Brain Research 1697 (2018) 34–44

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/bres

Research report

N-terminal and central domains of APC function to regulate branch number, length and angle in developing optic axonal arbors *in vivo*

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ARTICLE INFO

Article history:
Received 9 August 2017
Received in revised form 7 May 2018
Accepted 28 May 2018
Available online 29 May 2018

Keywords:
Optic axon arbors
Terminal branching
Bifurcation angle
Optic neurons
Adenomatous Polyposis Coli
Xenopus laevis

ABSTRACT

During formation of neuronal circuits, axons navigate long distances to reach their target locations in the brain. When axons arrive at their target tissues, in many cases, they extend collateral branches and/or terminal arbors that serve to increase the number of synaptic connections they make with target neurons. Here, we investigated how Adenomatous Polyposis Coli (APC) regulates terminal arborization of optic axons in living *Xenopus laevis* tadpoles. The N-terminal and central domains of APC that regulate the microtubule cytoskeleton and stability of β-catenin in the Wnt pathway, were co-expressed with GFP in individual optic axons, and their terminal arbors were then imaged in tectal midbrains of intact tadpoles. Our data show that the APCNTERM and APCβ-cat domains both decreased the mean number, and increased the mean length, of branches in optic axonal arbors relative to control arbors *in vivo*. Additional analysis demonstrated that expression of the APCNTERM domain increased the average bifurcation angle of branching in optic axonal arbors. However, the APCβ-cat domain did not significantly affect the mean branch angle of arbors in tecta of living tadpoles. These data suggest that APC N-terminal and central domains both modulate number and mean length of branches optic axonal arbors in a compensatory manner, but also define a specific function for the N-terminal domain of APC in regulating branch angle in optic axonal arbors *in vivo*. Our findings establish novel mechanisms for the multifunctional protein APC in shaping terminal arbors in the visual circuit of the developing vertebrate brain.

1. Introduction

Establishment of ordered neuronal connectivity during embryonic development is critical for proper nervous system function. Accordingly, aberrant development of neural networks is thought to underlie many neurological and cognitive disorders. The retino-tectal projection of lower vertebrates, such as tadpoles of the frog *Xenopus laevis*, is an accessible neuronal circuit that is ideal for studying mechanisms underlying the development of axonal projections *in vivo*. During formation of the retino-tectal projection, optic axons navigate from the eye to their target tissue in the brain, the optic tectum. When optic axons invade their target, they elaborate terminal arbors that make synaptic connections with neurons in specific regions of the tectum (Alsina et al., 2001; Harris et al., 1987; Sakaguchi and Murphey, 1985). Distinct morphological features of optic axonal arbors, such as branch number, length, and angle, are important for their function, potentially influencing the number and pattern of synaptic connections they can make in the tectum (Alsina et al., 2001; O'Rourke and Fraser, 1990). However, questions remain, about both the molecular mechanisms that sculpt developing optic axonal arbors *in vivo*, and the relationships between different branching features in growing optic axonal arbors. In previous work, we dissected the mechanisms of the Cadherin and Wnt signaling node, β-catenin, in regulating optic axonal arborization in *Xenopus laevis* tadpoles *in vivo* (Wiley et al., 2008; Elul et al., 2003). Here we address how APC, an intracellular signaling molecule in the Wnt pathway that modulates the function of β-catenin, regulates several branching parameters of developing optic axonal arbors *in vivo*.

APC is a large, multi-functional cytoplasmic protein first identified because of its association with hereditary colon cancer, and more recently, implicated in brain cancer and several neurological disorders (Bendelsmith et al., 2018; Azofra et al., 2016; Li et al., 2016; Jaiswal et al., 2005). The molecular mechanisms of APC functions are largely due to its critical role in the Wnt signaling

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https://doi.org/10.1016/j.brainres.2018.05.045
0006-8993/

pathway (Senda et al., 2005). In the Wnt signaling pathway, APC functions to modulate levels of ß-catenin. APC normally binds to β-catenin via its central domain. However, following activation of Wnt signaling, APC (along with other factors such as Axin) is uncoupled from β-catenin, which leads to an increase in β-catenin levels in the cytoplasm (Clevers and Nusse, 2012). Canonical Wnt signaling further results in increased β-catenin translocation into the nucleus, where it induces gene transcription together with TCF/LEF factors. In addition to its function in the Wnt signaling pathway, APC is also a microtubule regulator (Senda et al., 2005). In particular, the N-terminal domain of APC is known to affect microtubule organization by binding to the microtubule regulator KAP-3 (Chen et al., 2011; Senda et al., 2005). However, the APC N-terminal domain can also regulate oligomerization of APC, which may, in turn, modulate its activity in the Wnt signaling pathway (Chen et al., 2011; Senda et al., 2005).

A few studies have determined initial functions for APC in development of axonal projections and axon branching in neuronal systems. One paper demonstrated that APC, via modulation of β-catenin stability, regulates the overall projection of optic axons in the developing retino-tectal projection of *Zebrafish* (Paridaen et al., 2009). However, this study did not examine how APC modulation of β-catenin stability affected terminal arborization of individual optic axons in the developing retino-tectal circuit of *Zebrafish*. A second group showed that knockdown of APC in mice led to excessive collateral branches in cortical neurons cultured *in vitro* (Yokota et al., 2009). These researchers further demonstrated that expression of the N-terminal domain of APC that regulates indirect microtubule organization (and APC oligomerization) was responsible for modulating the numbers of branches of cortical neurons in culture (Chen et al., 2011). But, it is not known whether the APC N-terminal domain also regulates the number of branches or additional features of axon arbors in other types of neurons *in vivo*. In other studies, APC has also been shown to control axonal outgrowth and growth cone morphology in several types of neurons through altering microtubule regulation and organization (Purro et al., 2008; Koester et al., 2007; Votin et al., 2005; Zhou et al., 2004).

In this paper, we studied how distinct domains of APC that regulate cytoskeletal organization and β-catenin stability shape individual optic axonal arbors in intact, living *Xenopus* tadpoles. We overexpressed the N-terminal and central domains of APC in individual optic neurons in developing eyebuds of *Xenopus* embryos. We then examined how overexpression of APC N-terminal and central domains modulated the number, length and angle of branches in optic axonal arbors in tecta of *Xenopus laevis* tadpoles. The relationship between the number and mean length of branches in optic axonal arbors expressing the APC domains was also investigated. This work defines shared and specific functions for the N-terminal and central domains of APC in regulating diverse branching features of optic axonal arbors *in vivo*, and advances our understanding of the mechanisms shaping neuronal circuits in the developing vertebrate brain.

2. Results

2.1. Optic axons that express APCNTERM and APCβ-cat mutants project to tectum

APC is a multifunctional protein that regulates microtubule organization, as well as β-catenin stability in the canonical Wnt pathway. To study how APC modulates neuronal development, we constructed two truncated mutants of APC consisting of distinct domains (Fig. 1A). One mutant consisted of the N-terminal region of APC that mediates indirect microtubule regulation (and oligomerization of APC) (APCNTERM, Fig. 1A; Vleminckx et al.,1997). The second construct was comprised of the APC central domain that binds to, and destabilizes β-catenin (APCβ-cat, Fig. 1A; Vleminckx et al., 1997). Each of these mutants was combined with GFP and lipofected into developing optic neurons in eyebuds of one-day-old *Xenopus laevis* embryos (developmental stage 22). For controls, eyebuds of one-day-old embryos were lipofected with only GFP. Four days later, we imaged optic axons that either expressed GFP (controls), or an APC domain together with GFP (experimentals), in tectal midbrains of intact, living tadpoles (developmental stages 46/47; Fig. 1B).

We first examined whether optic neurons lipofected with the APC mutants were able to project axons to their primary target in the brain - the optic tectum. As shown in the representative images, optic axons overexpressing APCNTERM or APCβ-cat domains indeed arrived at, entered into, and arborized in, the dorsal tectum, as did control GFP expressing axons (Fig. 1B). To quantify these observations, we calculated the percentage of embryos lipofected with GFP, or GFP together with an APC domain, that displayed at least one green fluorescent optic axonal arbor in the optic tectum. We determined that approximately 60% of embryos lipofected with the control plasmid displayed GFP expressing axonal arbors in the tectum (n = 24 embryos lipofected with GFP). Additional analysis showed that ~50% of embryos that were lipofected with GFP together with the APCNTERM domain showed fluorescent optic axons in the tectum (n = 19 embryos lipofected with APCNTERM mutant). Lastly, 70% of embryos lipofected with GFP and APCβ-cat plasmids contained GFP-expressing optic axonal arbors in the tectum (n = 21 embryos lipofected with APCβ-cat domain). Therefore, following lipofection of GFP, and GFP together with APCNTERM or APCβ-cat mutants, in eyebuds of developing embryos, significant percentages of tadpoles displayed optic axonal arbors expressing GFP in the optic tectum. These analyses show that overexpression of the N-terminal and central domains of APC does not inhibit the projection of optic axons from the eye to the tectal midbrain in living tadpoles.

2.2. APC mutants decrease numbers of branches in optic axonal arbors in vivo

After optic axons arrive at and proceed to invade the optic tectum, they elaborate terminal arbors that make synaptic connections with target neurons (Alsina et al., 2001; Harris et al., 1987; Sakaguchi and Murphey, 1985). To determine how APC sculpts these terminal arbors, we examined images of GFP control, GFP-APCNTERM or GFP-APCβ-cat domain expressing optic axonal arbors in tectal midbrains of intact, living tadpoles, and quantified their number of branches (Fig. 1B, C; Wiley et al., 2008; Elul et al., 2003).

For baseline data, we first analyzed the number of branches in control optic axonal arbors in intact, living tadpoles at developmental stages 46/47. Control optic axonal arbors were moderately branched, with each arbor containing multiple (primary and secondary) branches (Fig. 1B, C). The numbers of branches in GFP expressing arbors ranged between 11 and 19 (Fig. 1B, C). On average, control, GFP-expressing arbors in stage 46/47 tadpoles contained ~16 branches (SE = 1.04, n = 12 GFP expressing control optic axonal arbors). These numbers of branches we calculated for control GFP arbors here are consistent with measurements we made on control optic axonal arbors in our earlier studies (Wiley et al., 2008; Elul et al., 2003). In our previous studies, control GFP-expressing optic axonal arbors also contained, on average, ~16 branches in stage 46/47 tadpoles (Wiley et al., 2008; Elul et al., 2003).

We next determined how expression of the APCNTERM domain that contains the indirect microtubule regulatory site of APC