ABSTRACT

The neurogenin (Ngn) transcription factors control early neurogenesis and neurite outgrowth in mammalian cortex. In contrast to their proneural activity, their function in neurite growth is poorly understood. Drosophila has a single predicted Ngn homolog, Tap, of unknown function. Here we show that Tap is not a proneural protein in Drosophila but is required for proper axonal growth and guidance of neurons of the mushroom body, a neuropile required for associative learning and memory. Genetic and expression analyses suggest that Tap inhibits excessive axonal growth by fine regulation of the levels of the Wnt signaling adaptor protein Dishevelled.

KEY WORDS: Neurogenin, Axonal growth, Mushroom body, Wnt signaling

INTRODUCTION

Proper function of the nervous system is based on the production of a diversity of neuronal and glial cells, as well as the precise targeting of their axons and dendrites. A key transcription factor (TF) family in the control of neuronal cell fate commitment and neurite guidance is the neurogenin (Ngn) family. Ngn proteins belong to a structurally and functionally conserved basic helix-loop-helix (bHLH) superfamily. Ngn proteins are sufficient to initiate neuronal cell fate in the central nervous system (CNS) (reviewed by Bertrand et al., 2002; Ma et al., 1998). By contrast, very little is known about the function of Ngn proteins in invertebrate systems. Gain-of-function analyses in Drosophila and vertebrate models suggest that during evolution a switch in proneural activity occurred between the Ngn and the highly related atonal family of bHLH TFs. Specifically, whereas Ngn proteins are necessary and sufficient for the induction of neurogenesis in vertebrates, they cannot do so in flies. Conversely, in flies atonal-type proteins can induce neurogenesis, but fail to do so in vertebrates (Quan et al., 2004). A crucial test of this ‘evolutionary proneural switch hypothesis’ is whether Drosophila Ngn genes act as proneural genes in flies and vertebrates.

In addition to their proneural function, Ngn genes play various crucial roles in the development of the vertebrate nervous system, including regulating the outgrowth and targeting of both axons and dendrites (Hand et al., 2005; Hand and Polleux, 2011; reviewed by Yuan and Hassan, 2014). This regulation of neurite growth is independent of proneural activity, as the phosphorylation of a single tyrosine in mouse Ngn2 is necessary to specify dendritic morphology without interfering with cell fate commitment (Hand et al., 2005). However, the mechanism by which Ngn proteins regulate neurite guidance is poorly understood.

The Drosophila genome encodes a single Ngn family member, based on the conservation of family-defining residues in the bHLH domains (Hassan and Bellen, 2000), named Target of Pox neuro (Tap). Previous work suggests that Tap is expressed in secondary progenitors of both neurons and glia, called ganglion mother cells, in the CNS (Bush et al., 1996), as well as in putative support cells in the peripheral nervous system (PNS) during embryogenesis (Gautier et al., 1997). A previously reported putative tap mutant allele (Ledent et al., 1998) was later shown to be a mutation in a different gene called blot (Johnson et al., 1999). Therefore, the function of the Drosophila Ngn Tap remains uncharacterized.

Here, we generated a null mutant allele of tap by replacing the single coding exon of the tap gene with Gal4. Using expression, gain-of-function and loss-of-function analyses we show that whereas ectopic expression of Tap in vertebrates can induce neurogenesis, tap is not a proneural gene in flies, consistent with the evolutionary proneural switch hypothesis. Instead, Tap is required to prevent overgrowth of axons during brain development, at least in part through the activity of the axonal Wnt-planar cell polarity (PCP) pathway, by fine tuning the levels of the Wnt signaling adaptor protein Dishevelled (Dsh).

RESULTS AND DISCUSSION

Tap is the only Ngn homolog in Drosophila

Drosophila Tap shares significant identity (~70%) in the bHLH domain with mouse and human Ngn1s (Fig. 1A), as compared with Drosophila Atonal or Scute. To test if Tap, like its vertebrate counterparts, has proneural activity, we injected tap mRNA into one cell of two-cell stage Xenopus embryos and assessed neurogenesis by staining for neuronal markers. Like mouse Ngn1, but unlike fly Atonal, Tap efficiently induces neurogenesis in this system (Fig. 1B-E). Conversely, when ectopically expressed in a classic Drosophila proneural assay TAP–like Ngn1, and in contrast to Atonal– fails to induce neurogenesis (Fig. 1F-I). These data suggest that Tap may not be a proneural protein in flies, even though it has...
neural induction potential in vertebrates as expected for a bona fide Ngn protein.

**Tap is widely expressed in the nervous system during development**

To investigate the function of Tap, we generated a mutant allele using ends-in homologous recombination (Rong and Golic, 2000) to replace the open reading frame of tap with an external driver, Gal4 (Fig. 2A). The tap<sup>Gal4</sup> allele, in homozygosity, serves as a driver to reveal the expression pattern of Tap. We made several attempts to generate a Tap antibody, but this was not successful. Tap<sup>Gal4</sup> was detected in a large number of cells in both the CNS and PNS throughout development (Fig. 2B-E). During embryogenesis, Tap is enriched in the ventral nerve cord and sparsely expressed in the PNS (Fig. 2B, Fig. S2), which is similar to the RNA distribution as revealed by in situ hybridization (Fig. S2). Postembryonically, Tap-positive cells were observed in many tissues, including optic lobe, mushroom body (MB), antenna lobe and subsesophageal ganglion (Fig. 2C-E).

**Tap is not required for specifying the neuronal or glial cell fate during embryogenesis**

Flies lacking Tap are mostly embryonic lethal, with a few escapers to early larval stages. This lethality can be rescued, including to adult viability, by re-expression of Tap (UAS::tap) in tap<sup>Gal4</sup> homozygous flies. We examined whether the number or fate of neuronal and/or glial cells are altered in tap mutant embryos. Surprisingly, we find no obvious morphological defects in the tap mutant embryos. The number and pattern of the neurons and glia in the PNS are intact. Although the cell number in the CNS is difficult to quantify, the general cellular pattern looked similar in mutant and control flies (Fig. 2F-I). These data suggest that Tap is not required for early neurogenesis in Drosophila embryos.

**tap mutants show MB β lobe midline crossing and an α lobe missing defect**

To characterize Tap function, we focused on the MB as a model system. Tap is expressed in the MB at both pupal and adult stages in a subset of α/β neurons (Fig. 3A-C) that form four clusters (Fig. 3C) and project their medial axons to the dorsal part of the β lobe (Fig. 3A,B). Tap expression is highest at early pupal stages and then declines. The phase of high level Tap expression correlates with the differentiation stage of α/β neurons, indicating that Tap might regulate the development of the α/β lobe.

To circumvent embryonic lethality we began by exploiting the heterozygous tap<sup>Gal4</sup> allele alone or in combination with two independent RNAi strains targeting distinct regions of tap. In wild-type Drosophila, axons of the medially projecting β lobes terminate near the midline but do not cross it (Strausfeld et al., 2003). In contrast to the wild-type MB morphology (Fig. 3D), tap loss-of-function brains exhibit β lobe fibers that extend across the midline (Fig. 3E), sometimes causing fusion of the two contralateral β lobes (Fig. 3F). The phenotype is variable in severity; we classified it as ‘normal’, ‘mild’ or ‘severe’ based on the thickness and density of the β lobe fibers crossing the midline. tap heterozygotes display an increase in the penetrance of mild defects, while both tap RNAi strains raise the incidence and severity of the β lobe midline crossing defect. When Tap is re-expressed, the defect can be rescued to control levels (Fig. 3J). This β axon midline crossing defect is developmental in origin as it can be observed at early pupal stages (Fig. S3B). Moreover, ectopic expression of Tap throughout the entire MB or in all the Tap’ neurons increases the distance between two contralateral β lobes, suggesting that Tap induces the retraction of β lobes and/or inhibits the axonal growth of β lobes (Fig. S3D,E).

In addition to the β axon midline crossing defect, an ‘α lobe missing’ phenotype was observed in Tap loss-of-function MBs (Fig. 3H,I). In some brains with a missing α lobe, the β lobe appears to branch into two bundles (Fig. 3I), suggesting that this α lobe
defect might be an axonal targeting defect rather than a growth defect. Unlike the β lobe defect, the penetrance of the α lobe missing phenotype varied considerably (Fig. 3J). Nonetheless, considering that the frequency of missing α lobes is still higher in the tap mutant flies than in wild-type and rescue strains, we conclude that Tap is essential for the growth and correct targeting of both lobes of α/β neurons.

**Tap is required cell-autonomously for the growth and targeting of the β lobe**

Since the branching and growth pattern of single α/β axons cannot be directly inferred from the morphology of the α/β lobes, a single-neuron level analysis, such as mosaic analysis with a repressible cell marker (MARCM), is necessary to clarify the targeting of α/β neurons (Lee et al., 2000). Therefore, tap null clones were generated in a tapGal4 heterozygous background using the MARCM technique. In both wild-type control and tapGal4 heterozygous backgrounds, a small minority of brains showed β lobe overgrowth and/or the α lobe missing defect as discussed above. Considering this, only brains with intact overall α/β lobe morphology were quantified for clonal axon phenotypes. Analysis of small MB clones revealed that none of the control clones showed any defects (Fig. 3K). By contrast, in 32% of tap null (tapGal4Gal4) embryos, β axons project beyond the β lobe domains (Fig. 3L,N). In severe cases, axons were observed to cross the midline and project to the contralateral β lobes. This defect can be rescued by re-introduction of tap specifically in the mutant clones (Fig. 3M,N). This suggests that Tap is required cell-autonomously for the development of the β axon branch. However, none of the mutant clones showed loss of α axon growth, suggesting that Tap plays a non-cell-autonomous role during α lobe development.
Tap regulates axonal growth and guidance through Dsh

In order to investigate the molecular mechanism by which Tap regulates axonal growth and guidance, we performed dominant interaction tests between Tap and well-established MB axonal guidance factors, particularly those whose loss of function causes β lobe overextension. Specifically, we asked whether heterozygosity for any of these genes strongly enhances the very mild phenotypes observed in tap heterozygotes. Specifically, we looked for phenotypes significantly greater than the sum of the two phenotypes. Among the candidate genes, drl (Moreau-Fauvarque et al., 1998), sli (our unpublished data) and Dscam1 (Hattori et al., 2007) did not show any obvious synergism with tap (Fig. 4A). However, loss of one copy of dsh strongly enhances both the α lobe guidance and β lobe overgrowth defects in the tap heterozygous background (Fig. 4A,B). This suggests that Dsh synergizes with Tap to regulate axon guidance and growth during MB development.

To test if Tap might regulate Dsh expression in vivo we overexpressed Tap or knocked it down in all neurons and measured Dsh protein and RNA levels. We find that Tap gain of function mildly increased Dsh levels, whereas Tap knockdown mildly decreased Dsh levels in vivo (Fig. 4C-E).
Dsh is a key Wnt signaling component and serves as a hub to regulate another receptor protein, frizzled 3, to regulate the polarity of growth cones in the filopodia (Shafer et al., 2011). Most of the known regulation of the Wnt-PCP pathway is via protein-protein interactions. For instance, Vangl2, a core receptor of the PCP pathway in mammalian cells, is found to antagonize Dvl, the mammalian homolog of Drosophila Dsh, and to post-translationally regulate another receptor protein, frizzled 3, to regulate the polarity of growth cones in the filodipodia (Shafer et al., 2011). Here we identify a transcriptional modulator of the PCP pathway during axonal growth. Both mRNA and protein expression of dsh are modified upon Tap manipulation, although the modification is mild, suggesting that Tap serves as a regulator rather than an activator of dsh. Our genetic data also point to a possible complex interaction between Tap and Dsh in MB α lobe growth. This suggests that the development of the α and β lobes are regulated independently. The fact that the null allele of dsh, but not the 'PCP-specific' allele, causes this effect could suggest the involvement of the canonical Wnt pathway in α lobe growth.

In contrast to the potential conservation of its function in neurite growth, Tap does not act as a proneural protein in flies. Despite the obvious differences in morphology between invertebrates and vertebrates, the fundamental mechanisms underlying neurogenesis are conserved. In particular, the induction of neurogenesis by bHLH proteins has been conceptually defined as a module that can be adapted to various contexts (Schlosser and Wagner, 2004). Our data, together with previous studies, suggest that during the evolution of vertebrates and invertebrates from their last common ancestor, the ancestral vertebrate neuroectoderm, but not that of invertebrates, became responsive to the neuroinductive activity of neurogenic-like proteins. It will be very interesting to determine whether the activity of ancestral-like proneural proteins, such as those found in sponges, resembles that of neurogenin, achaete-scute proteins. Crucially, this is not encoded as a change in the inductive capacity of the invertebrate neurogenins per se, as demonstrated by the neurogenic activity of Tap in Xenopus. Whether this is related to the dorsoventral axis switch in the location of the neuroectoderm
(dorsal in vertebrates, ventral in *Drosophila*) remains to be determined.

**MATERIALS AND METHODS**

**Fly husbandry and transgenic lines**
Flies were kept at 25°C or 29°C on standard medium. Experiments involving RNAi or genetic interaction were performed at 29°C, and remained at 25°C. Transgenic lines are listed in the supplementary Materials and Methods.

**Xenopus embryonic microinjection**
*ato*, *tap* and mouse *Ngn1* mRNAs were injected into a single blastomere of *Xenopus* embryos at the two-cell stage. Whole embryos were in situ hybridized with an *N-tubulin* probe as described (Quan et al., 2004).

**Cloning and gene targeting**
Fragments comprising the 5′ homologous recombination arm of the *tap* ORF, *Gal4* and the 3′ recombination arm of the *tap* ORF were amplified using the primers listed in the supplementary Materials and Methods and subcloned into pCRII(Em) vector (a gift of the B. Dickson laboratory, Janelia Farm Research Campus). *tap* targeting was achieved by ends-in homologous recombination (Fig. S1) (Rong and Golic, 2000).

**Immunohistochemistry**
Embryos and dissected tissues were stained using a published protocol (Hassan et al., 2000; Langen et al., 2013). Flat preparations of embryonic fillets at stage 17 were generated and stained on poly-lysine-coated glass following the protocol described by Featherstone et al. (2009). Images were acquired using a Leica TCS SP8 or SP5 and processed with ImageJ (NIH). Antibodies are detailed in the supplementary Materials and Methods.

**In situ hybridization**
Embryos and L3 brains were hybridized as described (Hassan and Vaessin, 1997). *tap* cDNA was used to generate the digoxigenin-labeled antisense probe.

**Western blot**
Proteins from 20 adult fly brains of each genotype were resolved and probed using the protocol of Okray et al. (2015). Antibodies are detailed in the supplementary Materials and Methods.

**qRT-PCR**
qPCR was performed according to the protocol of Li et al. (2013). A hundred adult fly heads were collected for each genotype. *Rp49* (*Rpl32*) and *RpS13* were used as reference genes, and qbase+ (Biogazelle) was used to process the data.

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**Competing interests**
The authors declare no competing or financial interests.

**Author contributions**
L.Y. conceived and designed the study, collected and analyzed the data, and wrote the manuscript. S.H. generated the *tap* mutant and collected data. Z.O. helped with the genetic interaction screen. X.R. performed the microinjection of *Xenopus* embryos. N.D.G. carried out the western blot analyses. A.C. and J.Y. carried out the cloning and provided technical assistance. E.B. conceived and guided the gain of function experiments. B.A.H. and X.-J.Q. conceived and supervised the study, and wrote the manuscript. All authors read and approved the final manuscript.

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**Supplementary information**
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