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Original research article

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.

1. 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/

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progenitor cells and regulating cell fate determination. Through cell-cell interaction between Notch receptors and Delta ligands, Notch acts 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.

2. Material and methods

2.1. Animal husbandry and ethical procedure

Zebrafish (*Danio rerio*) were maintained in Aquatic Housing Systems (Aquaneering, San Diego, USA) at 28 °C under a 14-h light/10-h 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 the ethical committee in Gothenburg and followed the guidelines of the Swedish National Board for Laboratory Animals.

2.2. 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.

2.3. 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.

2.4. 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. As vehicle control 1 % DMSO was used. During drug exposure, embryos were protected from light and incubated in dark at 28.5 °C until 24 or 48 hpf.

2.5. 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%