Response to Comment on "Nervy Links Protein Kinase A to Plexin-Mediated Semaphorin Repulsion"

The comment by Ice *et al*. (*1*) questions conclusions we drew concerning the function of the *Drosophila* protein Nervy (*2*). They state that the polyclonal antibody anti-ETO Ab-1, directed against the mammalian ortholog of Nervy ETO/MTG8, does not recognize *Drosophila* Nervy. We purchased the polyclonal antibody in question in July 2002 from EMD Biosciences because it was generated against a region that is highly conserved between the human Nervy/MTG/ETO protein and *Drosophila* Nervy (including one portion where 19 out of 21 amino acids are identical) (*2*, *3*). Our extensive analyses (*2*, *3*) revealed that the ETO Ab-1 antibody specifically recognized a single prominent band of the size expected for *Drosophila* Nervy on blots of wild-type *Drosophila* embryo lysates. Furthermore, this band was absent in lysates derived from *nervy* mutant embryos [fig. S2D in (*2*); also see (*3*)]. More recently, we noted lot-to-lot variation in this reagent and reported this observation to EMD Biosciences in June 2003. We also reported the specific batch of the ETO antibody that we used in our original study (*2*, *3*). In their comment, Ice *et al*. (*1*) report that they supplied these lots of ETO antibody to EMD Biosciences but rule out any possible lot-to-lot variation in these polyclonal antibodies. However, they did not detect Nervy in *Drosophila* embryonic lysates [see figure 1A, right panel, in (*1*)], whereas we detected a single prominent band that was absent in lysates from *nervy* mutant embryos (*2*). Therefore, the assertion of Ice *et al*. that the band we observed was a background band serendipitously reduced in the *nervy* mutant is not consistent with their observations, because they did not see prominent band using their lots of anti-ETO Ab-1 on wild-type *Drosophila* embryo lysates. The faint bands Ice *et al*. observed with their lots of ETO antibody upon long exposure [figure S1 in (*1*)] are only evident in Cos-7 cell extracts, not in *Drosophila* lysates [figure 1A in (*1*), right panel]. Apparent lot-to-lot differences in the anti-ETO used by Ice *et al*. can be seen by comparing bands in figure S1, A and B, in (*1*). We also performed multiple, carefully controlled coimmunoprecipitation (co-IP) studies using our purchased lot of anti-ETO. We observed that Nervy associates with an epitope-tagged neuronal cell surface receptor, Plexin A (PlexA), and with protein kinase A (PKA) but not with the neuronal protein enabled; Nervy did not coimmunoprecipitate in the absence of epitope-tagged PlexA [figures 1 and S2 in (*2*); (*3*)]. Therefore, it is extremely unlikely that a single serendipitously detected protein resulting from a background association with the anti-ETO would (i) be observed only at a size identical to Nervy, (ii) be able to selectively coimmunoprecipitate PlexA and PKA, (iii) be selectively recognized as a band of the same size as Nervy from a PlexA co-IP experiment, and (iv) be absent from *nervy* mutant embryos.

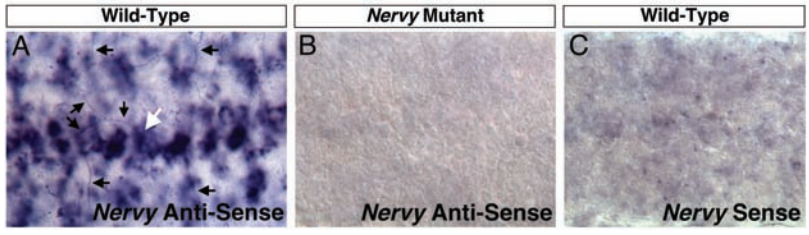

Fig. 1. *Nervy* (*Nvy*) antisense probes reveal *Nvy* transcript in axons of wild-type embryos but not *Nvy* mutant embryos. Filleted preparations of stage 16 *Drosophila* embryos (anterior, left). (**A**) In situ hybridization with antisense *Nvy* shows *Nvy* transcript within neurons and their axons (arrows), some of which can be seen here emanating from a neuronal cell body (ventral unpaired median neuron, white arrow). (**B**) In situ hybridization with antisense *Nvy* in a *Nvy* [PDFKG1 (*2*)] mutant embryo shows no evidence of *Nvy* in neuronal cell bodies or axons. (**C**) In situ hybridization with sense *Nvy* probe shows no specific staining of neurons or axons. Methods as in (*2*, *3*).

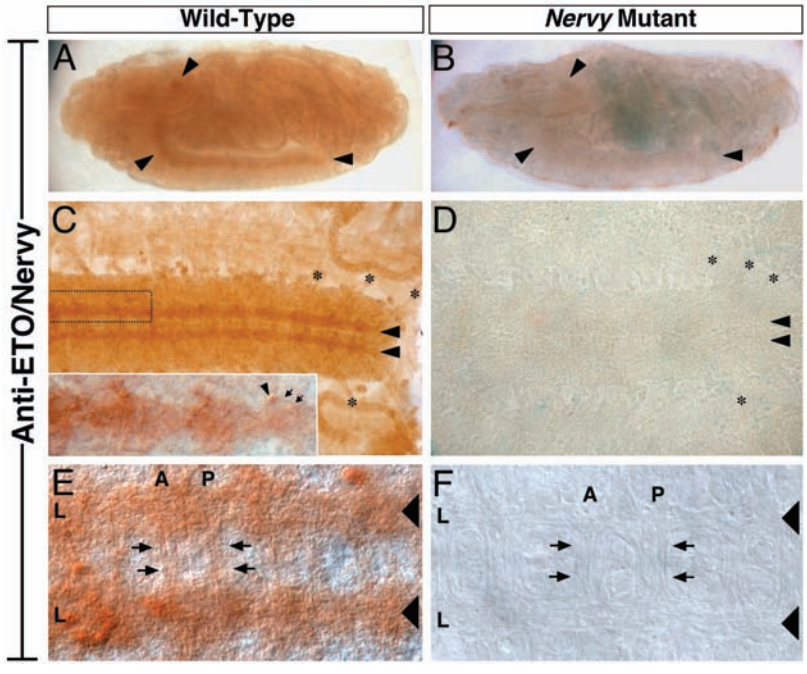

Fig. 2. Immunohistochemistry with ETO/Nvy antisera shows that in wild-type, but not in the *Nvy* mutant, the *Drosophila* embryonic nervous system can be stained. Stage 16/17 embryos immunostained with anti-ETO/Nvy Ab-1 (Lot D16428-1, EMD Biosciences) in 2003 and reported in (*2*, *3*) observed at low (**A** and **B**), intermediate (**C** and **D**), and high (**E** and **F**) power. The nervous system (arrowheads) immunostains in wild-type embryos [(A), (C), and (E)] but not in *Nvy* [PDFKG1 (*2*)] mutant embryos [(B), (D), and (F)]. Select immunostained axonal processes are indicated in (C) [arrows in the inset, emanating from a neuron (arrowhead)] and in (E) (arrows indicating multiple commissural axons). Axons were not immunostained in the *Nvy* mutant. Arrows in (F) indicate position of anterior and posterior commissures, visualized by Nomarski optics. Anterior is to the left. A, anterior commissure; P, posterior commissure; L, longitudinal connectives; asterisks, motor nerve roots. Methods as in (*2*, *3*).

Ice *et al*. (*1*) also question whether Nervy/MTG proteins are expressed in the cytoplasm and in axons. Although Nervy/MTG proteins are best known for their nuclear localization, other research groups report that mammalian Nervy/MTG proteins are indeed present in the cytosol (*4*–*10*). The nuclear or cytosolic localization appears to be dependent on both cell type and developmental stage (*4*–*10*). Of particular importance to Nervy/MTG protein localization is that MTG proteins are also reported to be present in

axons and at synapses (*5*, *6*). We found that the *nervy* transcript is also present in axons. Antisense probes for the *nervy* transcript strongly label neurons and their processes, including motor axons, and this labeling is absent when using a *nervy* sense-control or the antisense *nervy* probe in *nervy* mutant embyros [Fig. 1 and figure 1C, C^1, and C^2 in (*2*)]. Immunohistochemical staining of wild-type embryos using our purchased anti-ETO (*2*, *3*) also identified central nervous system neurons and their axonal processes [Fig. 2, C and E; figure 1D in (*2*); (*3*)]. This axonal staining was absent in *nervy* mutant embryos [Fig. 2, B, D, and F; see also (*2*, *3*)]. The inability of Ice *et al*. (*1*) to observe immunostaining of neuronal processes with their antibody directed against *Drosophila* Nervy could reflect differences in immunostaining protocols (*11*) or differences among the epitopes recognized by their anti-Nvy polyclonal antibody and the ETO antibody used in our study (*2*). It does not, however, bear on the accuracy of our carefully controlled immunostaining results with a different immunological reagent.

Our other observations (*2*) are also consistent with Nervy functioning in axons through its interactions with PlexA as a PKA (A kinase) anchoring protein (AKAP). Our large-scale yeast interaction screen first identified Nervy as a specific interacting partner with the axon guidance receptor PlexA (*2*). *Nervy* mutant analyses and extensive genetic studies revealed no gross abnormalities indicative of major changes in gene expression but, instead, showed axon guidance phenotypes similar to mutations in PlexA signaling components (*2*). Nvy/MTG proteins have a consensus PKA binding motif and are cytoplasmic AKAPs (*8*–*10*). Antibodies directed against Nervy immunoprecipitate PKA, and an antibody directed against the Myc epitope coimmunoprecipitates PKA when Myc-Nvy is expressed in neurons (*2*). The consensus PKA binding motif in Nervy is critical for its role in axon guidance, and genetic analyses and co-IP experiments between PKA and PlexA also support a role for PKA in PlexA signaling (*2*). Our molecular, genetic, in situ hybridization, immunolocalization, and immunoprecipitation data, along with published work from others, support that Nervy can be found in the cytoplasm, interacts with the cytoplasmic domain of the plasma membrane receptor PlexA, and directly modulates PlexA signaling by functioning as an AKAP. Our results in no way exclude the possibility that Nervy serves important roles in neuronal nuclei, but they support a critical role for direct PlexA/Nervy interactions in directing axon guidance decisions.

Jonathan R. Terman
Center for Basic Neuroscience
Department of Pharmacology
The University of Texas
Southwestern Medical Center
NA4.301, 5323 Harry Hines Boulevard
Dallas, TX 75390, USA
E-mail: jonathan.terman@utsouthwestern.edu
and *Howard Hughes Medical Institute*
Department of Neuroscience
Johns Hopkins University School of Medicine
1001 PCTB, 725 North Wolfe Street
Baltimore, MD 21205, USA

Alex L. Kolodkin
Howard Hughes Medical Institute
Department of Neuroscience
Johns Hopkins University School of Medicine
E-mail: kolodkin@jhmi.edu

References and Notes

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11. Ice *et al.* (*1*) state in their supporting online material that "embryos were fixed and prepared for confocal microscopy as previously described." We did not use confocal microscopy or immunofluorescence in our studies [(*2*) and this work].
12. We thank the NIH National Institute of Mental Health (J.R.T) and National Institute of Neurological Disorders and Stroke (A.L.K) for support of this work.

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References

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