*A*,*B*). We further assessed the expression of *hdc* mRNA by *in situ* hybridization in 7 dpf larval brains. These results (Fig. 3*A*) confirmed the findings observed in the immunostained brains; at 7 dpf there were significantly fewer cells expressing *hdc* mRNA in *psen1*$^{-/-}$ fish than in WT fish (Student's t test, $p \le 0.001$, $n = 24$/group). This lower number of histaminergic neurons was not associated with apoptotic cell death, as activated caspase-3 immunohistochemistry could not show any
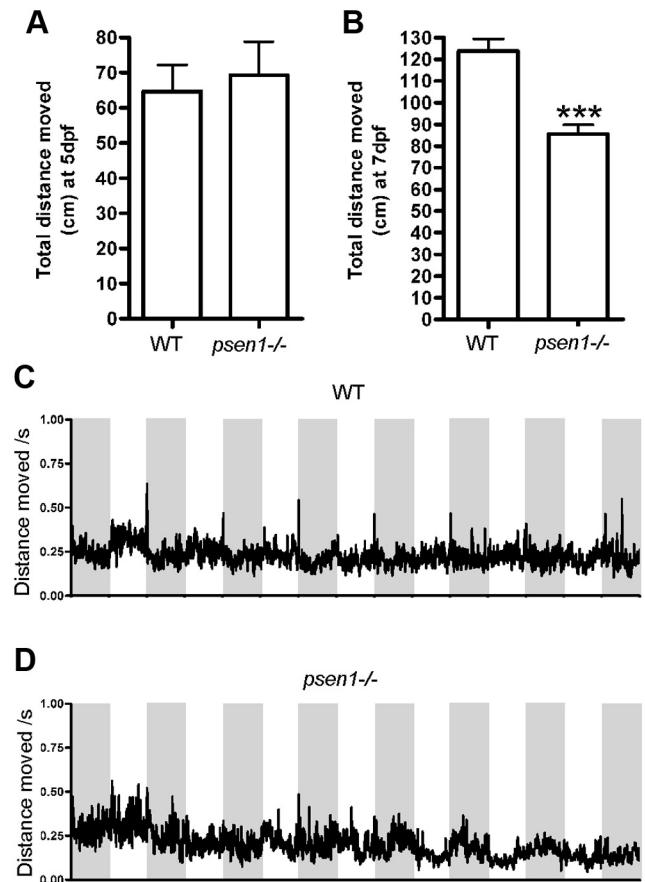
difference between WT and mutant line at 7 dpf (Fig. 3*B*). To ensure that the effect seen was specific to the histaminergic system and not a general defect, we also studied the dopaminergic, serotonergic, and galaninergic neurotransmitter systems. Tyrosine hydroxylase 1 immunohistochemistry did not reveal any major difference in the dopaminergic neuron populations (Fig. 3*C*) between WT and mutant fish at 7 dpf. *Galanin* mRNA, as observed by quantitative RT-PCR, was also unchanged between the mutant and WT (results not shown), as was the expression of the galanin peptide at 7 dpf (Fig. 3*D*). Both serotonin (Fig. 3*E*) and galanin (Fig. 3*F*) immunoreactivity in the posterior hypothalamus was unaltered between genotypes in adult animals. These results show that the *psen1*$^{-/-}$ affects specifically the development and maturation of the histaminergic system.

Behavior

Histamine is known to mediate several behaviors that are altered in AD. Here we studied a repertoire of behaviors that have earlier been documented as histamine dependent (Peitsaro et al., 2003; Peitsaro et al., 2007; Sundvik et al., 2011).

In larvae, we started by assessing the basic locomotor activity. At 5 dpf, when there was no difference in the number of histamine neurons (Fig. 2), there was also no difference in the distance
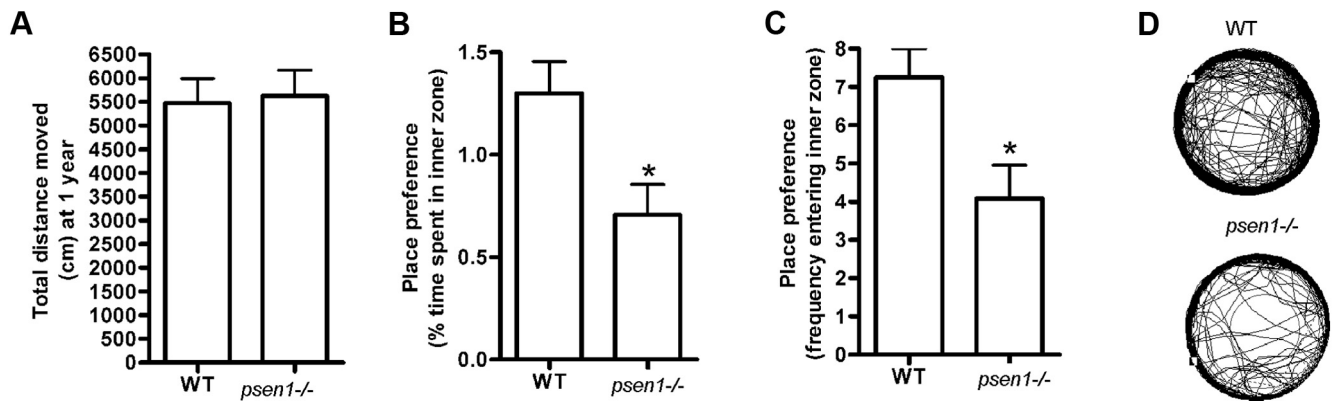
Figure 5. Behavior of adult zebrafish. *A*, Basic locomotor activity is not different in WT and *psen1*$^{-/-}$ adult fish. *B*, Place preference of *psen1*$^{-/-}$ fish compared with WT fish is altered, rendering the *psen1*$^{-/-}$ fish less willing to enter the inner zone of an arena, expressed as time spent in inner zone. *C*, The frequency of entering the inner zone is also reduced in *psen1*$^{-/-}$ fish compared with WT fish. Mean values ± SEM are indicated in all graphs ($n = 13$–16, * $p \leq 0.01$, Student's *t* test). *D*, Characteristic swim tracks of two individual fish.

moved between *psen1* mutants and control larvae (Fig. 4*A*). At 7 dpf, an age when histamine neuron numbers were decreased in the mutant fish (Fig. 2), we observed also a decrease in total distance moved in *psen1*$^{-/-}$ fish (Fig. 4*B*). The place preference of the larval zebrafish was altered, and *psen1*$^{-/-}$ spent more time in the inner zone of the arena compared with WT siblings at 5 dpf ($p \leq 0.01$, Student's *t* test; mean ± SEM of WT, 18.79 ± 3.486, $n = 18$; mean ± SEM of psen1$^{-/-}$ larvae, 38.49 ± 5.859, $n = 16$), although no significant change in histamine neuron number was observed. At 7 dpf, when the psen1$^{-/-}$ larvae showed a reduction in locomotor activity, the mutants showed a preference for the edges of the arena as they avoided the inner zone ($p \leq 0.05$, Student's *t* test; mean ± SEM of WT, 33.42 ± 6.453, $n = 18$; mean ± SEM of psen1$^{-/-}$ larvae, 15.52 ± 4.389, $n = 16$). Hence, either the lower number of histamine neurons or the reduced spontaneous locomotion affected the place preference at 7 dpf. Since the histamine neuron number was lower in *psen1*$^{-/-}$ at a young age, we assessed the response of larvae to short pulses of dark and light, and found that *psen1*$^{-/-}$ fish responded in the same manner as *hdc*-deficient zebrafish (Sundvik et al., 2011). The *hdc*-deficient fish lack histamine almost completely (Sundvik et al., 2011), whereas the *psen1* mutants displayed a significant decrease in histamine neuron number. This difference might explain why the dark-induced flash response was partially absent in the *psen1*$^{-/-}$ fish (Fig. 4*C*,*D*, repeated measures two-way ANOVA, $p = 0.0014$; $F = 1.72$, DF$n = 50$, DF$d = 2300$ for the initial dark-induced flash response). The locomotor activity, i.e., total distance moved, of adult *psen1*$^{-/-}$ zebrafish was not different in comparison to the WT fish (Fig. 5*A*). The *psen1*$^{-/-}$ fish exhibited, however, a difference from control fish in their place preference: they avoided the inner zone of the tank (Fig. 5*B*). Also, the frequency of entering the inner zone was altered as *psen1*$^{-/-}$ adult fish entered the inner zone of the tank significantly fewer times than adult WT fish (Fig. 5*C*). This was also observed when the movement pattern of the fish was studied. Representative tracks of the movement are presented (Fig. 5*D*).

Development of histaminergic system is dependent on γ-secretase activity

psen1 is thought to mainly affect Notch signaling during development in zebrafish as *psen1* morphants exhibit a similar phenotype as that observed in Notch pathway mutants (Nornes et al., 2008, 2009). Inhibition of the γ-secretase complex by DAPT has therefore been used in several reports to modify the Notch sig-
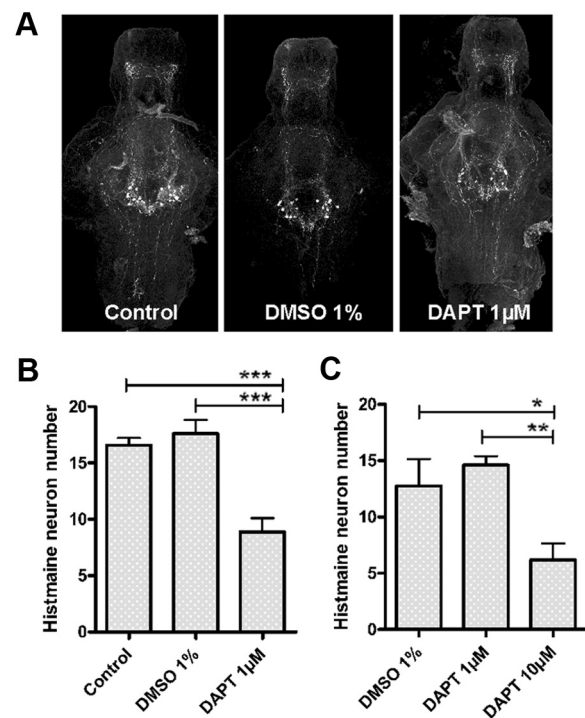

Figure 6. γ-Secretase inhibitor DAPT impairs the development of the histaminergic neurons. *A*, Treatment of zebrafish larvae from 1–5 dpf with DAPT impaired the development of histaminergic neurons as visualized with immunohistochemistry. *B*, Quantification reviled significantly fewer histamine neurons following DAPT treatment of WT larvae. Mean values ± SEM ($n = 7$–10, ***$p \leq 0.001$, one-way ANOVA, Tukey's *post hoc* test). *C*, Also a shorter treatment (48 h) with DAPT at a later stage of development, from 6–8 dpf, impaired the development of histamine neurons ($n = 8$–10, *$p \leq 0.05$, **$p \leq 0.01$, one-way ANOVA, Tukey's *post hoc* test). All graphs show mean values ± SEM.

naling pathway (Nornes et al., 2009; Yang et al., 2010, Dias et al., 2012). We found that inhibiting the activity of γ-secretase with 1 μM DAPT from 1–5 dpf affects the development of the histaminergic neurons. The histamine neuron number was lower in DAPT-treated animals than nontreated animals or those treated with the solute of DAPT, DMSO (Fig. 6*A*). Quantification revealed significantly fewer histamine neurons following DAPT treatment (***$p \leq 0.001$, one-way ANOVA, Tukey's *post hoc* test) compared with both untreated control and DMSO-treated larvae (Fig. 6*B*). Also, a shorter treatment (48 h) with a higher dose of
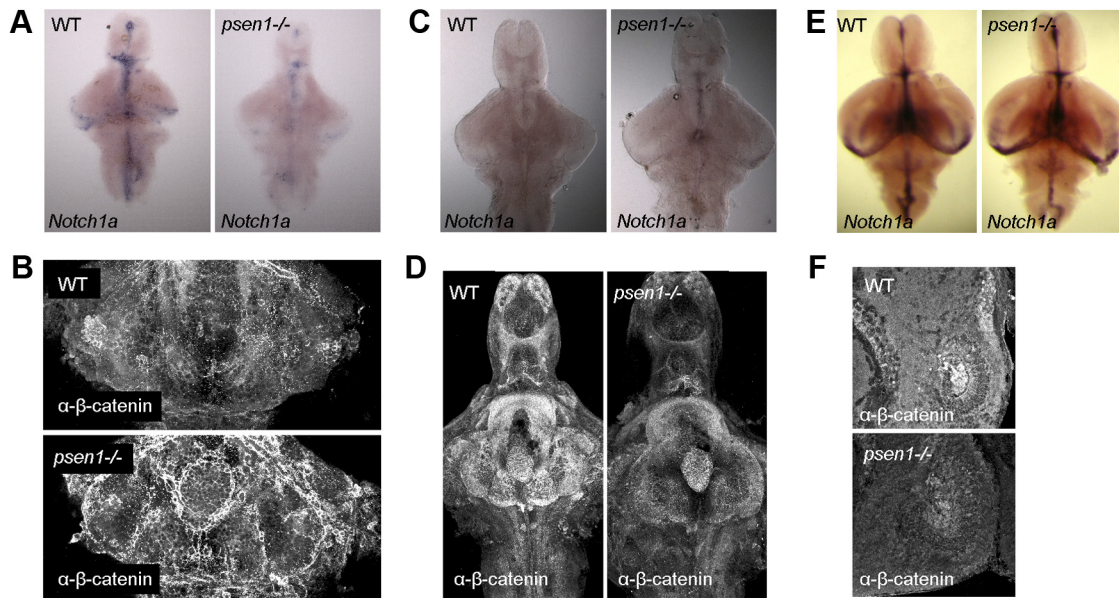
Figure 7. Signaling pathways affected by γ-secretase. **A**, **B**, Reduced *Notch1a* mRNA (**A**) and increased β-catenin (**B**) immunoreactivity in 7 dpf *psen1*$^{-/-}$ larvae when compared with WT fish. $n = 6 – 9$/group. **C**, **D**, Increased *Notch1a* mRNA (**C**) and reduced β-catenin (**D**) immunoreactivity at 14 dpf in *psen1*$^{-/-}$ larvae when compared with WT fish. **E**, **F**, At 1 month of age the *Notch1a* mRNA expression pattern and intensity were similar in the two groups (**E**), whereas at ∼2.5 years the β-catenin immunoreactivity was lower in the posterior hypothalamus of the *psen1*$^{-/-}$ animals compared with WT animals (**F**); $n = 4 – 7$/group in **C–F**.

DAPT (10 μM) at a later stage of development, from 6–8 dpf, impaired the development of histamine neurons compared with DMSO control group (*$p \leq 0.05$, **$p \leq 0.01$, one-way ANOVA, Tukey's *post hoc* test, Fig. 6C). Although there were significantly fewer histamine neurons in the animals treated with DAPT, the smaller number of neurons did not cause an impairment of the dark-induced flash response (repeated measures two-way ANOVA, $p = 0.7183$, $F = 0.91$, DF$n = 98$, DF$d = 1519$) when compared with WT animals. The reduction of the number of histamine neurons obtained with DAPT treatment (1–5 dpf) was not as large as that seen in *psen1*$^{-/-}$ mutants. Notch and Wnt/β-catenin are major signaling pathways important during embryonic development and in the adult brain (Inestrosa and Arenas, 2010; Ables et al., 2011). PSEN1 is necessary for degradation of β-catenin (Kang et al., 2002), and an impairment of PSEN1 function causes an increase in β-catenin (Xia et al., 2001). We therefore assessed further the expression of *Notch1a* mRNA by *in situ* hybridization and β-catenin immunoreactivity in 7 dpf larval brains, as both signaling pathways are regulated by γ-secretase function. *Notch1a* mRNA was strongly reduced in the *psen1*$^{-/-}$ larval brains compared with the level observed in WT zebrafish brains (Fig. 7A). Immunohistochemistry of β-catenin, revealed an increase in the β-catenin immunoreactivity in hypothalamus of *psen1*$^{-/-}$ fish compared with WT fish (Fig. 7B). At 14 dpf, *Notch1a* mRNA was slightly increased in the *psen1*$^{-/-}$ animals compared with WT fish (Fig. 7C), and finally at 1 month of age the *Notch1a* mRNA was similarly expressed in both genotypes (Fig. 7E). The β-catenin immunoreactivity was reduced in *psen1*$^{-/-}$ animals compared with WT animals at 14 dpf (Fig. 7D), and the same effect was observed in the posterior hypothalamus of adult *psen1*$^{-/-}$ animals when compared with WT animals (Fig. 7F). Taken together, these results indicate that γ-secretase activity is essential for the development of histaminergic neurons.

Discussion

We found that, in a zebrafish mutant carrying a premature stop codon in the *psen1* gene, the histaminergic neurotransmitter sys-

tem was significantly altered during the development and in adulthood. The change in the histamine system had measurable behavioral consequences in both larval and adult zebrafish. In agreement with these findings, we found that a dysfunctional γ-secretase complex regulates the development of histaminergic neurons via Notch signaling.

The ortholog of human PSEN1 in zebrafish was identified over a decade ago (Leimer et al., 1999). In contrast to *psen1* knockout mice, which die at birth (Shen et al., 1997), *psen1*$^{-/-}$ mutant zebrafish are viable and fertile. Unlike the *psen1* knockout mice, which have major skeletal and CNS deficits (Shen et al., 1997), we did not observe any major defects in the gross morphology of *psen1*$^{-/-}$ zebrafish in comparison with WT zebrafish. Inhibition of *psen1* function in zebrafish with morpholino oligonucleotides (MOs) induces defective brain development (Nornes et al., 2003). Transient disruption of *psen2* function with a MO has been suggested to have a stronger effect on Notch signaling in zebrafish embryos compared with mammals (Nornes et al., 2009). This might explain why the *psen1*$^{-/-}$ zebrafish does not show the same gross morphology as *psen1* knockout mice. The cholinergic neurotransmitter system is profoundly affected in AD patients (Whitehouse et al., 1982). Several postmortem studies have shown that other systems are also affected in brains of AD patients, including the histamine system in which a reduction in histamine levels (Panula et al., 1998) and accumulation of neurofibrillary tangles (Saper and German, 1987; Airaksinen et al., 1991) have been observed. We found that at a young age *psen1*$^{-/-}$ zebrafish had fewer histaminergic neurons compared with WT zebrafish, and this was associated with a disruption in a specific histamine-mediated behavioral response. The *psen1*$^{-/-}$ larval fish were less observant of their environment and did not consistently exhibit the dark-induced flash response as did WT larvae. These results are supported by previous studies that show that histamine is a wakefulness promoting factor, a mediator of vigilance and cognitive performance (Haas and Panula, 2003; Haas et al., 2008; Sundvik et al., 2011). Cognitive impairments are

a major health risk and have a multifaceted etiology closely associated with aging in humans. The brains of *psen1* knockout mice are significantly smaller (Shen et al., 1997), a result indicative of an impairment of neurogenesis and neuronal proliferation in *psen1* deficient animals. We found that in *psen1*$^{-/-}$ fish the *Notch1a* mRNA expression was reduced compared with WT controls. In WT fish, γ-secretase inhibitor DAPT induced the same phenotype as that observed in *psen1*$^{-/-}$ fish; there were fewer histaminergic neurons at the developmental stage when *psen1*$^{-/-}$ and/or DAPT-treated fish were compared with WT and/or control. Nevertheless, DAPT treatment did not abolish the dark-induced flash response as in *psen1*$^{-/-}$ mutants or in *hdc* morphants (Sundvik et al., 2011). This is likely due to a smaller reduction in histamine cell numbers than that seen in *psen1*$^{-/-}$ mutants or *hdc* morphants, but the role of unidentified additional factors cannot be excluded. This is, to our knowledge, the first report that describes a factor that affects the development of histaminergic neurons. The intracellular domain of Notch functions as a transcription factor and is essential in the early development as noted for the first time a century ago (reviewed in Artavanis-Tsakonas et al., 1999). We detected *Notch1a* mRNA along the midline rostrocaudal axis in the telencephalic ventricular zone, hypothalamus, and cerebellum of the zebrafish brain at 7, 14, and 28 dpf. These regions are the neurogenic and proliferative regions of the zebrafish brain (Kaslin et al., 2008; Kizil et al., 2011). In the *psen1*$^{-/-}$ zebrafish the expression of *Notch1a* mRNA in these zones was strongly reduced at 7 dpf, suggesting an alteration of neurogenesis and proliferation. The abnormalities in the histaminergic system were likely due to an impairment of the neurogenesis and/or proliferation rather than cell death in the *psen1*$^{-/-}$ fish, because we did not observe increased activation of caspase-3 in the mutants. Furthermore, we assessed other neurotransmitter systems (dopaminergic, serotonergic, and galaninergic) in the *psen1*$^{-/-}$ and WT fish to ensure that the effect observed was specific to the histaminergic neurotransmitter system and not a general effect on several neurotransmitter systems. Notch and Wnt/β-catenin signaling are involved in development of the dopaminergic system in zebrafish (Mahler et al., 2010), but we did not observe any major difference in the dopaminergic neurotransmitter system between the studied genotypes. Taken together, these results show that the phenotype of the *psen1*$^{-/-}$ fish is partially similar to that reported for *psen1*$^{-/-}$ mammalian models (reduced notch1 signaling, Jack et al., 2001) and increased β-catenin (Xia et al., 2001), and partially similar to the results for *psen1* MO-treated zebrafish embryos (van Tijn et al., 2011), as the Notch signaling pathway was altered in both zebrafish models. Intriguingly, we thereby found that the development of the histaminergic neurons is mediated by γ-secretase and most likely via Notch signaling. We then went on to assess the histaminergic system in the adult *psen1*$^{-/-}$ zebrafish, as neurodegenerative diseases such as AD manifest in late life and the whole pathology may not be apparent during early development. We found that in young adult *psen1*$^{-/-}$ zebrafish the histaminergic neuron number was significantly increased compared with WT control fish, whereas the serotonergic and galaninergic systems were unaltered at adult age. This change in neuron number was associated with a histamine-mediated behavior previously reported for adult zebrafish (Peitsaro et al. 2003), as we found that fish with higher histamine neuron numbers had an altered place preference when placed in an open arena. These individuals preferred to spend the time close to the edges of the arena. Adult zebrafish with reduced brain histamine level after treatment with α-FMH (α-fluoromethylhistidine, an inhibitor of the histamine-

producing enzyme HDC) also exhibit an altered place preference, as they spend more time in the middle of an open arena compared with saline-injected control animals (Peitsaro et al., 2003). Therefore, the increase in histamine neuron numbers in the adult *psen1*$^{-/-}$ zebrafish is most likely accountable for this specific change in place preference. Although no significant difference in histamine neuron number was observed at 5dpf, there was a difference in place preference behavior between the mutants and control fish, suggesting that already a subtle change in network balance can affect behavior. At this time it is not possible to exclude the role of other, unknown contributing factors unrelated to histamine. Since the reduced number of histamine neurons in 7 dpf fish and the increased number of neurons in adult fish were both associated with increased thigmotaxis, the mechanism of thigmotaxis may be different in larvae and adults. In adults it has been shown to be histamine dependent (Peitsaro et al. 2003). The mechanism underlying this increase in histamine neuron number in adult *psen1*$^{-/-}$ zebrafish is unknown, but it could be due to a change in proliferation or neurotransmitter respecification.

These results shed new light on the role of *psen1,* an important disease gene, in neurogenesis of an essential transmitter phenotype. The study identifies *psen1* as a key regulator of histaminergic neuron development. The results reveal a life-long brain plasticity in both the structure of the histaminergic system and its functions induced by altered *Notch1* activity as a consequence of *psen1* mutation. A related *Notch1*-dependent mechanism has been previously associated with damage-induced regeneration of motoneurons (Dias et al., 2012). The new histaminergic neurons in aging zebrafish brain may arise as a result of phenotypic plasticity (Spitzer, 2012) or represent newly differentiated stem cells. This study also shows the importance of studying adult animals in addition to developmental stages in characterization of phenotypes of disease gene mutants.

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