β-Amyloid precursor protein-b is essential for Mauthner cell development in the zebrafish in a Notch-dependent manner

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Abstract

Amyloid precursor protein (APP) is a transmembrane glycoprotein that has been the subject of intense research because of its implication in Alzheimer's disease. However, the physiological function of APP in the development and maintenance of the central nervous system remains largely unknown. We have previously shown that the APP homologue in zebrafish (*Danio rerio*), Appb, is required for motor neuron patterning and formation. Here we study the function of Appb during neurogenesis in the zebrafish hindbrain. Partial knockdown of Appb using antisense morpholino oligonucleotides blocked the formation of the Mauthner neurons, uni- or bilaterally, with an aberrant behavior as a consequence of this cellular change. The Appb morphants had decreased neurogenesis, increased notch signaling and *notch1a* expression at the expense of *deltaA/D* expression. The Mauthner cell development could be restored either by a general decrease in Notch signaling through γ-secretase inhibition or by a partial knock down of Notch1a. Together, this demonstrates the importance of Appb in neurogenesis and for the first time shows the essential requirement of Appb in the formation of a specific cell type, the Mauthner cell, in the hindbrain during development. Our results suggest that Appb-regulated neurogenesis is mediated through balancing the Notch1a signaling pathway and provide new insights into the development of the Mauthner cell.

Keywords

APP function; Mauthner cell; Development; Zebrafish; Hindbrain

Introduction

Amyloid precursor protein (APP) is a single-pass transmembrane protein that has been the subject of intense research because of its strong links to Alzheimer’s disease (AD). APP

undergoes complex post-translational processing to liberate peptides of varying length, for most of which the cellular function is yet to be determined (van der Kant and Goldstein, 2015). One of these, the amyloid β (Aβ) peptide is generated by the sequential cleavage of APP by β- and γ-secretases. The 42 amino acid long Aβ peptide aggregates into senile plaques in AD brains, which is suggested to induce tau phosphorylation and finally neurodegeneration, *i.e.*, the complete neuropathology of AD (Hardy and Higgins, 1992). As a consequence, most research has focused on regulating APP cleavage and revealing the downstream pathological processes. Interestingly, recent data suggest a possible involvement of two other β- and γ-secretase-cleaved APP fragments in neurodegeneration i.e. including the extracellular soluble fragments (sAPP) and the intracellular (AICD) domain (Ghosal et al., 2009; Lazarov and Demars, 2012). Beyond its contribution to pathological processes, APP has been implicated in neurite outgrowth and synapse formation (Small et al., 1994; Wang et al., 2009; Young-Pearse et al., 2008), neuronal migration (Young-Pearse et al., 2007), intracellular signaling (Octave et al., 2013; Vogt et al., 2011) and proliferation of neuronal progenitor cells (NPCs) (Itoh et al., 2009). However, the precise physiological function of APP in development and maintenance of a healthy brain remains uncertain and it is yet to be shown whether APP dysfunction *per se* plays a role in neurodegeneration.

Notch is a type I transmembrane receptor that, similarly to APP, depends on γ-secretase-mediated cleavage for the release of the intracellular signaling domain (NICD). Notch signaling has an evolutionary conserved role in maintaining neural stem/progenitor cells and regulating cell fate determination. Through cell-cell interaction between Notch receptors and Delta ligands, Notch act to convert initially homogeneous progenitor cells to different neurogenic fates. Here, cells expressing Delta differentiate to a neuronal fate and prevent neighboring cells from becoming neurons in a process known as lateral inhibition (reviewed by (Artavanis-Tsakonas et al., 1999; Schweisguth, 2004; Shimojo et al., 2011)). Zebrafish and mice lacking

Notch or Delta have excessive differentiation of neurons, while overexpression inhibits neurogenesis (Chitnis, 1995; Haddon et al., 1998; Nikolaou et al., 2009). Thus, Notch is important to maintain the balance between progenitors and neuronal cells.

Not only is the processing of Notch and APP similar, but emerging data also suggests a crosstalk between these two proteins (Fischer et al., 2005; Merdes et al., 2004). As with Notch, an involvement of APP in neural proliferation and differentiation is supported by *in vivo* studies showing that transgenic mice overexpressing human APP have increased cell proliferation, while APP knockout mice display reduced cell differentiation (Hu et al., 2013; Jin et al., 2004; Lopez-Toledano and Shelanski, 2007). It has been suggested that these functions might be regulated through an interaction between Notch and APP (Lazarov and Demars, 2012; Zhou et al., 2011). However, mechanisms behind this interaction remain unclear possibly due to different spatiotemporal requirements of the interaction or redundancy of other APP and Notch proteins (Chen et al., 2006; Fassa et al., 2005; Fischer et al., 2005; Kim et al., 2011; Roncarati et al., 2002).

In this study, we address the role of Appb in the formation of specific reticulospinal neurons in the hindbrain. We have previously shown a requirement of Appb in axonal out-growth and synapse formation of spinal cord motor neurons (Abramsson et al., 2013), supporting previous findings on the role of APP for the formation of neuromuscular junctions (Wang et al., 2005; Wang et al., 2009). Here we show that Appb is essential for Mauthner cell (M-cell; a reticulospinal, RS, neuron) development and hence for the establishment of the escape response circuit. As knockdown of Appb resulted in changed Notch/Delta expression and thus decreased neurogenesis, the M-cell phenotype could be rescued by repression of Notch1a signaling. In conclusion, these results show that Appb negatively regulates Notch1a activity to promote M-cell development.

Material and Methods

Animal husbandry and ethical procedure

Zebrafish (*Danio rerio*) were maintained in Aquatic Housing Systems (Aquaneering, San Diego, USA) at 28°C under a 14-hour light/10-hour dark cycle. Fish were fed twice daily a diet of live-hatched brine shrimp (Artemia) and flake fish food. Breeding of wild-type AB, *Tg(isl1:GFP)* (Higashijima et al., 2000) and *Tg(Appb:GFP)* (Lee and Cole, 2007) fish were carried out under standard conditions; embryos were collected in the morning and raised at ~28.5°C in embryo medium (Westerfield, 2007). Embryos were staged by hours post-fertilization (hpf) or days post-fertilization (dpf) as described previously (Kimmel et al., 1995) and fixed at the desired time points. Before fixation, embryos were anesthetized in 0.02% tricaine (Sigma-Aldrich) for all the experiments. All procedures for experiments were performed in accordance with animal protection standards of the University of Gothenburg and were approved by the ethical committee in Gothenburg.

Morpholino microinjections

A morpholino antisense oligonucleotide (MO) approach was applied to perform transient knockdown of gene expression. An antisense MO (Gene Tools, Philomath USA) targeting zebrafish *appb* (splice acceptor site *appb* MO; 5′-CTCTTTTCTCTCTCATTACCTCTTG-3′) was used as in (Abramsson et al., 2013), where specificity of the knockdown was verified. Notch1a UTR and ATG MOs (*notch1a* MO; 5′-GCCTCGGCGTTACAACTTCTTTAA-3′, 5′-TTCACCAAGAAACGGTTCATAACTC-3′) were mixed together and used as described earlier (Tsutsumi and Itoh, 2007). The knockdown efficacy of the *appb* and *notch1a* MOs was previously described (Abramsson et al., 2013; Tsutsumi and Itoh, 2007). Borosilicate injection needles were prepared using P-97 Flaming/Brown micropipette puller (Sutter Instrument,

Novato, USA). MO injection of 1 nl/embryo was performed directly into the cell at the one cell stage with a FemtoJet® microinjector (Eppendorf AG, Hamburg, Germany). For knockdown experiments, 2.5-3 ng of *appb* MO and 2 ng of *notch1a* MO were injected. As controls, embryos injected with an equal amount of standard control MO (Control MO; 5′-CCTCTTACCTCAGTTACAATTTATA-3′) or uninjected embryos were used.

mRNA rescue experiment

The *appb* plasmid was linearized with *BscI* and full-length *appb* mRNA was synthesized using the mMessage Machine in-vitro transcription kit (Invitrogen) and purified using phenol/chloroform extraction as previously described (Abramsson et al., 2013). For the rescue experiments, embryos were injected with mRNA (50 pg) and/or *appb* MO and examined at 48 hpf for the M-cell phenotypes.

DAPT treatment

Notch signaling was chemically inhibited by DAPT (N-[N-(3,5-Difluorophenacetyl-l-alanyl]-S-phenylglycine-t-butyl ester), a γ-secretase blocker (Geling et al., 2002; Song et al., 2010). DAPT (Calbiochem, La Jolla, CA) was reconstituted in 100% DMSO (dimethyl sulfoxide) to make a stock concentration of 10 mM. Embryos were dechorionated at 6 hpf with 0.5 mg/mL pronase (Sigma-Aldrich) on an agarose-coated Petri dish. To avoid precipitation of DAPT, four-well plates were placed on a shaker (at maximum required speed) containing fish water (60 mg Instant Ocean salt/L distilled water) and DAPT was added while pipetting to achieve a final concentration of 50 µM in 1% DMSO. One % DMSO was used as a vehicle control. During drug exposure, embryos were protected from light and incubated in dark at 28.5ºC until 24 or 48hpf.

Whole-mount antibody immunofluorescence and retrograde labeling

When needed, embryos were incubated in 0.003% PTU (1-phenyl-2-thiourea, Sigma-Aldrich) at 22 hpf to prevent pigmentation. For neurofilament RMO44 antibody stain, embryos were fixed in 2% trichloroacetic acid (Sigma Aldrich, #91230) at 48 hpf for 3 h at room temperature, washed in phosphate-buffered saline (PBS) and blocked in 0.5% Triton X-100, 10% normal goat serum, 0.1% bovine serum albumin (BSA) in PBS for 1 h. Antibody labeling was performed using monoclonal mouse anti-neurofilament 160 RMO44 antibody (Sigma Aldrich, #N2787) followed by goat anti-mouse Alexa Fluor 488 (Invitrogen, #A-11001) as secondary antibody at 1:1000 and 1:500 dilutions, respectively, and incubated overnight (ON) at 4°C. Brains were dissected out (Turner et al., 2014) and flat-mounted in a sandwich of large and small cover slips (Rath et al., 2012) with 80% glycerol. Staining with rabbit anti-phospho-histone H3 (pH3) (Millipore) has been used for proliferation assay, and was performed on embryos fixed in 4% paraformaldehyde (PFA) at 4°C ON, washed in PBS and processed as previously described (Abramsson et al 2013). Double-labeling of M-cell and Appb was performed with the 3A10 antibody in *Tg(Appb:GFP)* fish line. To examine the GFP expression in *Tg(Appb:GFP)* embryos with respect to M-cell, fixed embryos were incubated with a mixture of mouse monoclonal 3A10 (Developmental Studies Hybridoma Bank, University of Iowa, USA) and polyclonal rabbit anti-GFP (Molecular Probes). The procedure was followed as described previously (Hatta, 1992). Alexa Fluor 488 rabbit anti-GFP (Molecular Probes) and Alexa 568-conjugated goat anti-mouse (Molecular Probes) antibodies were used to visualize the signal. Specimens mounted in a cover slip sandwich were imaged using Zeiss LSM710 confocal microscope (Carl-Zeiss, Jena, Germany). Images were analyzed and produced using ImageJ software (National Institute of Health, USA) and Adobe Photoshop CS6 (Adobe).

Live embryos were stained for apoptotic cells with acridine orange (AO, Invitrogen, #A-3568). Dechorionated anaesthetized embryos were placed in 5μg/ml AO diluted in embryo medium for 30 min at room temperature in the dark. Embryos were then washed twice in embryo medium and analyzed under confocal microscope for AO positive cells in the hindbrain.

For retrograde labeling, rhodamine dextran (10,000 MW, Invitrogen, D-1824) was injected into the spinal cord (O'Malley et al., 1996) of anaesthetized animals at 3 dpf. Embryos were imaged at 4 dpf under confocal microscope for dye labeling in the hindbrain.

RNA in situ hybridization

For whole mount *in situ* hybridization (WMISH), embryos were fixed in 4% PFA in PBS at the desired time points, dehydrated in methanol and stored in -20^{o}C until used. WMISH procedure was performed as in (Thisse and Thisse, 2008) with slight modifications. In brief, antisense digoxigenin-labeled RNA probes were synthesized from linearized DNA template against *appb* (Musa et al., 2001), neurogenin 1 (*neurog1*) (Korzh et al., 1998*), deltaA, deltaD* (Haddon et al., 1998), hairy-related 6 (*her6*) (Pasini et al., 2001), *notch1a, notch 1b* (Ke et al., 2008), *krox-20* (Oxtoby and Jowett, 1993), *hoxb1a* (Prince et al., 1998) and *fgf3* (Kiefer et al., 1996). pBS zfhoxb1a (gift from Victoria Prince, Addgene plasmid # 27519). Probes were purified using Illustra MicroSpin G-50 Columns (GE Healthcare Life Sciences). Embryos were rehydrated gradually in PBSTw (0.1% Tween-20 in PBS), permeabilized in protease (Sigma-Aldrich) and re-fixed in 4% formalin. Prehybridization/probe hybridization reactions were carried out at 70°C as optimal temperature and the color reaction was performed using NBT/BCIP substrate (Roche, Country). Eventually, embryos were deyolked and mounted in glycerol as described above. Expression patterns of stained embryos were imaged using a Nikon stereomicroscope

and produced using Adobe Photoshop CS6 (Adobe). For all the evaluations, at least 25-30 embryos were observed.

Behavioral monitoring and electrical stimulation-induced escape response

Control and *appb* MO-injected embryos at 2 dpf (day post-fertilization) were immobilized in 1.7% low melting agarose (Sigma-Aldrich), in a custom-made wax chamber on a glass slide. Agarose was removed gently from tail region to make the tail free to move. Weak electrical stimulations (5 V) were supplied to the head region to induce the escape behavior. Ten electrical stimulations were supplied, one per second, using tungsten electrodes connected to a 2100 pulse stimulator (AM systems Inc, Sequim, WA). Three trials were performed for each animal with 10 min time intervals. The numbers of tail responses were recorded with NIS-Elements (Nikon) and counted manually.

Statistical analysis

Statistical analysis was performed using GraphPad Prism® 6 software. The tail flip response, cell proliferation, apoptotic cells and rhombomere measurements were evaluated statistically using unpaired two-tailed *t*-test. Statistical significance was set at the $p < 0.05$ level.

Results

Distinct expression pattern of appb in hindbrain during development

The expression of *appb* mRNA is localized to axial structures and is most prominent in neural tissues during development (Abramsson et al., 2013; Musa et al., 2001). Strong expression of *appb* mRNA was found in clusters of cells of the hindbrain organized in a segmented pattern. This, in combination with indications that lack of Appb results in defect axonal branching and

thinner M-cell axons (Abramsson et al., 2013; Joshi et al., 2009), led us to examine the role of *appb* in the hindbrain. We started by analyzing the expression of *appb* during rhombomere formation to address the temporospatial changes in expression level (Fig: 1). Detection of *appb* mRNA using *in situ* hybridization showed strong expression of *appb* in clusters of cells in the lateral region of each rhombomere (Fig: 1B-D). This pattern emerged at 19.5 hpf and overlapped with RS neurons showing a ladder-like pattern in the hindbrain (Maves et al., 2002; Wada et al., 2005). We therefore went on to investigate whether these neurons are affected by changes in Appb level.

Loss-of-function of Appb affects rhombomere 4-specific Mauthner cell development

The ladder-like patterning of RS neurons in the hindbrain of the zebrafish can be visualized using retrograde labeling (O'Malley et al., 1996). The largest of these neurons are the M-cells symmetrically positioned on each side of the rhombomere 4 (Hanneman et al., 1988; Metcalfe et al., 1986; Will, 1991). Each M-cell sends their axons contralaterally to the midline of the hindbrain that then descends caudally along the spinal cord (Fig 2A) where it makes connections with inter- and motor-neurons via axon collaterals (Korn and Faber, 2005; Miller et al., 2015). In response to knockdown of Appb, we found a significant loss in the M-cell formation using retrograde labeling (Fig: S1). To verify that the absence of M-cells was not caused by insufficient backfilling of the axons, we also performed immunostaining of RS neurons using the neurofilament RMO44 antibody (Fig: 2A-B'). In control MO-injected embryos; bilateral M-cells were observed in 100% of embryos (Fig: 2A, D). In contrast, the M-cells were absent, either unilaterally (~42%) or bilaterally (~46%) in Appb morphants (Fig: 2B-B'; Table 1) while other RS neurons were grossly unaffected. This finding indicates that Appb is directly or indirectly essential for M-cell formation. Hence, in this study we focused on the M-cells as they were the most dramatically affected neuronal cell type in *appb* morphants.

Immunostaining with RMO44 or 3A10 was used hereafter since this method is less sensitive to technical variation.

Injection of mRNA encoding full-length *appb* rescued the M-cell phenotype of approximately 64% of the *appb* MO knockdown embryos (Fig: 2C-D). Injections of 50 pg *appb* mRNA only did not affect the M-cell number (Fig: 2D). These findings support that the morphant phenotype is a specific effect of *appb* knockdown.

Activation of Mauthner cell circuitry leads to decreased tail response in morphants

We next asked whether the change in the M-cell number observed in *appb* MO-injected embryos resulted in impaired physiological functions. The M-cell circuit plays a vital role in the fast escape response in zebrafish (Eaton et al., 2001; Korn and Faber, 2005). The bilaterally paired M-cells fire in response to sensory stimulation and initiate a strong, rapid contralateral muscle contraction (Eaton et al., 2001), which allows a rapid turn away from threats.

It was previously reported that the head-tactile input activates M-cell-dependent escape before 75 hpf (Kohashi et al., 2012). To mimic this response, we made use of a behavioral test set-up, where weak electrical stimuli were supplied to embryos at 2 dpf to activate the M-cell induced tail flip response (Fig: 3A). We found that most of the controls responded to 10 out of 10 stimuli (Fig: 3B; n=9); whereas a significantly decreased number of tail responses were observed in Appb morphants (Fig: 3B; n=16), in spite of normal muscular structure and function in Appb morphants (Abramsson et al., 2013). These data support that the M-cell is required for the proper tail flip responses in the set-up used here and that the electrical stimulation-induced tail flip response is generated by the presence of M-cell and its activation at 2 dpf. However, we could not differentiate the response of embryos with one M-cell from that of those without M-cells. It is well established that in the absence of M-cell, segmental homologues of the M-

cell in the more posterior hindbrain may drive the escape behavior (O'Malley et al., 1996). Nevertheless, these homologues give rise to responses with longer latency (Burgess and Granato, 2007; Kohashi and Oda, 2008). We conclude that loss of Appb function leads to M-cell mediated behavioral deficits by the.

Knockdown of Appb leads to increased Notch signaling and reduced neural differentiation

At 7.5 hpf, when the M-cell is suggested to form as the first of the reticulospinal neurons (Hanneman et al., 1988), the Appb expression level is high in axial structures (Musa et al., 2001). Previous studies have implicated APP in neurogenesis at both embryonic and adult stages (reviewed by (Lazarov and Demars, 2012). We therefore asked if the reduction in M-cell number result from a reduced neurogenesis in the hindbrain. We analyzed the expression of the proneural genes *neurog1* and *deltaA/D* that are expressed in differentiating neurons and found a reduced expression of *neurog1* and both *delta/D* genes in the hindbrain of *appb* knockdowns as compared to controls (Fig: 4A-C & D-F). These results shows that the generation of neuronal progenitor cells is restricted and suggests that Appb promotes neurogenesis in the hindbrain, possibly through an interaction with the Notch-Delta pathway. The singling out of one M-cell on each side of the hindbrain is mediated via Notch-Delta through a mechanism known as lateral inhibition (Haddon et al., 1998). The importance of this process is clearly shown by zebrafish mutants with repressed Notch signaling such as *deadly seven* (Notch1a) and *mindbomb* (E3 ubiquitin ligase) that resulted in an over production of neurons and thus supernumerary M-cells (Gray et al., 2001; Itoh et al., 2003; Schier et al., 1996). Conversely, activation of Notch1 suppresses expression of *neurog1*, and induces the expression of *her* genes, which eventually causes a reduction in the number of primary neurons (Takke et al., 1999). To address if the Notch pathway was affected we therefore examined the expression of *her6*, a downstream responsive gene of Notch signaling (Pasini et al., 2001) and the gene

expression of *notch1a/b*. We found an increase in the expression of both *her6* (Fig: 5A, D & S2A, D) and *notch 1a* (at 24 hpf, Fig: 5B, E and at 48 hpf Fig: S2B, E) in morphants compared to controls. However, the expression of *notch1b* was not clearly changed at 24 hpf (Fig: 5C, F) and only slightly increased at 48 hpf (Fig: S2C, F). The decreased Delta expression in combination with an increased Notch1a expression and signaling indicates that Appb regulates neurogenesis at least partly through the Notch-Delta pathway.

Lateral inhibition implies that a decrease in Delta expression follows with an increased Notch expression and activity. Therefore, an increased expression of *notch1a* and *her6* is consistent with the Appb morphants showing an increase in Notch signaling.

Finally, it is also possible that the change in M-cell number might be caused by changes in cell death or proliferation. We stained embryos at 2 dpf with AO to label cells undergoing apoptosis. Quantification of AO positive cells displayed no significant difference in the hindbrain of Appb morphants compared to control MO (Fig: S3A-C), indicating that the reduction of proliferation was not due to cell death. However, quantification of embryos stained with pH3 to label cells in M-phase showed a significant decrease in the number of dividing cells in the hindbrain of Appb morphants as compared to staged controls (Fig: 6A-C). Although this result is disparate with the general view of decreased differentiation would allow for an increase in proliferation, APP and its processed forms is involved in promoting proliferation of neuronal stem cells both *in vitro* and *in vivo* (Bolos et al., 2014; Caillé et al., 2004; Demars et al., 2011; Ohsawa et al., 1999). In addition, a few studies also show that increased Notch activity in progenitors inhibits proneural expression, which in turn prevents proliferation and differentiation (Lewis et al., 2009; Reynolds-Kenneally and Mlodzik, 2005).

Taken together, these data show that a reduction in Appb level decreases neurogenesis and proliferation in the hindbrain. The observed changes in Notch signaling and Delta expression suggest that the reduction in M-cells likely is due to disturbed lateral inhibition.

Balanced Notch signaling restores the Mauthner cell number

Our results suggest that M-cell development in the Appb morphants might be suppressed by increased Notch activity. Therefore, we next asked if this phenotype could be rescued by inhibiting Notch signaling. First, Notch cleavage was blocked using the γ-secretase inhibitor, DAPT. The γ-secretase complex cleaves Notch to produce an intracellular fragment, NICD, similarly to the processing of APP to generate the AICD fragment. As reported previously (Song et al., 2010), DAPT increases the number of M-cells in zebrafish. We observed that treating embryos with 50 µM DAPT from 6hpf indeed normalized the M-cell phenotype in Appb morphants back to control levels (Fig: 7B-C, ~67%). Notably, in control embryos, the same dose of DAPT resulted in an increased number of M-cells (Fig: 7A & C, ~53%). Subsequently, we asked if DAPT treatment not only rescues M-cell formation, but also the expression of *ngn1, delta/D* and *her6* in Appb morphants. To analyze this, we treated the embryos with DAPT as described above and performed mRNA *in situ* hybridization for these genes. As expected, DPAT treatment of *appb* MO embryos rescue the expression of *ngn1, delta/D* and *her6* back to the levels comparable to the control MO embryos (Fig: S4).

Several of the Notch family members are expressed in the hindbrain, however; only Notch1a has been shown to be important for M-cell development (Almeida et al., 2011; Gray et al., 2001). To confirm that the effect of DAPT treatment was Notch-dependent and to address which of the Notch receptors were involved, we knocked Notch1a down as previously described (Tsutsumi and Itoh, 2007). Embryos injected with *notch1a* MO and control MO had supernumerary M-cells (>2 cells), as previously described in the Notch1a zebrafish mutants *deadly seven* and Notch1a morphants (Almeida et al., 2011; Gray et al., 2001) (Fig: 8A & C; ~81%). Interestingly, in embryos injected with *appb* MO together with *notch1a* MO with the purpose of balancing the Notch signaling, we found that ~61% of the embryos had a normal M-

cell number as compared to ~17% of the *appb* MO embryos (Fig: 8B-C). Thus, these data show that Notch activity in Appb morphants can be balanced by inhibiting cleavage of Notch by γ-secretase or by decreasing Notch1a levels, indicating that Appb mediates its function in M-cell development through regulating Notch1a activity.

Knockdown of Appb causes alterations in rhombomere size

M-cell development is restricted to rhombomere 4 and is therefore absent in mutants devoid of rhombomere 4. We therefore examined hindbrain segmentation in *appb* MO to exclude that the M-cell phenotype is a side-effect of rhombomeric organization. To test this, we performed *in situ* hybridization using the r3/r5 marker *krox20* (Oxtoby and Jowett, 1993) on control MO and *appb* MO-injected fish. We found no change in the organization of rhombomere r3-r5 but a significant decrease in the anterior-posterior length of r3 and r4 was observed. However, r5 length was comparable to that of controls (Fig: 9 A, B, G).

To address if the identity of r4 is maintained unchanged in Appb morphants, we used the r4 specific markers; *hoxb1a* and *fgf3*. We did not observe any changes in the expression or specificity of either gene in morphants compared to control (Fig: 9C-F). However, as with *krox20*, a shorter length of r4 in *appb* morphants was observed with both *hoxb1a* and *fgf3*. The facial branchiomotor neurons of the hindbrain form early in rhombomere 4 and migrate to rhombomere 7 where they extend axons back to r4 and out to their targets (Chandrasekhar, 2004). The complex development of these neurons is sensitive to changes in hindbrain segmentation. We therefore used the Tg*(isl:*GFP*)* fish line that expresses GFP in the branchiomotor neurons to analyze the differentiation of facial motor neurons (Fig. 9H-I). At 24 hpf we could not detect any change in the spatial arrangement of these neurons. Thus, while the

shorter lengths of r3 and r4 in appb MO might be a consequence of decreased neurogenesis, the loss of M-cells is likely not a result of changed r4 identity.

Appb is expressed at 10 hpf but not in the mature Mauthner cell

In the zebrafish, as in mice, APP begins to be expressed during early development at time points overlapping with the Mauthner cell development (Abramsson et al., 2013; Musa et al., 2001; Salbaum and Ruddle, 1994). To address this spatial expression in detail we analyzed the mRNA expression of Appb with *in situ* hybridization at 10hpf. We found Appb expression in axial structures throughout the embryo and particularly strong expression in the forming hindbrain region (Fig10A1, A2, arrow). As with rodent and human APP, Appb is expressed in several different tissues in addition to neurons. To analyze if Appb is expressed in the mature Mauthner cell we took advantage of the Tg(*Appb*:GFP) transgenic fish line expressing GFP under the control of the *appb* promoter (Lee and Cole, 2007) and performed double labeling of GFP and the Mauthner cell specific antibody 3A10 (Hatta, 1992). Confocal imaging of the dissected hindbrains indicates that the Mauthner cell expresses no or low levels of Appb as compared to surrounding cells (Fig. 10B).

Discussion

In this study, we report that Appb is required for M-cell development in zebrafish. Our data shows that changes in Appb levels affect Notch expression and signaling in the hindbrain Accordingly, decreasing Notch1a activity in *appb* morphants restores neurogenesis and normalizes the M-cell number. This suggests that Appb balances Notch1a signaling to promote neural cell development. To our knowledge, this is the first study that shows an essential requirement of APP for the development of a specific neuronal cell type.

The role of APP during development has been difficult to study likely due to redundancy of other APP family members. However, a comprehensive knowledge on the physiological function of APP is fundamental in deciphering the pathological processes underlying AD. Due to the teleost-specific genome duplication (3R), the zebrafish have two APP homologues, *appa* and *appb*. While the viable phenotype of the *appa* knockdown in zebrafish is similar to APP knockout mice, lack of *appb* is embryonic lethal due to gastrulation defects (Joshi et al., 2009). Despite this apparent dissimilarity in phenotype, studies by us and others support a conserved function of Appb in zebrafish (Abramsson et al., 2013; Song and Pimplikar, 2012). Together this makes zebrafish a unique model to study the *in vivo* function of Appb during development.

In the present study we found that, generation of a hypomorph loss of gene function through partial Appb knockdown is sufficient to inhibit M-cell development without major effects on other reticulospinal neurons. In the hindbrain, the M-cell is the first neuron to form at around 7.5 hpf, slightly before rhombomere 4 becomes visible. As mentioned above, several mutations with abnormalities in Mauthner cell have defective segmentation. In these mutants, the Mauthner cells are either missing due to the lack of rhombomere 4 or mislocalized as a result of changed identity of other rhombomeres. Although rhombomere 4 is changed in size, its maintained identity makes this an unlikely cause of the Mauthner cell defect in *appb* morphants.

The other major group of genes that are important for Mauthner cell development act in the Notch –Delta pathway, The dependence of the Mauthner cell on lateral inhibition through Notch-Delta signaling is well described (Gray et al., 2001; Haddon et al., 1998). Intriguingly, Appb morphants displayed both increased Notch expression and activity as indicated by the elevated expression of its downstream target *her6*. While Notch signaling is essential for maintaining NPCs in the developing brain (Lewis, 1998), over-activation of Notch prevents neural differentiation through down-regulation of the Delta expression (Nikolaou et al., 2009;

Schweisguth, 2004). The sensitivity of the M-cell to changes in Notch signaling has been well described by genetic and chemical manipulations (Gray et al., 2001; Haddon et al., 1998; Song et al., 2010); inhibition of Notch results in an overproduction of M-cells, whereas fewer M-cells form if Notch signaling is increased. This is in accordance with our data where the effect of an over-activation of Notch could be restored by DAPT treatment to normalize proneural gene expression and thus M-cell number.

The absence of markers for the early development of the M-cell restricts our ability to follow processes preceding 18 hpf. However, the detected decrease in the expression of the proneural gene *neurog1* and the Notch ligands *deltaA/D* at later stages indicates that Appb promotes neurogenesis *in vivo* by interacting with the Notch-Delta pathway. Although a general reduction in Notch restored the expression of proneural genes and the M-cell phenotype in morphants the broad effect make it Consistent with this hypothesis, the expression of Notch1a, known to be essential for M-cell development, but not Notch1b, was found to be increased in Appb hypomorphs. This suggested that Appb might act by inhibiting Notch1a activity in NPC such that the defective M-cell development might be rescued by a general decrease in Notch1a levels. The rescue of Mauthner cells when Notch1a levels were restored and the normalized expression of proneural genes in morphants with a general decrease in Notch signaling. Indeed, a partial knockdown of Notch1a rescued M-cell development of the Appb morphants. The mechanism by which Appb conveys its effect on M-cell development could either be parallel to or in the same pathway as Notch. The increase in Notch signaling in Appb morphants however suggests that Appb acts in the same pathway as Notch1a. Notch activation increases the transcription of *her/hes* genes that in turn repress the proneural genes such as *neurog1* and Delta genes. Consistent with this we found a lower expression of both *neurog1* and *deltaA/D* that thus maintains the expression of Notch in progenitor cells to keep them in an undifferentiated state. These data support previous studies in which mice overexpressing APP exhibit NPC with

greater potential to differentiate into neurons, while APP knock-out mice showed decreased neural differentiation (Hu et al., 2013). The molecular mechanism behind this process has been unclear, however our data suggests that at least part of the Appb-stimulated neurogenesis is mediated through functional interaction with Notch.

Finally, it is interesting to note that embryos with reduced Appb levels exhibit phenotypes such as somite, motor neuron and vascular defects (Abramsson et al., 2013; Cameron et al., 2012; Joshi et al., 2009) which are also observed in Notch-Delta signaling mutants (Gore et al., 2012; Gray et al., 2001; Schier et al., 1996). This implies that these phenotypes might be the effect of Appb on Notch signaling during development of these tissues. In conclusion, the reduced neurogenesis resulting from a change in Appb-Notch ratio is therefore a likely explanation for defective M-cell formation in Appb morphants.

In contrast to the clear effect of *appb* knockdown on M-cell development, the formation of other RS neurons was mainly unaltered. Considering that the *des* mutant exhibits defects in several other RS neurons, it is likely that the partial knockdown of Appb creates a milder phenotype than that seen in the *des* mutants. Since Appb has a spatiotemporal expression that allows for its involvement in both M-cell development and the development of other RS neurons, the more severe effect of *appb* knockdown on M-cells suggests that RS neurons might require less Appb or that other factors make them less susceptible. Another explanation could be that the initial M-cell determination proceeds normally and that the critical period of Appb function is at later stages in the developmental process. Our observations that there are no general changes in cell death or facial motor neuron differentiation in Appb morphants suggest that Appb is required in very early stages of M-cell formation. In support for an early role of Appb are results from *in vitro* studies that show an increased expression of APP in NPCs as they mature into neurons and that APP promotes neural differentiation of embryonic stem cells

(Freude et al., 2011; Porayette et al., 2009). However, the lack of markers for the early M-cell development prevents us from concluding on the exact time point when Appb is needed.

Contrary to the well-established role for Notch in neurogenesis and neuronal differentiation in mammals, the mechanism by which APP regulates these processes is still not clear. Although a crosstalk between APP and Notch has been described both *in vitro* and *in vivo*, their combined function in the context of neurogenesis *in vivo* has not been shown before (Kim et al., 2011). One of the suggested mechanisms is that APP and Notch act as competitive substrates for γ-secretase cleavage (Berezovska et al., 2001; Lleo et al., 2003), implying that a changed expression level of one affects the cleavage of the other. Accordingly, decreased Appb levels would result in an augmented cleavage of Notch and thus increased intracellular signaling. Our findings support this hypothesis, since M-cell formation was rescued after reducing γ-secretase activity with DAPT or *notch1a* knockdown. However, we cannot rule out other mechanisms by which APP could affect Notch cleavage or activation such as physical interaction (Chen et al., 2006) or inhibition of NICD signaling through a direct or indirect interaction with AICD (Roncarati et al., 2002).

In conclusion, we provide strong evidence of a cell-specific requirement of Appb in zebrafish M-cell development that is mediated through competitive regulation of Notch1a signaling. Although the exact interaction between Appb and Notch1a is yet to be determined, this opens up for new views on the function of Appb and its homologues during neurogenesis. It is tempting to speculate that slight changes in expression or cleavage of APP, caused either by mutations in APP itself or in proteins mediating its cleavage, are likely to have an effect on Notch signaling that might give rise to substantial neuronal changes over time. This hypothesis is supported by findings of changed Notch levels in brains of individuals with AD (Nagarsheth et al., 2006) or Down's syndrome (Fischer et al., 2005; Lockstone et al., 2007) harboring three copies of APP. Finally, cells and organs affected by reduced Appb level have also been

described as affected in Notch-Delta mutants. Thus it is likely that APP might interact with Notch in other tissues as well since Appb is expressed in many organs.

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