Baz, Par-6 and aPKC are not required for axon or dendrite specification in *Drosophila*

Melissa M Rolls & Chris Q Doe

Par-3/Baz, Par-6, and aPKC are evolutionarily conserved regulators of cell polarity, and overexpression experiments implicate them as axon determinants in vertebrate hippocampal neurons. Here we examined their mutant and overexpression phenotypes in *Drosophila melanogaster*. We found that mutants neurons had normal axon and dendrite morphology and remodeled axons correctly in metamorphosis, and that overexpression did not affect axon or dendrite specification. Baz/Par-6/aPKC are therefore not essential for axon specification in *Drosophila*.

Neurons are highly polarized cells that establish two distinct compartments— axonal and somatodendritic—during their development. These differ on many levels: from their ability to synthesize proteins, to the organization of the cytoskeleton, to the proteins in the plasma membrane[1]. Par-3 (Baz in *Drosophila*), Par-6 and aPKCλ/aPKCζ (atypical protein kinase C, aPKC in *Drosophila*) have been proposed as axon determinants in vertebrate neurons[2,3]. In cultured hippocampal neurons, Par-3, Par-6 and aPKC concentrate at the tips of growing axons when they grow beyond unpolarized processes[2,3]. Overexpression of Par-3 or Par-6 proteins[3], or expression of a truncated dominant-negative Par-3 protein[2], results in additional long axon-like processes at this stage. These results suggest that Par-complex proteins may be axon determinants. This hypothesis has not been directly tested, however, either by examining the mutant phenotype for any of these genes, or by *in vivo* analysis of their neuronal function. If, as suggested by overexpression experiments, Par-3 and Par-6 are indeed crucial to axon specification, one might expect axons not to form in their absence. Here we used null mutants to eliminate *Drosophila* Baz, Par-6 and aPKC

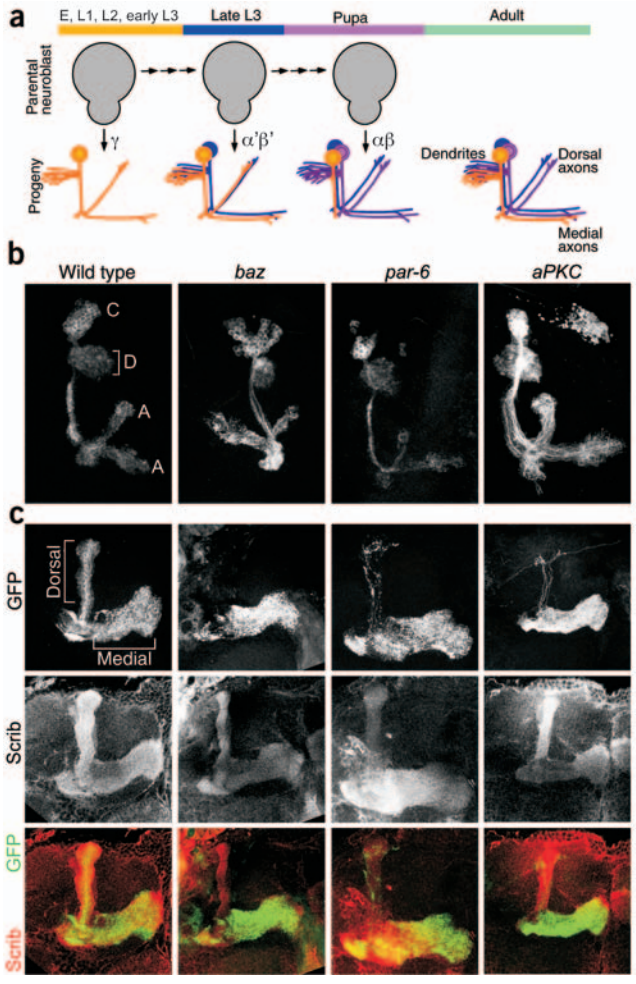

Figure 1 *Baz, par-6,* and *aPKC* mutant neurons form normal axons and dendrites *in vivo*. (**a**) Timeline and classes of mushroom body neurons (adapted from ref. 7). Four neuroblasts per hemisphere generate mushroom body neurons continuously from the embryonic (E) to pupal stages. The γ-neurons (orange) are produced first; dendrites emerge close to the cell body and to the branched dorsal and medial axon lobes. At pupariation, γ axons are pruned back to the branch point and then regrow into the medial lobe only (far right). The α'β' neurons (blue) are produced at the end of the third larval instar (L3), and αβ neurons (pink) at the beginning of the pupal stage. The α'β' and αβ neurons always project axons to both dorsal and medial axon lobes. (**b**) GFP-marked neuronal clones created in the first instar and scored 3 d later in late third instar larvae. Left panel, wild-type clone. Right panels, *baz, par-6,* and *aPKC* null-mutant clones (n = 8 for *baz*, n = 7 for *par-6* and n = 24 for *aPKC*). GFP-actin marks neurons in wild-type, *baz* and *par-6* clones; mCD8-GFP marks the *aPKC* clone. Dorsal axon lobes point up, and medial axon lobes to the right, except for *baz* clone where it points left. Position of cell bodies (C), dendrites (D) and two axons lobes (A) are indicated in the wild-type clone. **(c)** GFP-marked neuronal clones created in the first instar and scored in 2- to 5-d-old adult brains. Left panel, wild-type clone. Right panels, *baz, par-6,* and *aPKC* null mutant clones (n = 2 for *baz*, n = 4 for *par-6* and n = 12 for *aPKC*). Only dorsal and medial axon lobes are shown. GFP clone marker (upper panels); Scrib highlights axon lobes and shows that whole dorsal and medial lobes are present in these compressed confocal z-series (middle panels); merged images (lower panels). Mutant alleles used were baz^4, $aPKC^{K06403}$ and $par\text{-}6^{\Delta 226}$. Clones were generated using standard methods[7]. Brains were dissected from wandering third instar larvae, or from 2- to 5-d-old adults using standard methods[7]. Crosses to generate MARCM clones available upon request.

Institute of Neuroscience and Institute of Molecular Biology, Howard Hughes Medical Institute, University of Oregon, Eugene, Oregon 97403, USA. Correspondence should be addressed to C.Q.D. (cdoe@uoneuro.uoregon.edu).

Published online 7 November 2004; doi:10.1038/nn1346

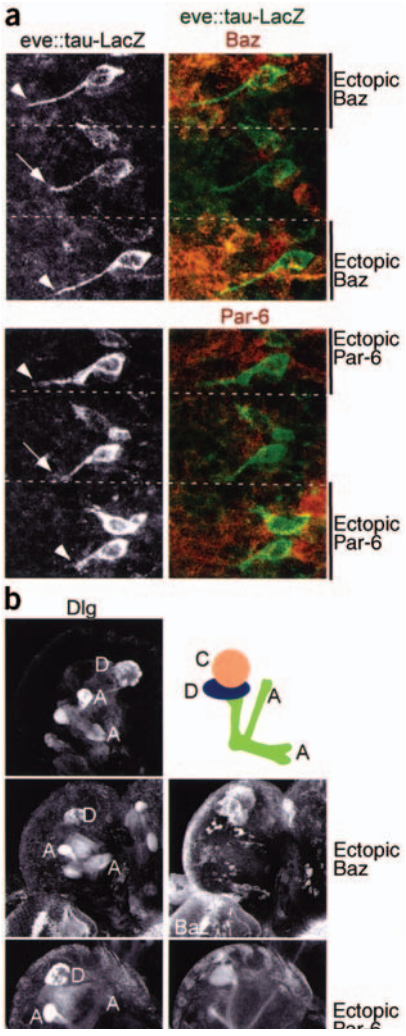

Figure 2 Overexpression of Baz or Par-6 does not affect axon morphology or number. (**a**) Baz or Par-6 overexpression in alternating segments of the embryonic CNS, before and during aCC pioneer motor neuron axon extension, generated no difference in aCC axon number and outgrowth (arrows; *n* = 28 for Baz, *n* = 14 for Par-6). The aCC motor neuron was identified by an *eve::tau-lacZ* transgene, which is expressed in only three neurons per hemisegment. Embryos are *paired-Gal4/UAS-baz* or *UAS-par-6; eve::tau-lacZ/+* shown at stage 12. The *eve::tau-lacZ* line was provided by Miki Fujioka and Jim Jaynes (Thomas Jefferson University).(**b**) Baz and Par-6 overexpression in developing larval mushroom body neurons does not affect axon or dendrite morphology. Dlg axon/dendrite marker in wild-type brains (left) and overexpression brains (right). D, dendrites; A, dorsal and medial axon lobes. Larvae are *OK107-Gal4 / UAS-Baz* or *UAS-Par-6*, shown at third larval instar. Similar results were observed when the *201Y-Gal4* driver was used to drive *baz* or *par-6* expression (data not shown). *201Y-Gal4* and *OK107-Gal4* obtained from Bloomington stock center. Antibody staining done as described[4], with the addition of guinea pig anti-Baz (used at 1:500).

from developing neurons *in vivo*, and then assayed the neurons for axon specification and outgrowth.

We used previously characterized null alleles of *baz*, *par-6*, and *aPKC* for our analysis[4–6], and used the MARCM (mosaic analysis with a repressible cell marker) system to make GFP-marked homozygous mutant clones[7,8]. We assayed the mushroom body neurons, a group of several hundred interneurons that have been extensively characterized for axon and dendrite outgrowth and remodeling, and that are generated from just four precursors (neuroblasts) that produce neurons from the embryonic through pupal stages (**Fig. 1a**)[7,8]. We induced homozygous mutant clones in mushroom body neuroblasts at the first larval instar and assayed homozygous mutant neuronal progeny 3 d later in third larval instar brains, or 7–10 days later in adult brains (**Fig. 1b** and **c**).

Wild-type third larval instar neuronal clones showed the expected morphology, with dendrites filling the calyx adjacent to the cell bodies and to branched dorsal and medial axon lobes (**Fig. 1b**, left). To our surprise, *baz*, *par-6*, and *aPKC* null-mutant neurons all showed normal axon and dendrite morphology (**Fig. 1b**, right). We next assayed mutant clones in adults to score the development of the latest-born neurons (α'β' and αβ, formed during late third-instar and pupal stages[7,8]). Because these neurons project to both dorsal and medial axon lobes in adults, whereas the first-born γ-neurons do not project to the adult dorsal lobe (**Fig. 1a**), we could be confident that we were scoring only the late-born α'β' and αβ axons. Wild-type adult neuronal clones showed α'β' and αβ neurons extending into the dorsal axon lobe (**Fig. 1c**, upper left). In mutant clones, α'β' neurons showed completely normal axon projections that reached the tip of the dorsal lobe (**Fig. 1c**, right). We observed fewer α'β' and αβ neurons, as expected, due to the failure of mutant neuroblasts to generate a full lineage. The *aPKC* mutant clones were about one quarter the size of wild-type clones[4], consistent with the proliferation arrest that occurs at about the time that the neuroblast stops generating γ neurons and starts generating α'β' neurons[9]. This small number of α'β' neurons allowed us to score axon morphology with single-cell resolution. We saw clearly that individual mutant-α'β' neurons had normal axon projections. Thus, we conclude that genetically null mutant *baz*, *par-6* or *aPKC* neurons show no defects in axon specification or outgrowth.

We were concerned, however, that the stability of the Baz, Par-6 and aPKC proteins might be such that any protein present in the parental neuroblast at the time of clone induction would persist for many days and be inherited by many or all of the hundred genetically mutant neuronal progeny, where it might be present at sufficiently high levels to promote axon specification. To address this possibility, we assayed Par-6 and aPKC protein levels in mutant clones at the third larval instar, 3 d after clone induction, and found no detectable protein in *par-6* or *aPKC* mutant neuroblasts or neurons (**Supplementary Fig. 1** online, and ref. 4); thus α'β' and αβ neurons born subsequently must have initiated axon and dendrite formation in the absence of the proteins. In addition, we have observed that all three proteins (Baz, Par-6 and aPKC) are partitioned into the neuroblast—and out of the differentiating neurons—during each asymmetric neuroblast division[4]. This would make it virtually impossible for residual protein to be inherited passively by the mutant neuronal progeny. We conclude that Baz, Par-6 or aPKC are not required for axon or dendrite specification in mushroom body interneurons.

We next tested whether Baz, Par-6 and aPKC are involved in axon remodeling during metamorphosis. In wild-type brains, γ-neuron axons are pruned back in the early pupal stage, and later grow back into the medial lobe only[7] (**Fig. 1a**). We found that *baz*, *par-6*, and *aPKC* null-mutant neurons showed a normal pattern of γ-axon remodeling, with adult-specific projections into the medial lobe only (**Fig. 1c**). Because the remodeling occurred many days after clone induction and after the third larval instar when no protein is detectable in clones, we could infer that no protein was present in the neurons during axon remodeling. We conclude that Baz, Par-6 and aPKC are not required for axon outgrowth during metamorphosis.

Overexpression of mammalian Par-3 (Baz) or Par-6 leads to growth of extra axon-like processes in cultured hippocampal neurons[2,3]. To determine whether a similar effect occurs in *Drosophila* neurons *in vivo*,

we overexpressed Baz or Par-6 in alternating segments of the embryo during axon specification, and assayed axon development in the aCC motor neuron using the aCC axon marker *eve::tau–lacZ*[10]. Wild-type aCC neurons had no detectable Baz or Par-6 during axon outgrowth (data not shown), but when overexpressed, these proteins were clearly detectable in the CNS (**Fig. 2a**). Overexpression of Baz or Par-6 had no effect on the number, orientation or growth of aCC axons, however (arrowheads, **Fig. 2a**). In addition, we overexpressed Baz or Par-6 proteins at higher-than-normal levels in larval mushroom body neurons using a Gal4 driver expressed at the time of axon initiation (OK107) or a Gal4 driver expressed slightly later (201Y). In both cases we found no defect in axon or dendrite morphology (**Fig. 2b**). We conclude that overexpression of Baz or Par-6 in neurons does not promote axon specification or block dendrite formation.

We did not find a role for Baz, Par-6 or aPKC in axon specification, nor did we observe these proteins to be targeted to the tips of growing axons *in vivo* or *in vitro* (data not shown). We did, however, observe a polarized localization of the proteins in mature sensory or central neurons. In sensory neurons, Baz was localized at the dendrite tips whereas Par-6 and aPKC were localized around the dendrite in the support cells (**Supplementary Fig. 2a**). In mushroom body interneurons, Baz was axon specific, Par-6 was enriched in young axons and present at low levels throughout central interneurons, and aPKC was perisynaptic (**Supplementary Fig. 2b**). Rather than being co-localized as has been observed in epithelia, in *Drosophila* neuroblasts and in vertebrate neurons[11], each of the three proteins had a distinct subcellular distribution in these mature neurons. We propose that all three proteins have distinct functions in mature neurons.

We have shown that *Drosophila* Baz, Par-6 and aPKC are not required for axon specification *in vivo*, and that their overexpression has no effect on axon specification or outgrowth. In contrast, overexpression of Par-3 or Par-6 in cultured mammalian hippocampal neurons results in multiple axon-like processes, leading to the hypothesis that these proteins are axon determinants[3]. How can we reconcile these apparently paradoxical results? One possibility is that vertebrate neurons require Par-complex proteins for axon specification, whereas *Drosophila* neurons do not. If this is the case, it would be interesting to learn how different molecular pathways in mammals and flies generate the same functional subcellular domain (the axon). Another possibility is that neither fly nor vertebrate neurons use Par proteins to specify axon identity *in vivo*; cultured hippocampal neurons are separated from normal external polarity cues and may use a different mechanism for axon specification. Polarity cues from surrounding cells may also inhibit neurons *in vivo* from changing polarity in response to extra Par-3 or Par-6, explaining the different effects of overexpressing these proteins in *Drosophila* and in hippocampal neurons. A third possibility is that the overexpression experiments, where proteins are present at higher-than-normal levels, do not reflect the *in vivo* functions of the proteins. Loss-of-function and overexpression experiments that examine vertebrate neurons *in vivo* or in slice preparations will be crucial for fully understanding the role of Par complex proteins in vertebrate axon specification.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank Howard Hughes Medical Institute (C.Q.D.) and the Damon Runyon Cancer Research Foundation (M.M.R.) for funding, and the Bloomington Stock Center for fly stocks. A. Wodarz kindly provided the antibodies. We thank S. Siegrist for comments on the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 6 August; accepted 7 September 2004
Published online at http://www.nature.com/natureneuroscience/

1. Craig, A.M. & Banker, G. *Annu. Rev. Neurosci.* **17**, 267–310 (1994).
2. Nishimura, T. *et al*. *Nat. Cell Biol.* **6**, 328–334 (2004).
3. Shi, S.H., Jan, L.Y. & Jan, Y.N. *Cell* **112**, 63–75 (2003).
4. Rolls, M.M., Albertson, R., Shih, H.P., Lee, C.Y. & Doe, C.Q. *J. Cell Biol.* **163**, 1089–1098 (2003).
5. Petronczki, M. & Knoblich, J.A. *Nat. Cell Biol.* **3**, 43–49 (2001).
6. Wodarz, A., Ramrath, A., Kuchinke, U. & Knust, E. *Nature* **402**, 544–547 (1999).
7. Lee, T., Lee, A. & Luo, L. *Development* **126**, 4065–4076 (1999).
8. Jefferis, G.S., Marin, E.C., Watts, R.J. & Luo, L. *Curr. Opin. Neurobiol.* **12**, 80–86 (2002).
9. Lee, T., Winter, C., Marticke, S.S., Lee, A. & Luo, L. *Neuron* **25**, 307–316 (2000).
10. Fujioka, M., Emi-Sarker, Y., Yusibova, G.L., Goto, T. & Jaynes, J.B. *Development* **126**, 2527–2538 (1999).
11. Henrique, D. & Schweisguth, F. *Curr. Opin. Genet. Dev.* **13**, 341–350 (2003).