Development/Plasticity/Repair

Genomic Analysis of *Drosophila* Neuronal Remodeling: A Role for the RNA-Binding Protein Boule as a Negative Regulator of Axon Pruning

Eric D. Hoopfer,[1,2]* Andrea Penton,[1]* Ryan J. Watts,[1] and Liqun Luo[1,2]

[1]Department of Biology and [2]Neurosciences Program, Howard Hughes Medical Institute, Stanford University, Stanford, California 94305

Drosophila **mushroom body (MB) γ neurons undergo axon pruning during metamorphosis through a process of localized degeneration of specific axon branches. Developmental axon degeneration is initiated by the steroid hormone ecdysone, acting through a nuclear receptor complex composed of USP (ultraspiracle) and EcRB1 (ecdysone receptor B1) to regulate gene expression in MB γ neurons. To identify ecdysone-dependent gene expression changes in MB γ neurons at the onset of axon pruning, we use laser capture microdissection to isolate wild-type and mutant MB neurons in which EcR (ecdysone receptor) activity is genetically blocked, and analyze expression changes by microarray. We identify several molecular pathways that are regulated in MB neurons by ecdysone. The most striking observation is the upregulation of genes involved in the UPS (ubiquitin–proteasome system), which is cell autonomously required for γ neuron pruning. In addition, we characterize the function of Boule, an evolutionarily conserved RNA-binding protein previously implicated in spermatogenesis in flies and vertebrates.** ***boule*** **expression is downregulated by ecdysone in MB neurons at the onset of pruning, and forced expression of Boule in MB γ neurons is sufficient to inhibit axon pruning. This activity is dependent on the RNA-binding domain of Boule and a conserved DAZ (deleted in azoospermia) domain implicated in interactions with other RNA-binding proteins. However, loss of Boule does not result in obvious defects in axon pruning or morphogenesis of MB neurons, suggesting that it acts redundantly with other ecdyonse-regulated genes. We propose a novel function for Boule in the CNS as a negative regulator of developmental axon pruning.**

Key words: **axon degeneration; mushroom body; neural development; ecdysone; ecdysone receptor; ubiquitin proteasome system; metamorphosis**

Introduction

Pruning of exuberant neuronal connections is a widely used mechanism in metazoan development for achieving the mature pattern of neural connectivity (Luo and O'Leary, 2005; Ding et al., 2007). In the mammalian nervous system, specific axonal projections are selectively pruned through a process of localized axon degeneration, retraction, or a combination of the two (Nakamura and O'Leary, 1989; Bagri et al., 2003; Bishop et al., 2004; Portera-Cailliau et al., 2005; Hoopfer et al., 2006). During *Drosophila* metamorphosis, the nervous system undergoes extensive remodeling as the fly transitions from the larval to adult stage. For example, mushroom body (MB) γ neurons prune larval axon branches and dendrites and later reextend processes to form the adult-specific connection pattern (Lee et al., 1999). MB pruning occurs through a spatially restricted process of axon degeneration that requires the cell-autonomous activity of the ubiquitin–proteasome system (UPS) (Watts et al., 2003), and nearby glia that engulf and degrade γ neuron fragments via the endosomal/lysosomal pathway (Awasaki and Ito, 2004; Watts et al., 2004).

Axon pruning of MB γ neurons is triggered by the steroid hormone ecdysone, which regulates gene expression through cell-autonomous actions of a nuclear receptor heterodimer consisting of ultraspiracle (USP) and ecdysone receptor B1 (EcRB1) (Lee et al., 2000). Ecdysone appears to be a general regulator of developmental axon pruning in *Drosophila* (Schubiger et al., 2003; Kuo et al., 2005; Marin et al., 2005; Williams and Truman, 2005; Roy et al., 2007). However, the genes that are regulated by ecdysone to initiate pruning remain mostly unknown. Classic studies of the ecdysone-dependent puffing patterns of the larval salivary gland polytene chromosomes (Ashburner, 1974) (for review, see Thummel, 2002) identified a set of primary-response genes that are direct targets of ecdysone receptor (EcR), including transcription factors that regulate the expression of secondary-response genes. Recent microarray studies have described developmental and ecdysone-dependent genome-wide transcriptional

Received Feb. 14, 2008; revised April 7, 2008; accepted April 28, 2008.

This work was supported by a fellowship from the National Science Foundation (E.D.H.), an Epilepsy Program Training Grant (A.P.), and National Institutes of Health Grant R37-NS041044 (L.L.). L.L. is a Howard Hughes Medical Institute investigator. We thank S. Wasserman, M. Fuller, and Exelixis for reagents; H. Su, A. Gontang, and W. Hong for assistance; T. Oro for the use of the Laser Capture Microdissection facility; and P. Vitorino and members of the Luo Laboratory, especially B. Tasic and M. Spletter, for comments and discussion.

*E.D.H. and A.P. contributed equally to this work.

Correspondence should be addressed to Liqun Luo at the above address. E-mail: lluo@stanford.edu.

E. D. Hoopfer's present address: Division of Biology 216-76, California Institute of Technology, Pasadena, CA 91125.

R. J. Watts's present address: Department of Neurodegeneration, Genentech, Inc., South San Francisco, CA 94080.

DOI:10.1523/JNEUROSCI.0677-08.2008

 0270-6474/08/286092-12$15.00/0

changes in whole animals or cultured larval organs at the onset of metamorphosis (White et al., 1999; Arbeitman et al., 2002; Li and White, 2003; Beckstead et al., 2005). However, MB γ neurons account for only a few percent of total neurons in the brain; thus, EcR-regulated gene expression changes in γ neurons may be obscured by changes in gene expression in the whole tissue/organism.

Here, we use laser capture microdissection in combination with microarrays to analyze gene expression changes in MB neurons at the onset of axon pruning. Among the genes that are developmentally regulated in response to ecdysone signaling, we show that EcR upregulates genes involved in many cellular pathways such as UPS-mediated protein degradation, including genes encoding regulatory subunits of the proteasome previously shown to be required for axon pruning (Watts et al., 2003). We then focus on the role of the RNA-binding protein Boule, which is downregulated by ecdysone in MB neurons at the onset of axon pruning. Increased expression of Boule in MB γ neurons inhibits axon pruning. We suggest that Boule may act as a negative regulator of γ neuron axon pruning by regulating mRNA translation.

Materials and Methods

RNA isolation and microarray analysis. Flies were raised on standard fly food. To select for third-instar larvae ~18 h before puparium formation (BPF), before the late larval ecdysone pulse that initiates pruning, 0.05% bromophenol blue was added to the food and we selected wandering third-instar larvae with dark blue guts (Andres and Thummel, 1994). Pupae were staged by selecting newly formed white prepupae, which we define as 0 h after puparium formation (APF). Isolation of RNA from MB neurons was accomplished using laser capture microscopy. Fifteen micrometer frozen sections were cut from larvae or pupae, dehydrated through an ethanol series, and fixed in 100% xylene for 5 min. MB neurons expressing mCD8::GFP were microdissected using the Arcturus LCM microdissection system (model ASLMD). Each capture consisted of ~100 cell bodies, and 40 captures were pooled to obtain each replicate. Total RNA was extracted using the PicoPure RNA isolation kit from Arcturus, linearly amplified (two rounds) using the RiboAmp HS RNA amplification kit (Arcturus), and labeled using the GeneChip IVT labeling kit from Affymetrix. Approximately 22 ng of starting total RNA yielded up to 70 μg of amplified cRNA. Amplified cRNA was hybridized to Affymetrix *Drosophila* Genome 1 microarrays.

Normalization of probe signal intensity levels across arrays was done using the robust multichip average (Irizarry et al., 2003) implemented in Expression Console Software (Affymetrix). Significance analysis of microarrays (SAM) (Tusher et al., 2001) was used to identify genes that showed statistically different expression between conditions outlined in Figure 1*A*. For each comparison, a δ value was chosen to give a false discovery rate <1% and only genes above a 1.5-fold change in expression level were included. Microarray data from this study can be accessed at the National Center for Biotechnology Information Gene Expression Omnibus website (accession numbers GSE10012, GSE10013, and GSE10014; http://www.ncbi.nlm.nih.gov/geo/).

Fluorescent in situ *hybridization and immunohistochemistry.* Larvae or pupae were staged in the same manner used for the microarray experiments. Fluorescent *in situ* hybridization was done essentially as described by Spletter et al. (2007). Probes were amplified from cDNA generated from either wild-type or EcRDN-expressing brains dissected from 0 h pupae, cloned, and sequenced to verify identity [primer sequences are listed in supplemental Table S5 (available at www.jneurosci.org as supplemental material)]. Briefly, larvae or pupae were cryosectioned at 15 μm and sections were fixed and hybridized to digoxygenin (DIG)-labeled RNA probes. Sense and antisense probes were generated from the same plasmid. Sections were incubated with horseradish peroxidase (HRP)-conjugated anti-DIG antibody (1:200–1:2000; Dako) and rabbit anti-green fluorescent protein (GFP) antibody (Invitrogen). HRP-DIG antibody signal was amplified using an HRP-dependent tyramide amplification kit from PerkinElmer followed by secondary goat anti-rabbit Alexa 488 antibody (Invitrogen) and goat Cy3-conjugated streptavidin antibody (1:500; Jackson ImmunoResearch). *In situ* hybridizations were repeated three independent times, with $n > 5$ animals for each round.

Fly brains were dissected, fixed, and processed for whole-mount immunostaining as previously described (Lee et al., 1999). The following antibodies were used: rat monoclonal anti-mouse CD8 α subunit (1:100; Caltag); mouse monoclonal 1D4 (1:50; anti-FasII) and mouse monoclonal mAbdac2-3 (1:30; anti-Dac) (both from Developmental Studies Hybridoma Bank); mouse monoclonal M5 anti-FLAG (1:100; Sigma-Aldrich); rabbit polyclonal anti-Boule preabsorbed against *w* embryos (1:500; kind gift from S. Wasserman, University of California, San Diego, La Jolla, CA) (Cheng et al., 1998).

Fly strains and transgene construction. The following GAL4 lines were used in this study: *yw; UAS-mCD8::GFP; OK107-GAL4* (OK107-GAL4) and *yw; FRTG13, UAS-mCD8::GFP, 201Y-GAL4* (201Y-GAL4). For the microarray experiments OK107-GAL4 virgins were crossed to either *yw* [wild-type (wt) control] or *w; UAS-EcR-W650A* (EcRDN) males. MARCM clones of MB neurons were generated by heat-shocking the following genotypes as described by Lee et al. (1999): *hsFLP122, UAS-mCD8::GFP, FRT19A/usp^3,FRT19A; UAS-mCD8::GFP/+; OK017-GAL4/+* (see Fig. 4*D*); *hsFlp122, UAS-mCD8::GFP/X;201Y-GAL4,UAS-mCD8::GFP/+;bol^{40}, FRT2A;tub-GAL80,FRT2A* (see Figs. 4*G*, 7*E*); *hsFlp122, UAS-mCD8::GFP/X; UAS-mCD8::GFP/+; bol^{40},FRT2A;tub-GAL80,FRT2A; OK107-GAL4/+* (see Fig. 7*C*,*D*,*F–I*). Boule transgenic flies (described below) used in Figure 6 are as follows: *yw* (wt control), *UAS-bolA::FLAG$^{12.2}$* (BolA), *yw; UAS-bolPM1::FLAG$^{8.2}$;UAS-bolPM1::FLAG$^{8.1}$* (2× PM1) or *yw;UAS-bolΔDAZ::FLAG$^{9.3}$* (bolΔDAZ). These flies were crossed to either OK107-GAL4 (see Fig. 6*B*,*G–I*) or *hsFlp122, UAS-mCD8::GFP; 201Y-GAL4,UAS-mCD8::GFP* (see Fig. 6*C–F*).

Point mutations were made using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Primers were designed to introduce nucleotide substitutions that result in an amino acid substitution from K73L (PM1) or Y75A (PM2), respectively. The deletion of eight core amino acids of the RRM domain was done using the same strategy, except that primers were designed to flank the sequence encoding amino acids 73–80. These mutations were produced in the coding region for *boule-A*, which was PCR amplified from pBluescript-Boule (gift from M. Fuller, Stanford University, Stanford, CA) with primers designed to flank the gene with *Eco*RI and *Acc*65I restriction sites, which were used for cloning into the pUAST vector. In addition, the 5′-primer contained the native Kozak sequence for Boule-A (GCAGAG) upstream of the transcriptional start site, whereas the 3′-primer contained three FLAG epitope sequences followed by a stop codon. The bolΔDAZ transgene was constructed by PCR amplification of the *boule-A* coding sequence for amino acid residues 1–158, with the native Kozak sequence upstream of the ATG and followed by 3×FLAG epitopes and a stop codon. Expression was also verified by Western blot of larval brain extracts using M5 α-FLAG mouse monoclonal antibody (1:5000; Sigma-Aldrich) and reprobed with rabbit polyclonal anti-GFP (1:2000; Invitrogen).

The *bol^{40}* mutant was created by Flp-mediated recombination of FRT-containing piggyBac insertions (pBac{WH1}f00724 and pBac{WH2}f00596) flanking the coding region of *boule* (see Fig. 7*A*), as described by Parks et al. (2004). Deletions were identified by two-sided PCR with a 5′-genomic primer flanking the deletion site and a 3′-primer for WH1, and a 5′-primer for WH2 with 3′-genomic primer flanking the 3′-end of the deletion. Deletion of *boule* transcripts was also verified by reverse transcription (RT)-PCR (see below).

RT-PCR. RT-PCR for all *boule* transcripts was done using a combination of 5′-primers located in exon 1 (common to transcripts A and D) or exon 3 (common to B and C), and 3′-primers in exon 10 (common to A and C) or exon 11 (common to B and D). Additionally, primers that amplify α-tubulin were used as controls. Primer sequences are listed in supplemental Table S5 (available at www.jneurosci.org as supplemental material). RNA was isolated from adult flies and reverse transcribed according to the manufacturer's protocol with Superscript II RT (Invitrogen) using oligo-dT for priming. cDNA equivalent to ~50 ng of total RNA isolated from adult flies was used for amplification at 58°C for 25 cycles using Platimun Pfx DNA Polymerase (Invitrogen) according to the manufacturer's protocols.

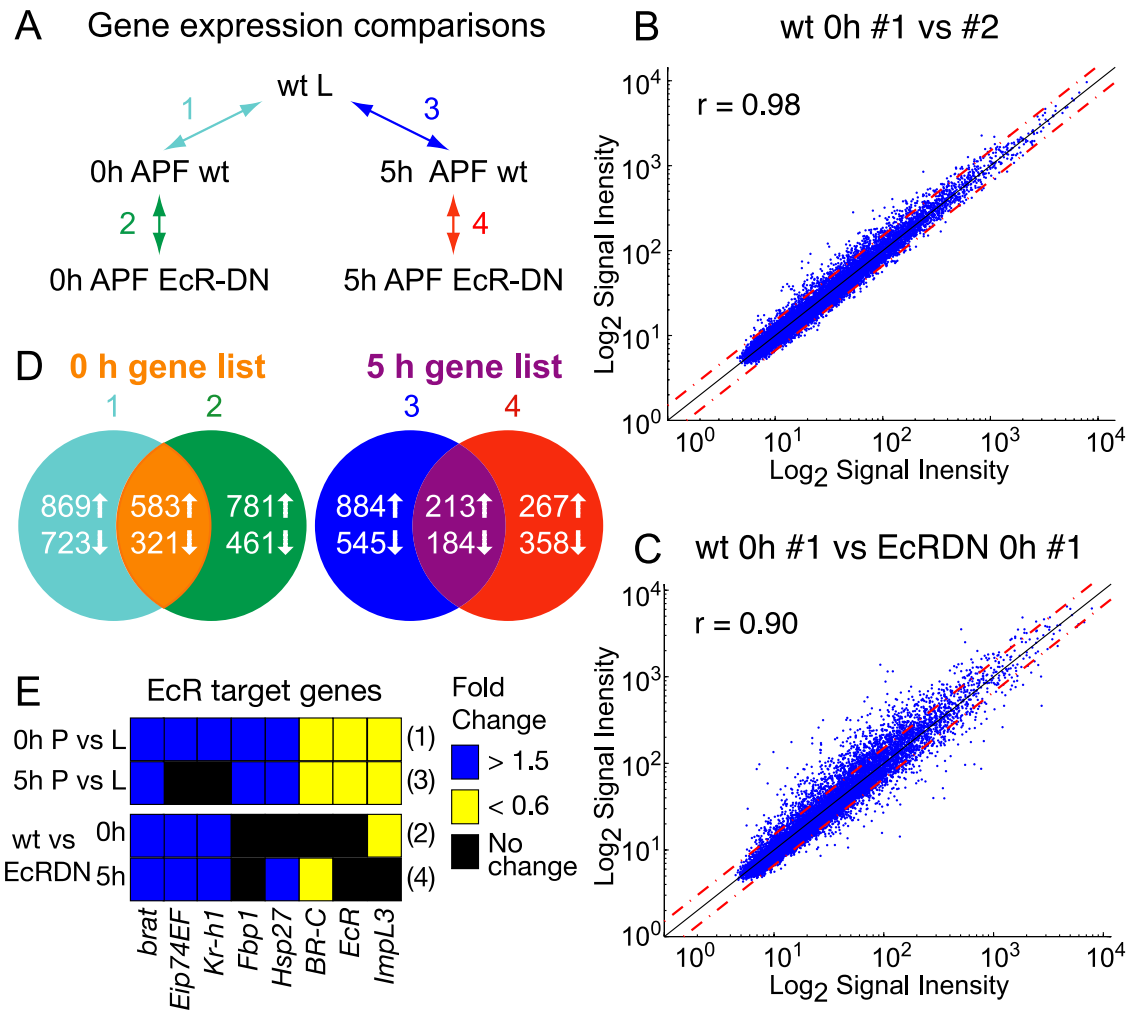

Figure 1. Microarray analysis of ecdysone-regulated gene expression in MB γ neurons. ***A***, Microarray experimental design. To assess changes in gene expression in MB γ neurons before and after the initiation of axon pruning, we compared wt third-instar larvae (L) 18 h BPF with newly formed pupae at 0 and 5 h APF. Ecdysone-dependent changes in gene expression were assessed by comparing wt MB γ neurons with those expressing EcRDN at both 0 and 5 h APF. All samples were laser captured from cryostat sections of brains expressing *UAS-mCD8::GFP* with OK107-GAL4 in the absence or presence of *UAS-EcRDN*. ***B***, Normalized probe signal intensities for two biological replicates of wt γ neurons at 0 h APF are plotted against each other. The red dashed lines represent a 1.5-fold difference in signal intensity. The degree of correlation between the two replicates is reflected by the Pearson's correlation coefficient (*r*). ***C***, Comparison of normalized signal intensities for individual samples from wt and EcRDN-expressing MB γ neurons at 0 h APF. ***D***, Venn diagram depicting the number of genes differentially expressed between the conditions outlined in ***A*** and the overlap between genes that show both developmental and EcR-dependent changes in expression at 0 or 5 h APF (for genes, see supplemental Tables S1, S2, available at www.jneurosci.org as supplemental material). ***E***, Expression changes of a select subset of known ecdysone-regulated genes detected by microarray analysis. Fold changes in gene expression in pupal MB neurons at 0 or 5 h APF (P) compared with larvae (L), or wt P compared with EcRDN P at 0 and 5 h APF. The numbers to the right of the rows refer to the comparison from ***A***.

Results

Microarray analysis to identify target genes of the ecdysone receptor during MB neuronal remodeling

To identify potential targets of the EcR that regulate MB neuronal remodeling, we analyzed global gene expression changes in wt and EcR mutant MB neurons before and after the onset of axon pruning. EcR is required for viability, reflecting its essential role in a variety of larval tissues throughout development. Thus, we expressed a dominant-negative form of the EcR (EcRDN) (Cherbas et al., 2003) specifically in MB neurons using the GAL/UAS system. Expression of EcRDN in MB γ neurons strongly inhibits axon pruning (Cherbas et al., 2003; Awasaki and Ito, 2004; Hoopfer et al., 2006); however, expression of EcRDN with the γ-specific 201Y-GAL4 is lethal in early pupal stages, likely because of expression outside of the nervous system. To avoid caveats associated with general developmental arrest, we used the brain-specific pan-MB OK107-GAL4 to drive expression of EcRDN, which also inhibits γ neuron axon pruning but does not result in lethality. In wandering third-instar larvae and newly formed pupae, the MB is primarily composed of γ and α′/β′ neurons (Lee et al., 1999). We used laser capture microdissection to isolate RNA from MB neurons visualized by OK107-GAL4 driven a membrane-bound GFP (mCD8::GFP) (supplemental Fig. S1*A*,*B*, available at www.jneurosci.org as supplemental material). RNA from 40 laser-captured samples was pooled together, amplified, labeled, and hybridized to Affymetrix *Drosophila Genome 1* microarrays. To verify that laser capture enriched for MB RNAs, we used real-time PCR to quantify levels of *eyeless*, which is specifically expressed in larval MB neurons in the central brain (Noveen et al., 2000). We found that *eyeless* transcripts are enriched >1000-fold in laser-captured MB neurons compared with adjacent unlabeled neurons or whole fly.

The experimental design of the microarray experiments is summarized in Figure 1*A*. To identify genes that are differentially expressed during the course of MB remodeling, we compared RNA isolated from wt controls at three time points: (1) staged

third-instar larvae at ~18 h BPF before the late larval pulse of ecdysone that initiates axon pruning; (2) at 0 h APF, after the ecdysone pulse; and (3) at 5 h APF when the first morphological signs of pruning can be detected (Watts et al., 2003). RNA from EcRDN-expressing neurons was isolated at 0 and 5 h APF, and compared with similarly aged wt neurons to identify genes that show EcRDN-dependent changes in gene expression. For each condition, we analyzed three to seven replicates. Biological replicates showed a strong correlation in signal intensity (Pearson's coefficient $r = 0.98$) (Fig. 1*B*), whereas wt and EcRDN neurons at the same time point showed a lower correlation ($r = 0.90$) (Fig. 1*C*). We used SAM analysis (Tusher et al., 2001) to identify genes that show statistically significant differences in expression between conditions. Additionally, we restricted our analysis to genes that have differences in gene expression of 1.5-fold and above. In this manner, we identified genes that are differentially expressed in wt and EcRDN-expressing neurons, and in wt neurons before and after the ecdysone pulse. Genes that show increased expression in pupal neurons compared with larval neurons, and increased expression in wt pupal neurons versus EcRDN pupal neurons, are likely to be ecdysone-induced genes; conversely, genes with decreased expression in pupal versus larval neurons, and decreased expression in wt versus EcRDN pupal neurons, are likely to be ecdysone-repressed genes (Fig. 1*D*). We find 583 putative ecdysone-induced genes at 0 h APF and 213 genes at 5 h APF, and 321 putative ecdysone-repressed genes at 0 h APF and 184 genes at 5 h APF [for list of genes, see supplemental Tables S1, S2 (available at www.jneurosci.org as supplemental material)]. For a subset of these genes (17), we analyzed differential expression at the appropriate time points by fluorescent *in situ* hybridization (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Approximately 88% (15 of 17) showed a similar developmental or EcR-dependent regulation of gene expression to that predicted from microarray analysis.

Interestingly, we identify several known ecdysone-regulated genes, such as *EcR* itself (Andres et al., 1993; Beckstead et al., 2005), *Broad-complex* (*BR-C*) (DiBello et al., 1991), *E74* (Burtis et al., 1990), *brat* (Beckstead et al., 2005), and *Kr-h1* (Pecasse et al., 2000; Beck et al., 2004), among others (Fig. 1*E*), suggesting that these global EcR targets are also targets of ecdysone in MB neurons. However, most of these genes are not essential for axon pruning, because previous studies indicate that MB γ neurons homozygous mutant for *E74*, *BR-C*, and *Kr-h1* prune axons normally (Lee et al., 2000; Shi et al., 2007).

Global analysis of ecdysone-regulated gene function during MB neuronal remodeling

To gain insight into the functional classes of genes regulated by EcR in the MB, we examined which functionally related groups of genes were enriched within the population of differentially expressed genes. The Gene Ontology (GO) consortium provides a detailed annotation of genes with respect to their molecular functions, subcellular localization and the biological processes in which they are involved (Ashburner et al., 2000). We used the functional annotation tool DAVID (Dennis et al., 2003) to identify functional classes of genes that are statistically overrepresented in the population of ecdysone-regulated genes compared with the total set of genes represented on the microarray. Because many GO terms share redundant sets of genes, we restricted our preliminary analysis to a set of 179 GO terms (GO essential slim) selected by Tomancak et al. (2007). A summary of the GO terms that are statistically overrepresented in the upregulated and downregulated gene populations is shown in Figure 2*A*.

We find several ontologies that are overrepresented in the population of genes that show EcR-dependent upregulation at 0 and 5 h APF [for details, see supplemental Table S3 (available at www.jneurosci.org as supplemental material)]. Among the functional classes of enriched genes were those that encode cytoskeletal-binding proteins, components of programmed cell death and autophagy, and regulators of transcription. For example, we observe an upregulation of many autophagy-related genes by ecdysone (Fig. 2*A*). However, knockdown of ATG-5, ATG-7, or ATG-12 expression in MB γ neurons by RNA-mediated interference does not inhibit axon pruning (O. Schuldiner and L. Luo, unpublished observations), but expression of the same transgenes does inhibit autophagy in the *Drosophila* fat body (Scott et al., 2004), suggesting that the autophagy pathway may not be essential for axon pruning. Additionally, we find that several genes encoding structural constituents or regulators of the cytoskeleton are differentially regulated in MB neurons at the onset of pruning, including regulators of actin (Fig. 2*B*) and microtubule (MT) dynamics (Fig. 2*C*). The selective disruption of the MT cytoskeleton in γ neuron axon branches, but not in the primary axon branch, is one of the earliest markers of axon degeneration in *Drosophila* neurons (Watts et al., 2003), and is also an early step in Wallerian axon degeneration of mammalian neurons (Zhai et al., 2003). Interestingly, we observe differential regulation of MT stabilizing and destabilizing proteins such as *stathmin* and *spastin*, *APC*, *Eb1*, *CLIP-190*, and *Mapmodulin* (Fig. 2*C*).

The population of genes downregulated by ecdysone is enriched for genes involved in energy production, protein translation, and metabolism (supplemental Table S4, available at www.jneurosci.org as supplemental material). Among the classes of genes most highly enriched are those encoding proteins associated with mitochondria and ribosomes, particularly genes involved in the production of precursor metabolites and energy, such as those involved in oxidative phosphorylation and glycolysis/gluconeogenesis, as well as structural constituents of cytoplasmic and mitochondrial ribosomes.

Both upregulated and downregulated gene sets show enrichment for genes encoding proteins that participate in synaptic transmission (Fig. 2*D*; supplemental Tables S3, S4, available at www.jneurosci.org as supplemental material). Interestingly, genes encoding different types of neurotransmitter receptors show differential regulation by ecdysone. For example, NMDAR1, serotonin receptor 2 (5-HT_2), and the α subunit of the nicotinic acetylcholine receptor (nAChRα) are upregulated, whereas the β subunit of nAChR, dopamine receptor, and $GABA_A$ and $GABA_B$ receptors are downregulated. Similarly, key enzymes involved in the synthesis of neurotransmitters such as acetylcholine, serotonin and dopamine, and glutamate are downregulated (*Cha*, *Ddc*, and *Got2*, respectively). Conversely, acetylcholine esterase, which degrades ACh, is upregulated in MB neurons. Together, these ecdysone-mediated transcriptional changes during MB remodeling suggest the possibility that MB neurons may change functionally in addition to structurally.

Ecdysone-mediated upregulation of the UPS in MB neurons

Genes involved in the UPS stood out as being among the most highly enriched class of upregulated genes (Fig. 2*A*; supplemental Table S3, available at www.jneurosci.org as supplemental material). Genes encoding subunits of the proteasome complex showed a 7.2-fold enrichment over the expected frequency ($p = 1.13E^{-14}$), as did genes involved in proteolysis (1.4-fold; $p =$

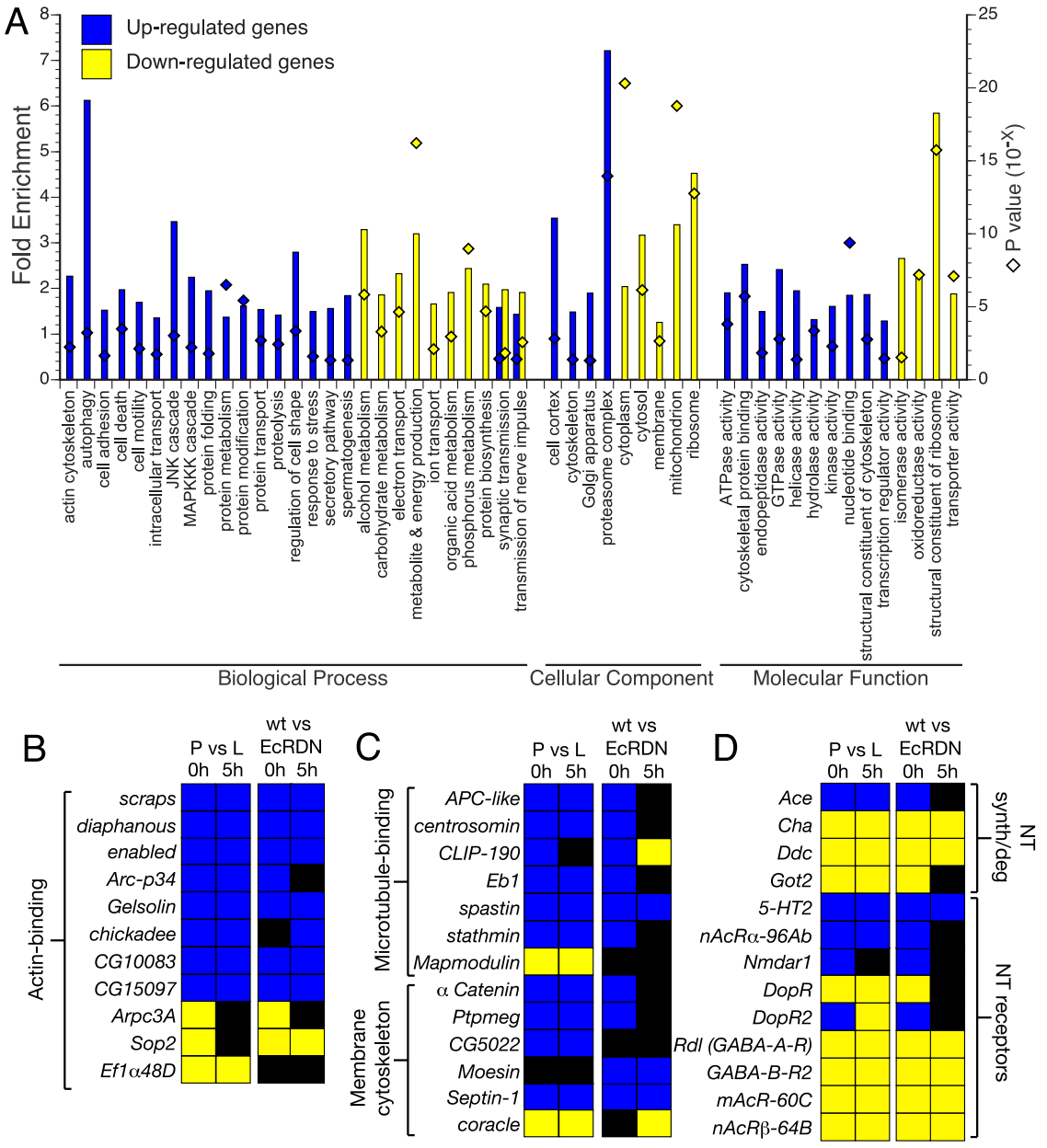

Figure 2. Functional characterization of differentially expressed genes. ***A***, Analysis of Gene Ontology categories for genes that show EcR-dependent upregulation or downregulation at 0 and/or 5 h APF (composite of genes from the orange and purple regions of the Venn diagrams in Fig. 1*D*). The graph represents GO categories that are significantly overrepresented ($p < 0.05$) in the two populations of differentially expressed genes. The bars indicate the fold enrichment (left *y*-axis) of the genes belonging to a particular GO term in the population of regulated genes, compared with the total population of genes on the DrosGenome1 array. The diamonds indicate the modified Fisher's exact *p* value (EASE score; right *y*-axis) for each category. Genes were classified by using a set of 179 GO categories for biological processes, cellular components, and molecular function (GO essential slim) (Tomancak et al., 2007). A given gene may belong to more than one group; for details, see supplemental Tables S3 and S4 (available at www.jneurosci.org as supplemental material). ***B***, ***C***, Genes encoding actin-binding (***B***) and microtubule-binding proteins or proteins associated with the membrane cytoskeleton (***C***) are differentially regulated by EcR in MB neurons. Gene functions are inferred from GO terms in Flybase and the study by Goldstein and Gunawardena (2000). Color convention for fold expression change is the same as in Figure 1*E*. ***D***, Regulation of genes encoding NT receptors and proteins involved in NT synthesis and degradation. Gene function is inferred from GO terms in Flybase.

$3.70E^{-3}$), or with endopeptidase activity (1.5-fold; $p = 0.0146$). We have previously shown that activity of the UPS is essential for MB axon pruning (Watts et al., 2003). Specifically, γ neurons fail to prune axons and dendrites if they are homozygous mutant for *Ubiquitin activating enzyme 1* (*Uba1*), which encodes the E1 of the UPS, or *Mov34* or *Rpn6*, which encode regulatory subunits of the proteasome. Here, we observe an upregulation of genes involved in all steps of UPS function (Fig. 3*A*,*B*), including ubiquitin itself, *Uba1*, ubiquitin-conjugating enzymes (E2s), ubiquitin ligases (E3s), and ubiquitin-specific proteases (UBPs). Furthermore, multiple subunits of the proteasome core complex and regulatory particle are also upregulated.

To further investigate this potential regulation of UPS components by EcR, we analyzed the expression of *Uba1* and *Rpn6* in wt or EcRDN MB neurons by *in situ* hybridization. Interestingly, *Uba1* mRNA expression is present in wt MB γ neurons in third-instar larvae and 0 h APF pupae (Fig. 3*C*,*D*), but is markedly decreased in EcRDN-expressing γ neurons at 0 h APF (Fig. 3*E*). In contrast, *Rpn6* mRNA shows increased expression in 0 h APF MB neurons compared with larvae (Fig. 3*F*,*G*), which is depen-

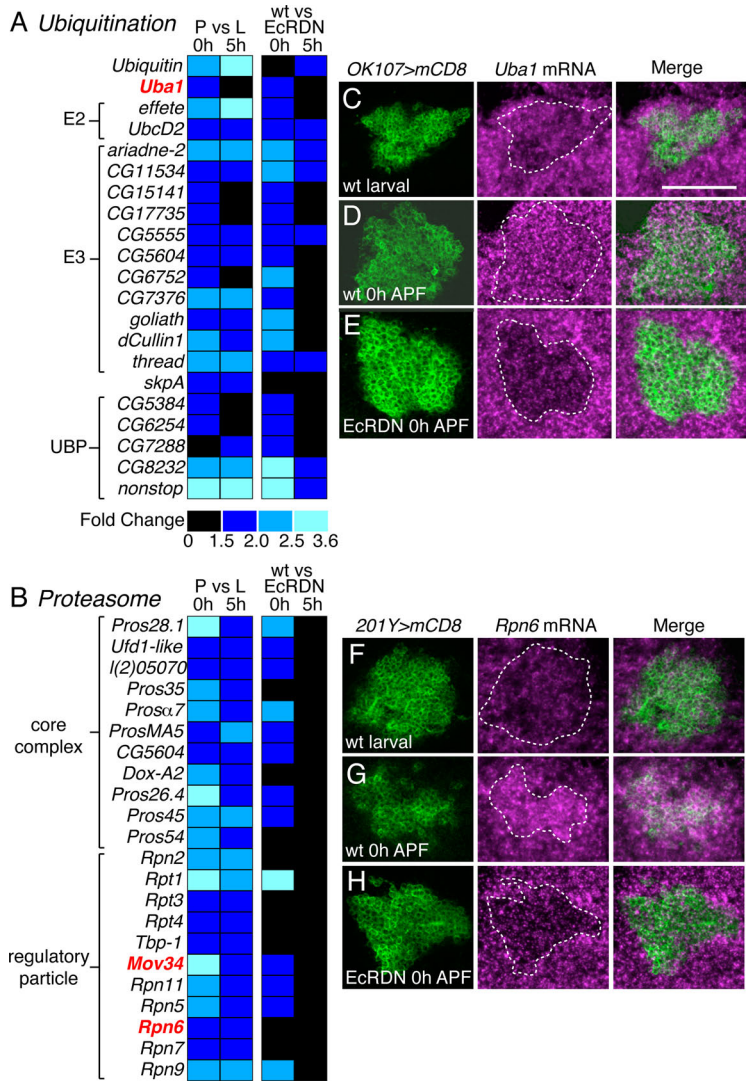

Figure 3. Ecdysone-dependent transcriptional upregulation of the UPS. ***A***, ***B***, Heat maps representing expression changes for genes involved in the ubiquitin–proteasome system. Genes in bold red print have been shown to be required for MB γ neuron pruning (Watts et al., 2003). ***C–E***, *Uba1* mRNA expression (magenta) in wt γ neurons (green) in early third-instar larvae before the ecdysone pulse that initiates pruning (***C***), just after the ecdysone pulse at 0 h APF (***D***), and in MB γ neurons at 0 h APF where EcR activity is blocked by expression of EcRDN (***E***). ***F–H***, *Rpn6* mRNA expression (magenta) in wt γ neurons (green) in early third-instar larvae (***F***), 0 h APF (***G***), and EcRDN-expressing γ neurons at 0 h APF (***H***). The panels show confocal *z*-projections from a 15 μm cryosection through MB neuron cell bodies. The dashed outline represents the extent of GFP-labeled MB neurons. Scale bar, 50 μm.

dent on EcR activity (Fig. 3*H*). Thus, it seems that EcR is necessary to maintain *Uba1* expression and to stimulate *Rpn6* expression. Together, these results indicate that ecdysone signaling positively regulates components of the UPS in MB γ neurons at the onset of axon pruning.

Boule expression is downregulated by EcR at the onset of MB pruning

Genes that are downregulated by EcR during metamorphosis might encode negative regulators of axon pruning. We focus on such a candidate, *boule*, which when overexpressed in MB neurons blocks axon pruning (see below). *boule* encodes a putative RNA-binding protein identified in *Drosophila* as being required for meiotic cell cycle progression during spermatogenesis, and had previously been characterized as being restricted to germ cells (Eberhart et al., 1996). However, our microarray data indicate that *boule* is expressed in MB neurons in early third-instar larvae and is downregulated 1.7-fold at 0 h APF. Furthermore, *boule* shows 2.3-fold higher expression in EcRDN versus wt neurons at 0 h APF (supplemental Table S2, available at www.jneurosci.org as supplemental material).

To verify the microarray results, we first analyzed the endogenous expression of *boule* in MB neurons. *boule* mRNA is expressed in MB γ neurons in early third-instar larvae, before the ecdysone pulse that initiates axon pruning (Fig. 4*A*), and is absent in γ neurons at 0 h APF (Fig. 4*B*). EcRDN expression blocks the downregulation of *boule* mRNA in γ neurons at 0 h APF (Fig. 4*C*). Sense controls done in parallel showed no specific staining in MB neurons in any of these conditions (supplemental Fig. S1*K–P*, available at www.jneurosci.org as supplemental material). To confirm that the downregulation of *boule* expression is attributable to endogenous activity of EcR, we analyzed *boule* mRNA expression in MB γ neuron clones homozygous mutant for usp^3, a loss-of-function allele of *ultraspiracle* (Lee et al., 2000). We generated MB neuroblast clones homozygous for usp^3 using the MARCM technique (Lee and Luo, 1999), which allows labeling of homozygous mutant clones in an otherwise unlabeled and heterozygous background. Similar to MB neurons expressing EcRDN, usp^3 MB neuroblast clones exhibit increased *boule* expression at 0 h APF (Fig. 4, compare *D*, *B*). We also see a similar result in MB γ neuron clones that are homozygous mutant for *baboon* (data not shown), a TGFβ receptor required for EcRB1 expression in γ neurons (Zheng et al., 2003). Together, these results indicate that *boule* expression is developmentally downregulated by EcR at the onset of MB axon pruning.

Immunostaining with a Boule polyclonal antibody (Cheng et al., 1998) shows that the Boule protein reflects the mRNA expression pattern; Boule is present in early larval MB neurons (Fig. 4*E*), but is undetectable by 0 h APF (Fig. 4*F*). The MB neuron staining in larvae is significantly decreased in γ neuron clones homozygous for a null mutation in *boule* (see Fig. 7; supplemental Fig. S2, available at www.jneurosci.org as supplemental material) compared with adjacent heterozygous MB neurons, confirming the specificity of the anti-Boule antibody (Fig. 4*G*). In comparison, protein staining for Dachshund, which is expressed in all MB neurons (Noveen et al., 2000), is the same within and outside of the *bol* mutant clone (Fig. 4G_2). These experiments validate that Boule protein is expressed in wt larval MB neurons and downregulated at the onset of metamorphosis.

Overexpression of Boule in MB γ neurons inhibits axon pruning

In parallel with the microarray screen for ecdysone-regulated genes in MB neurons, we simultaneously performed a functional

screen for genes that disrupt axon pruning when misexpressed in MB γ neurons (E. D. Hoopfer and L. Luo, unpublished observation). We identified EP3659, a P-element insertion containing multiple UAS sites (Rorth, 1996), as a potent inhibitor of axon pruning when crossed to pan-MB neuron OK107-GAL4 (Fig. 5, compare *A*, *B*). EP3659 is inserted upstream of the second transcriptional start site of the *boule* gene (see Fig. 7*A*), and is predicted to express both protein isoforms that are made by alternative splicing. Antibody staining confirms that EP3659 drives Boule protein expression in larval MB neurons when crossed to OK107-GAL4. Boule protein is localized to the cytoplasm of the cell body (Fig. 5*A*′,*B*′) and is not detectable in the axons or dendrites.

A developmental time course analysis of axon pruning with 201Y-GAL4 reveals that expression of Boule specifically in γ neurons is sufficient to inhibit pruning of dorsal and medial axon branches during metamorphosis (Fig. 5, compare C_1, C_2; D_1, D_2), and that these unpruned γ neuron axon branches persist in the adult (Fig. 5C_3,D_3). The following evidence suggests that the axon pruning phenotype induced by Boule overexpression is unlikely caused by nonspecific effects on general RNA stability or translation. First, overexpression of *Drosophila* poly(A)-binding protein in MB neurons does not inhibit axon pruning (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Second, in an overexpression screen, we identified several other RNA-binding proteins with diverse RNA-binding domains that disrupt MB axon morphogenesis when misexpressed, yet none inhibited axon pruning (E. D. Hoopfer and L. Luo, unpublished observations).

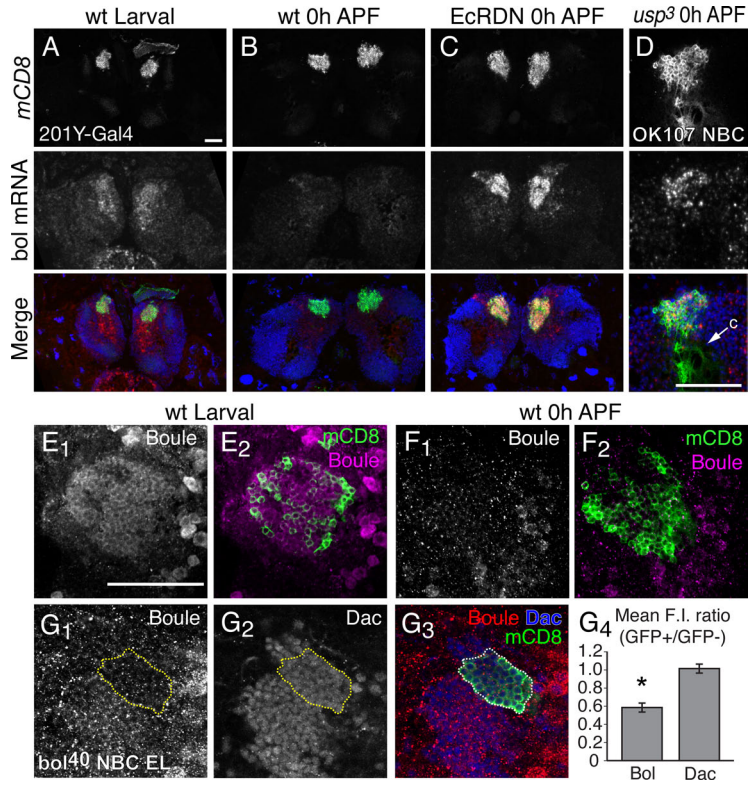

Figure 4. Boule expression is downregulated by ecdysone signaling in MB neurons at the initiation of metamorphosis. ***A–C***, *In situ* hybridization analysis of Boule mRNA expression in MB γ neurons. *Boule* mRNA expression in the brain of wt early third-instar larvae (***A***) and 0 h APF pupae (***B***). ***C***, Boule mRNA expression is elevated in EcRDN-expressing MB γ neurons at 0 h APF. The merged images show MB γ neurons in green, boule mRNA in red, and 4′,6′-diamidino-2-phenylindole (DAPI) nuclear staining in blue. ***D***, Boule mRNA expression in a MB neuroblast clone (NBC) that is homozygous for usp^3 at 0 h APF. The merge shows MB neurons labeled with mCD8::GFP (green), *boule* mRNA (red), and DAPI (blue). The arrow denotes calyx region (c), which is devoid of cell bodies. ***E***, ***F***, Boule protein expression (magenta) in MB γ neurons (green) in early third-instar larvae (***E***) and 0 h APF pupae (***F***), as detected by antibody staining with a rabbit polyclonal antibody against Boule. ***G***, Boule protein is decreased in bol^{40} mutant clones in early third-instar larvae. The panels show Boule (***G1***) and Dachshund (Dac) (***G2***) protein staining, with a merge (***G3***) showing the MARCM NBC in green, Boule in red, and Dac in blue. The yellow dashed line represents the extent of the bol^{40} clone marked by mCD8::GFP. ***G4***, Graph of the mean ratio of fluorescence intensity (F.I.) for Boule or Dac protein staining within the bol^{40} homozygous clones marked by GFP, or in adjacent heterozygous cells (GFP^-). Error bars represent SEM. The asterisk denotes a value of $p < 0.002$ (two-tailed unpaired *t* test; $n = 6$ MB NBCs). Residual staining with the Boule polyclonal antibody in bol^{40} clones is likely attributable to nonspecific antibody staining. Images in ***A–D*** show confocal *z*-stacks of 15 μm cryosections of larval or pupal brains showing MB neuron cell bodies marked with mCD8::GFP driven by 201Y-GAL4 or OK107-GAL4. Images for ***E–G*** are 1 μm optical sections taken from a confocal stack. $n \geq 12$ brains for each experiment. Scale bars, 50 μm.

Analysis of Boule function in MB axon pruning

Given the downregulation of *boule* in MB neurons at the onset of pruning and the inhibition of MB pruning by Boule overexpression, we hypothesize that Boule acts as a negative regulator of axon pruning. *Boule* and its metazoan homologues encode a family of RNA-binding proteins with a conserved function in meiotic cell cycle regulation from worms to humans (for review, see Reynolds and Cooke, 2005). Boule family members contain a conserved RNA binding domain with two conserved RNA recognition motifs (RRMs), and a deleted in azoospermia (DAZ) domain that has been suggested to mediate protein–protein interactions with poly(A)-binding protein as well as other RNA-binding proteins (Maegawa et al., 2002; Collier et al., 2005; Urano et al., 2005) (Fig. 6*A*).

To verify that it is overexpression of *boule* by EP3659 that blocks axon pruning, we generated a UAS transgene that drives expression of Boule-A with C-terminal FLAG tags (*UAS-bolA::FLAG*) (Fig. 6*A*). Previous work has shown that EP3659 drives expression of *boule* transcripts A and D (Joiner and Wu, 2004), resulting in expression of both protein isoforms. Furthermore, RT-PCR using cDNA laser-captured from MB neurons expressing EcRDN shows that *boule-A* is the primary transcript present in these neurons (data not shown). Overexpression of BolA::FLAG, hereafter referred to as Bol-A, in MB neurons using either 201Y-GAL4 or OK107-GAL4 inhibits axon pruning (Fig. 6*D*,*H*), indicating that *boule* overexpression in MB neurons is sufficient to block axon pruning.

To determine whether RNA-binding is necessary for Boule to inhibit axon pruning, we constructed a set of *UAS-BolA::FLAG* transgenes with a deletion of the RRM1 domain [*bolΔRBD(73–80)*], or with point mutations in conserved residues of the RRM1 domain [*bolPM1(K73L)* and *bolPM2(Y75A)*]. These point mutations have been shown to affect RNA binding specificity and function of other RRM1-containing proteins *in vivo* (Amrein et al., 1994; Lisbin et al., 2000; Wan et al., 2001). We screened transgenic insertions with OK107-GAL4 flies and assessed axon pruning and Boule transgene expression by anti-FLAG immunostaining (data not shown). Interestingly, all of the transgenic lines for

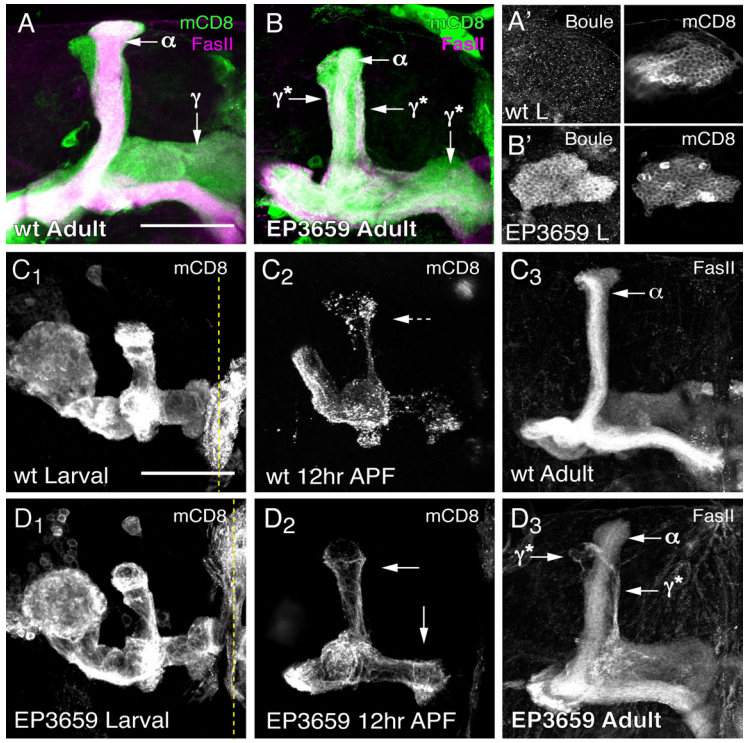

Figure 5. Boule expression inhibits axon pruning of γ neurons. ***A***, ***B***, Axons of wt (***A***) and EP3659-expressing (***B***) adult MB neurons. OK107-GAL4 drives expression of mCD8::GFP and EP3659 in all classes of MB neurons. Immunostaining against FasII (magenta) weakly stains γ neuron axons and strongly stains α/β neuron axons. FasII-positive axons outside of the α lobe are unpruned γ neurons (γ*). ***A′***, ***B′***, Single confocal section through OK107-GAL4 labeled cell bodies from wt (***A′***) or EP3659 (***B′***) larval MB neurons immunostained against Boule protein. ***C***, ***D***, Developmental axon pruning of wt γ neurons (***C***) and γ neurons expressing EP3659 (***D***). The γ neuron-specific driver 201Y-GAL4 marks γ neuron axons before axon pruning in third-instar larvae (***C***$_1$, ***D***$_1$), at the peak of axon pruning in 12 h APF pupae (***C***$_2$, ***D***$_2$), and in the adult (***C***$_3$, ***D***$_3$). The solid arrows denote intact axons; the dashed arrows denote degenerating axons; and the dashed yellow line denotes the midline. All images are confocal *Z* projections visualized using anti-mCD8 (green) for mCD8::GFP and anti-FasII (magenta) labeling. $n \geq 12$ brains for each time point. Scale bars, 50 μm.

*bol*Δ*RBD*, *bolPM1*, and *bolPM2* (>10 independent insertions screened for each) showed low protein expression compared with the *UAS-BolA::FLAG* transgene, suggesting that mutating the RNA-binding domain affects the maturation or stability of Boule protein.

Expression of two insertions of *UAS-bolPM1::FLAG* (2× PM1) gives similar levels of transgenic protein expression in MB neurons as one copy of a weakly expressed transgenic insertion of wt *UAS-BolA::FLAG*, as determined by Western blot of larval brain extracts (Fig. 6*B*). This allowed us to compare the activity of the wt and RNA-binding mutant Boule-A. Whereas wt MB γ neurons labeled with 201Y-GAL4 show no intact γ axons at 24 h APF (Fig. 6*C*, open arrowhead), MB γ neurons expressing wt Bol-A show a partial inhibition of axon pruning at 24 h APF (Fig. 6*D*, arrowhead denotes axons). In contrast, no inhibition of axon pruning is observed at 24 h APF in MB γ neurons expressing 2× PM1 (Fig. 6*E*) or 2× PM2 (data not shown). However, when the transgenes are driven with the strong pan-MB OK107-GAL4, both wt Bol-A and 2× PM1 inhibit axon pruning as evident by the ectopic FasII-positive axons in the dorsal lobe of adult MBs (Fig. 6*H*,*I*), whereas 2× PM2 does not show defects in axon pruning (data not shown). In summary, these data show that, at similar expression levels, the PM1 mutation in the RNA-binding domain of Boule reduces its ability to inhibit axon pruning; however, this effect can be compensated for by overexpression of mutant protein.

To test whether DAZ-mediated protein interactions are necessary for Boule function in axon pruning, we constructed a similar *UAS-BolA::FLAG* transgene with a truncation of the C-terminal 31 aa containing the DAZ domain (*UAS-Bol*Δ*DAZ::FLAG*). Expression of BolΔDAZ with 201Y-GAL4 or OK107-GAL4 shows no inhibition of axon pruning (Fig. 6*F*,*J*) despite comparable level of transgene expression as wt Bol-A (Fig. 6*B*). Thus, the DAZ protein interaction domain is required for Boule function in axon pruning.

boule is not required for normal MB morphogenesis or neuronal remodeling

Most studies of *boule* and its homologues suggest that Boule protein expression is restricted to the germline where it is required for germ cell production by regulating meiotic cell cycle progression (Eberhart et al., 1996; Karashima et al., 2000; Xu et al., 2001; Luetjens et al., 2004). However, we find that Boule is expressed in larval MB neurons and is sufficient to inhibit MB axon pruning. Similarly, a previous study also described Boule expression in the adult brain, where its overexpression causes defects in synaptic transmission in the retina (Joiner and Wu, 2004). Given these gain-of-function phenotypes in the nervous system, we next asked what was the consequence of losing Boule on MB axon pruning and neuronal morphogenesis.

Four different transcripts of *boule* are produced through alternative splicing and alternative use of two different promoters (Fig. 7*A*). The distal promoter is predicted to drive expression of transcripts B and C, whereas the proximal promoter drives expression of transcripts A and D. Male flies homozygous mutant for *bol*^{1}, a P-element insertion in *boule* that reduces Boule protein expression in the testes, are infertile (Eberhart et al., 1996). We analyzed the expression of *boule* in male and female flies by RT-PCR with primers specific for each of the four transcripts. As shown in Figure 7*B*, all four transcripts are present in adult males, whereas transcript B is absent in females, suggesting that B is testes specific. We find that *bol*^{1} males lack transcripts B and C, but still express A and D (Fig. 7*B*). This observation is confirmed by the absence of Boule protein in the testes of *bol*^{1} males (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Conversely, Boule expression is virtually identical in the brains of wt and *bol*^{1} males (supplemental Fig. S2, available at www.jneurosci.org as supplemental material), thereby strongly suggesting that the transcripts observed in the brain originate from the proximal promoter.

To assess the role of *boule* in the CNS, we created a null allele of *boule* by deleting the majority of its coding region using Flp-mediated recombination between two FRT-containing piggyBac-elements (Parks et al., 2004) (Fig. 7*A*). The resulting deletion mutant *Df(3L)bol*^{40} (*bol*^{40}) does not express transcripts A, C, or D, and expresses a truncated form of *boule-B*, which lacks the starting methionine as well as the RNA-binding and DAZ

domains (Fig. 7*B*). Similar to *bol*^{1} mutants, *bol*^{40} males, but not females, are infertile (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). In contrast to *bol*^{1} flies, which are homozygous viable, between 80 and 90% of *bol*^{40} homozygotes die before puparium formation; the escapers develop normally and survive into adulthood.

Given that Boule acts as a negative regulator of pruning and the endogenous protein is downregulated in MB neurons at the onset of pruning, one might hypothesize that loss of *boule* would cause early pruning in γ neurons or ectopic pruning in other MB neuron classes. This would suggest that Boule acts as a "master regulator" of axon pruning, and that loss of Boule is sufficient to initiate pruning in the absence of any other ecdysone-mediated regulators of pruning. To test this hypothesis, we generated clones of MB neurons that are homozygous for *bol*^{40} in an otherwise heterozygous organism using the MARCM technique. Mutant clones were induced at various stages of postembryonic development to selectively label wt or homozygous mutant MB neuroblast clones, which label all three neuron classes, or single-cell clones for each MB neuron class. We observed no gross morphological differences between wt and *bol*^{40} neuroblast clones (Fig. 7*C*,*D*). Because excess Boule inhibits pruning, we tested whether the loss of Boule may induce precocious pruning. We therefore analyzed neuronal remodeling of single-cell and two-cell clones of γ neurons during axon pruning. We observe no difference in the time course of axon pruning between wt controls (data not shown) (Watts el al., 2003) and *bol*^{40} mutant MB γ neurons (Fig. 7*E*). These results indicate that *boule* in MB γ neurons is not required for axon pruning (Fig. 7E_1–E_3) or general morphogenesis (Fig. 7E_4). Furthermore, we did not observe any difference in axonal morphology between wt and mutant α′/β′ neurons (Fig. 7*F*,*G*) or α/β neurons (Fig. 7*H*,*I*). Thus, loss of *boule* is not sufficient to cause precocious or ectopic pruning, suggesting that Boule acts redundantly with other ecdysone-regulated genes to control axon pruning.

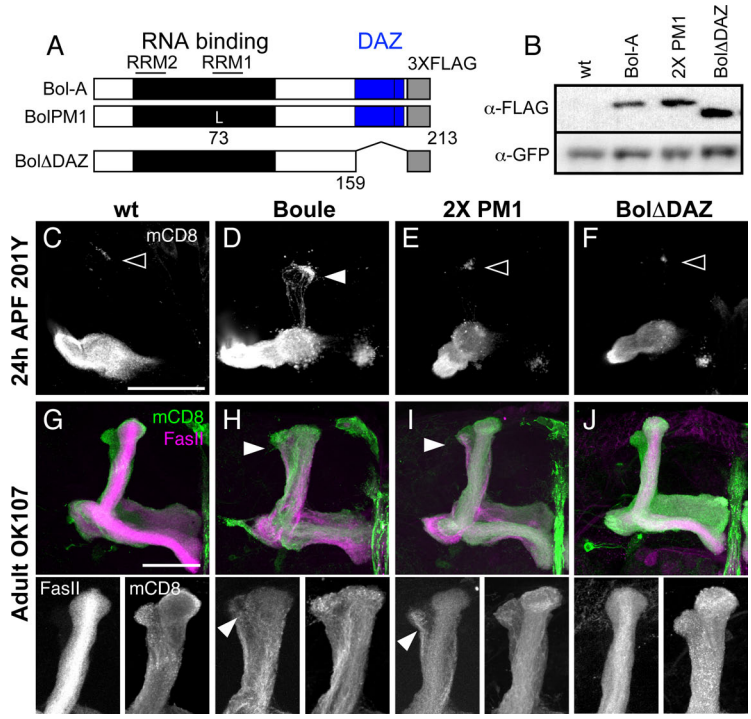

Figure 6. Mutations in the RNA-binding and DAZ domains reduce the ability of Boule to block axon pruning. ***A***, Schematic of *UAS-Boule-A::FLAG* transgenes. The wt transgene expresses Boule-A with three FLAG epitopes (gray box) at the C terminus (Bol-A). Boule contains a conserved RNA-binding domain (black box) composed of type 1 and type 2 RRMs (lines), and a DAZ repeat domain (blue box). The BolPM1 transgene has a point mutation (K73L) in the RRM1 domain of Bol-A, and the BolΔDAZ transgene is truncated at amino acid 159, thereby deleting the DAZ domain. ***B***, Western blot of brain extracts from larvae coexpressing mCD8::GFP and Boule-FLAG transgenic proteins in MB neurons driven by OK107-GAL4. The top panel shows staining with a monoclonal antibody against FLAG. The bottom panel shows the same blot after stripping and reprobing with anti-GFP as a control for protein loading. All larvae express mCD8::GFP. Larvae with two insertions of the BolPM1 transgene (2× PM1) or one insertion of BolΔDAZ have higher protein expression levels compared with larvae with one copy of the wt BolA transgene. ***C–F***, MB γ neuron axon pruning at 24 h APF, in which γ neurons are labeled with mCD8::GFP driven by 201Y-GAL4. At this time point, axons have completely degenerated in wt γ neurons (***C***), with few axon fragments remaining at the tips of the former dorsal lobe (open arrowhead). Transgenic expression of wt Bol-A in γ neurons (***D***) partially inhibits axon pruning, as evident by the GFP-positive axons in the dorsal lobe (arrowhead). MB γ neurons expressing 2× PM1 (***E***) or BolΔDAZ (***F***) mutant protein prune normally. ***G–J***, Axon pruning defects in adult MB neurons expressing Boule transgenes driven by OK107-GAL4. Transgenic protein expression levels were increased by using OK107-GAL4, which strongly expresses in all classes of MB neurons but has little expression outside of the CNS. The panels below ***G–J*** show enlarged images of the dorsal lobe, with FasII staining on the left and OK107-GAL4 driven mCD8::GFP on the right. MB γ neurons expressing transgenic wt Bol-A (***H***) or BolPM1 mutant protein (***I***) have a thick dorsal lobe with ectopic FasII-positive axons in the dorsal lobe (arrowheads), indicating a failure to prune. MB neurons expressing BolΔDAZ mutant protein (***J***) are similar to wt MBs (***G***). ***C–J*** show confocal *Z* projections of the MB axon lobes. $n > 8$ brains for each experiment. Scale bars, 50 μm.

Discussion

Ecdysone-regulated gene expression program for MB γ neuron remodeling

To identify genes that regulate developmental axon pruning, we used DNA microarrays to analyze ecdysone-dependent gene expression changes in MB neurons at the onset of metamorphosis. We identified 1038 genes that show ecdysone-dependent expression in MB neurons at the onset of neuronal remodeling at 0 h APF, or in the early steps of axon pruning at 5 h APF (supplemental Tables S1, S2, available at www.jneurosci.org as supplemental material). Approximately 32% of these were previously identified as being regulated by ecdysone at the onset of metamorphosis in the whole animal or the brain (Li and White, 2003; Beckstead et al., 2005). The large number of genes unique to our data set supports the assertion that we have enriched for MB specific ecdysone-regulated gene expression changes.

We find that distinct functional classes of genes are differentially regulated by ecdysone during MB axon pruning (Fig. 2). These classes give insight into the molecular pathways that regulate neuronal remodeling. For example, the upregulation of genes encoding regulators or structural constituents of the cytoskeleton provides candidate molecules that may be involved in remodeling the axon cytoskeleton during pruning. We also describe the differential regulation of several genes involved in synaptic transmission, including those involved in the synthesis and degradation of neurotransmitters (NTs) and receptors for excitatory and inhibitory NTs. This may reflect developmental changes in the functional properties of the MB neurons, or a response to pruning of their presynaptic partners (Marin et al., 2005). Given that

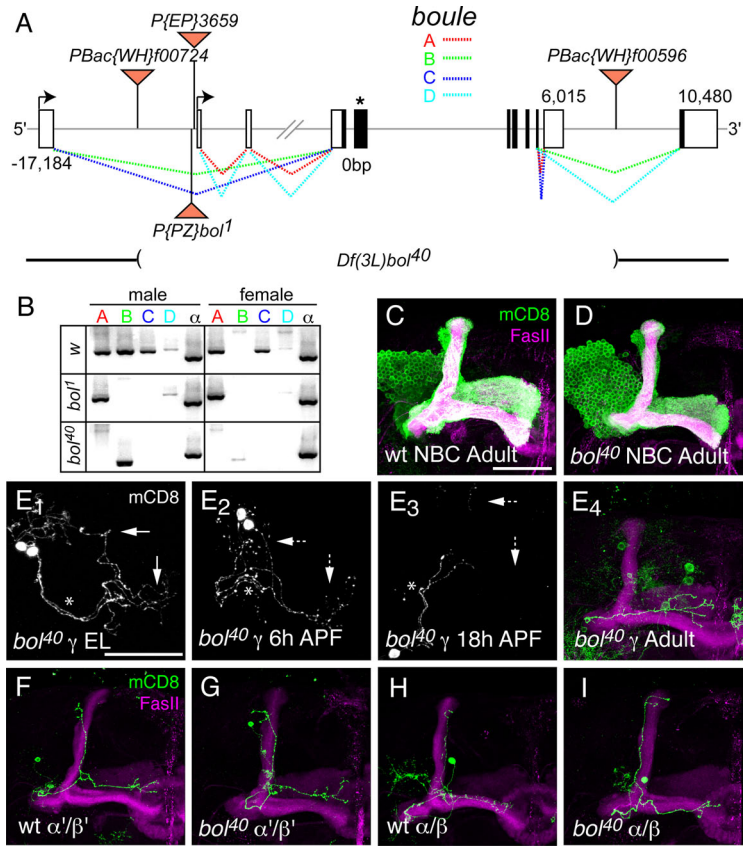

Figure 7. Loss-of-function analysis of Boule in MB neurons. ***A***, Schematic of *boule* gene illustrating alternative splicing of exons and transposon insertions. The *boule* gene is predicted to make four different transcripts through alternative splicing and alternative promoter use. The two promoters are represented by black arrows. *EP3659* is inserted upstream of the second transcriptional start site in the same orientation as *boule*. The bol^{1} P-element insertion is located just upstream of *EP3659*, but in the opposite orientation. Flippase-mediated recombination between two piggyBac elements containing FRT sites (*PBac{WH}f00724* and *PBac{WH}f00596*) was used to create a small deficiency in *boule* ($Df(3L)bol^{40}$), which deletes the majority of the coding sequence (see Materials and Methods). The coding sequence is shown in black, and the asterisk denotes the location of the RNA-binding domain. The dashed lines represent alternative splicing patterns with colors corresponding to individual splicing isoforms. ***B***, RT-PCR of *boule* transcript expression in w^{1118} (wt control), bol^{1}, and bol^{40} adult male and female flies. wt males express all four *boule* transcripts, whereas females do not express *bol-B*. bol^{1} homozygous mutant flies do not express *bol-B* or *bol-C*. No full-length transcripts are detected in bol^{40} homozygous mutant males or females. A truncated transcript of *bol-B*, most likely corresponding to the splicing of exon 1 to exon 11, is expressed in bol^{40} males. RT-PCR for ubiquitously expressed α-tubulin (α) mRNA is used as a positive control for RT-PCR. ***C***, ***D***, MARCM NBCs for wt control MB neurons (***C***) and bol^{40} mutant MB neurons (***D***) have grossly similar neuronal morphology. ***E***, Developmental time course analysis of γ neuron pruning in bol^{40} mutant single-cell or two-cell MARCM clones. As previously described for wt MB γ neurons (Watts et al., 2003), bol^{40} mutant γ neurons have intact dorsal and medial axon branches in the third-instar larva (***E***$_1$) and show initial signs of axon fragmentation at 6 h APF (***E***$_2$). By 18 h APF (***E***$_3$), the axon branches have degenerated back to the primary axon [marked with an asterisk (*)] and only the medial branch is reextended in the adult (***E***$_4$). The dashed arrows denote degenerating axons. ***F–I***, Single-cell MARCM clones of wt and bol^{40} mutant α′/β′ (***F***, ***G***, respectively) and α/β (***H***, ***I***, respectively) showing normal morphology in the adult. All panels show confocal *Z* projections of the MB neurons. $n \geq 12$ brains for each experiment. Scale bars, 50 μm.

little is known about the physiological properties of larval MB neurons, these genes may provide insight into potential functional differences between larval and adult MB neurons.

Ecdysone induces expression of the UPS in MB γ neurons at the onset of pruning

Of particular relevance to axon pruning is the upregulation of genes encoding components of the UPS (Fig. 3), because UPS activity is cell-autonomously required in various paradigms of *Drosophila* neuronal pruning (Watts et al., 2003; Kuo et al., 2005; Marin et al., 2005; Williams et al., 2006). Interestingly, genes in every step of UPS-mediated protein degradation are upregulated in an ecdysone-dependent manner (Fig. 3*A*,*B*). What is the functional significance of this transcriptional regulation for axon pruning? One possibility is that UPS function is increased to deal with an increased load of proteins that need to be degraded during pruning. We see a similar transcriptional coregulation of proteasome genes and the 20S maturase (*Pomp* in *Drosophila*) as observed in cultured *Drosophila* and mammalian cells in response to proteasome stress (Meiners et al., 2003; Lundgren et al., 2005). Additionally, we also see upregulation of the *Drosophila* homolog of the yeast ubiquitin-specific protease *Ubp6* (*CG5384*), which is associated with the proteasome (Lundgren et al., 2005) and has recently been shown to regulate ubiquitin homeostasis by preventing the degradation of ubiquitin (Hanna and Finley, 2007). Thus, MB γ neurons exhibit ecdysone-mediated induction of genes that may serve to increase overall UPS-mediated degradation.

We also observe an ecdysone-dependent upregulation of genes encoding components of the UPS that regulate the target specificity of degradation such as ubiquitin ligases (E3s) (Fig. 3*A*). Interestingly, multiple genes involved in programmed cell death, which have been shown to be involved in dendrite pruning in *Drosophila* sensory neurons, such as *effete*, *thread*, and *Ice* (Kuo et al., 2006; Williams et al., 2006), are upregulated by ecdysone in MB neurons at the onset of metamorphosis. However, genetic analysis suggests that none of these genes is required for axon or dendrite pruning in MB γ neurons by itself (E. D. Hoopfer, W. Hong, and L. Luo, unpublished observation). Thus, the molecular program used for pruning may differ by cell type, or may be more redundant in the CNS. Identification of the specific ubiquitin ligases and their substrates that are involved in MB pruning should yield insight into how these molecular programs differ. The ubiquitin ligases we identify in our microarray analysis (Fig. 3*A*) are attractive candidates for future investigation.

Boule as a negative regulator of axon pruning

We identified *boule* in two independent screens for genes involved in MB γ neuron pruning. Boule is downregulated in MB neurons by ecdysone at the onset of axon pruning, and overexpression of Boule in γ neurons is sufficient to inhibit axon pruning. Thus, we propose that Boule acts as a negative regulator of γ neuron pruning. How does Boule function to inhibit MB γ neuron pruning? Boule contains a highly conserved RNA-binding domain (Eberhart et al., 1996), and has been proposed to regulate meiotic entry during spermatogenesis by stimulating translation of the *Drosophila* Cdc25-type phosphatase *twine* through binding to the untranslated regions of *twine* mRNA (Maines and Wasserman, 1999). By using multiple insertions of *UAS-bolPM1::FLAG* to express BolPM1 at similar levels as the wt Bol-A transgenic

protein, we show that Bol-A blocks axon pruning to a greater extent than BolPM1 (Fig. 6); however, increased expression of BolPM1 can block axon pruning. A possible explanation for this dosage-dependent difference in the phenotype of BolPM1 may be attributable to the nature of the point mutation. A similar point mutation in *tra2* was shown *in vitro* to decrease RNA-binding specificity, without affecting RNA-binding affinity (Amrein et al., 1994). Thus, at low levels, BolPM1 may no longer efficiently interact with its RNA targets because of nonspecific interactions, but at higher levels binding may be saturated resulting in an inhibition of pruning.

Whereas a mutation in the RNA-binding domain reduces the ability of Boule to inhibit axon pruning, deletion of the DAZ domain abolishes the ability of Boule to block axon pruning. Biochemical analyses of vertebrate DAZ-like (DAZL) proteins have shown that the DAZ domain is essential for translational stimulation by mediating interactions with poly(A) binding proteins (Maegawa et al., 2002; Collier et al., 2005). Indeed, mouse DAZL protein has been shown to associate with poly(A)-bound polyribosomes in mouse testes (Tsui et al., 2000). In addition, human Boule can associate with other RNA-binding proteins, such as Pumilio-2, through its DAZ domain (Urano et al., 2005). Thus, our results suggest that *Drosophila* Boule may inhibit axon pruning by positively regulating the translation of RNAs through an interaction with poly(A)-binding protein or possibly other RNA-binding proteins.

Our loss-of-function data using a null allele of *boule* indicate that, whereas Boule expression is sufficient to inhibit γ neuron pruning, loss of Boule expression in MB neurons is not sufficient to initiate axon degeneration in the absence of other factors. This suggests that Boule acts redundantly with other positive regulators of axon pruning that are induced by ecdysone signaling, such as the UPS. Although we cannot rule out the possibility that the overexpression of Boule causes a neomorphic phenotype, the fact that Boule is expressed in MB neurons and downregulated by ecdysone signaling makes this possibility less likely. Regardless, its gain-of-function phenotype suggests that Boule must perturb the genetic program that regulates MB pruning; thus, the identification of the RNA targets of Boule in MB neurons should identify other molecular players in axon pruning. In the testes, Boule regulates translation of *twine* mRNA (Maines and Wasserman, 1999). We analyzed *twine* expression in pupal MB neurons using a *twine-lacZ* reporter that faithfully reflects *twine* translation in the testes (White-Cooper et al., 1998), and saw no *twine* expression in wt or Boule-overexpressing MB neurons (data not shown), suggesting the Boule has novel targets in MB neurons.

In summary, this study represents the first comprehensive analysis of the transcriptional program induced by ecdysone in a specific population of neurons in the *Drosophila* brain. Our results provide insight into the genetic program that underlies neuronal remodeling. We identify several molecular pathways that raise interesting hypotheses concerning the mechanisms that regulate both the morphological and functional remodeling of neurons during development. Several genes encoding ubiquitin ligases, regulators of cytoskeletal dynamics, and components of synaptic transmission are promising candidates for future investigation. Importantly, the transcriptional upregulation of UPS components by EcR provides a mechanistic link between ecdysone regulation and UPS activity in axon pruning. Last, we identify the RNA-binding protein Boule in two independent screens for genes involved in MB axon pruning. The function of Boule as a negative regulator of axon pruning suggests that, in addition to the transcriptional regulation of axon pruning by ecdysone signaling, Boule may represent an important point of posttranscriptional regulation for the initiation of axon pruning.

References

Amrein H, Hedley ML, Maniatis T (1994) The role of specific protein-RNA and protein-protein interactions in positive and negative control of pre-mRNA splicing by Transformer 2. Cell 76:735–746.

Andres AJ, Thummel CS (1994) Methods for quantitative analysis of transcription in larvae and prepupae. Methods Cell Biol 44:565–573.

Andres AJ, Fletcher JC, Karim FD, Thummel CS (1993) Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid-regulated transcription. Dev Biol 160:388–404.

Arbeitman MN, Furlong EE, Imam F, Johnson E, Null BH, Baker BS, Krasnow MA, Scott MP, Davis RW, White KP (2002) Gene expression during the life cycle of *Drosophila melanogaster*. Science 297:2270–2275.

Ashburner M (1974) Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. II. The effects of inhibitors of protein synthesis. Dev Biol 39:141–157.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25:25–29.

Awasaki T, Ito K (2004) Engulfing action of glial cells is required for programmed axon pruning during *Drosophila* metamorphosis. Curr Biol 14:668–677.

Bagri A, Cheng HJ, Yaron A, Pleasure SJ, Tessier-Lavigne M (2003) Stereotyped pruning of long hippocampal axon branches triggered by retraction inducers of the semaphorin family. Cell 113:285–299.

Beck Y, Pecasse F, Richards G (2004) Kruppel-homolog is essential for the coordination of regulatory gene hierarchies in early *Drosophila* development. Dev Biol 268:64–75.

Beckstead RB, Lam G, Thummel CS (2005) The genomic response to 20-hydroxyecdysone at the onset of *Drosophila* metamorphosis. Genome Biol 6:R99.

Bishop DL, Misgeld T, Walsh MK, Gan WB, Lichtman JW (2004) Axon branch removal at developing synapses by axosome shedding. Neuron 44:651–661.

Burtis KC, Thummel CS, Jones CW, Karim FD, Hogness DS (1990) The *Drosophila* 74EF early puff contains E74, a complex ecdysone-inducible gene that encodes two ets-related proteins. Cell 61:85–99.

Cheng MH, Maines JZ, Wasserman SA (1998) Biphasic subcellular localization of the DAZL-related protein boule in *Drosophila* spermatogenesis. Dev Biol 204:567–576.

Cherbas L, Hu X, Zhimulev I, Belyaeva E, Cherbas P (2003) EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. Development 130:271–284.

Collier B, Gorgoni B, Loveridge C, Cooke HJ, Gray NK (2005) The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. EMBO J 24:2656–2666.

Dennis Jr G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 4:P3.

DiBello PR, Withers DA, Bayer CA, Fristrom JW, Guild GM (1991) The *Drosophila* Broad-Complex encodes a family of related proteins containing zinc fingers. Genetics 129:385–397.

Ding M, Chao D, Wang G, Shen K (2007) Spatial regulation of an E3 ubiquitin ligase directs selective synapse elimination. Science 317:947–951.

Eberhart CG, Maines JZ, Wasserman SA (1996) Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. Nature 381:783–785.

Goldstein LS, Gunawardena S (2000) Flying through the *Drosophila* cytoskeletal genome. J Cell Biol 150:F63–F68.

Hanna J, Finley D (2007) A proteasome for all occasions. FEBS Lett 581:2854–2861.

Hoopfer ED, McLaughlin T, Watts RJ, Schuldiner O, O'Leary DD, Luo L (2006) Wlds protection distinguishes axon degeneration following injury from naturally occurring developmental pruning. Neuron 50:883–895.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249–264.

Joiner ML, Wu CF (2004) Nervous system function for the testis RNA-binding protein boule in *Drosophila*. J Neurogenet 18:341–363.
Karashima T, Sugimoto A, Yamamoto M (2000) *Caenorhabditis elegans* homologue of the human azoospermia factor DAZ is required for oogenesis but not for spermatogenesis. Development 127:1069–1079.
Kuo CT, Jan LY, Jan YN (2005) Dendrite-specific remodeling of *Drosophila* sensory neurons requires matrix metalloproteases, ubiquitin-proteasome, and ecdysone signaling. Proc Natl Acad Sci USA 102:15230–15235.
Kuo CT, Zhu S, Younger S, Jan LY, Jan YN (2006) Identification of E2/E3 ubiquitinating enzymes and caspase activity regulating *Drosophila* sensory neuron dendrite pruning. Neuron 51:283–290.
Lee T, Luo L (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22:451–461.
Lee T, Lee A, Luo L (1999) Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development 126:4065–4076.
Lee T, Marticke S, Sung C, Robinow S, Luo L (2000) Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. Neuron 28:807–818.
Li TR, White KP (2003) Tissue-specific gene expression and ecdysone-regulated genomic networks in *Drosophila*. Dev Cell 5:59–72.
Lisbin MJ, Gordon M, Yannoni YM, White K (2000) Function of RRM domains of *Drosophila melanogaster* ELAV: Rnp1 mutations and rrm domain replacements with ELAV family proteins and SXL. Genetics 155:1789–1798.
Luetjens CM, Xu EY, Rejo Pera RA, Kamischke A, Nieschlag E, Gromoll J (2004) Association of meiotic arrest with lack of BOULE protein expression in infertile men. J Clin Endocrinol Metab 89:1926–1933.
Lundgren J, Masson P, Mirzaei Z, Young P (2005) Identification and characterization of a *Drosophila* proteasome regulatory network. Mol Cell Biol 25:4662–4675.
Luo L, O'Leary DD (2005) Axon retraction and degeneration in development and disease. Annu Rev Neurosci 28:127–156.
Maegawa S, Yamashita M, Yasuda K, Inoue K (2002) Zebrafish DAZ-like protein controls translation via the sequence "GUUC." Genes Cells 7:971–984.
Maines JZ, Wasserman SA (1999) Post-transcriptional regulation of the meiotic Cdc25 protein Twine by the Dazl orthologue Boule. Nat Cell Biol 1:171–174.
Marin EC, Watts RJ, Tanaka NK, Ito K, Luo L (2005) Developmentally programmed remodeling of the *Drosophila* olfactory circuit. Development 132:725–737.
Meiners S, Heyken D, Weller A, Ludwig A, Stangl K, Kloetzel PM, Kruger E (2003) Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of mammalian proteasomes. J Biol Chem 278:21517–21525.
Nakamura H, O'Leary DD (1989) Inaccuracies in initial growth and arborization of chick retinotectal axons followed by course corrections and axon remodeling to develop topographic order. J Neurosci 9:3776–3795.
Noveen A, Daniel A, Hartenstein V (2000) Early development of the *Drosophila* mushroom body: the roles of eyeless and dachshund. Development 127:3475–3488.
Parks AL, Cook KR, Belvin M, Dompe NA, Fawcett R, Huppert K, Tan LR, Winter CG, Bogart KP, Deal JE, Deal-Herr ME, Grant D, Marcinko M, Miyazaki WY, Robertson S, Shaw KJ, Tabios M, Vysotskaia V, Zhao L, Andrade RS, et al. (2004) Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome. Nat Genet 36:288–292.
Pecasse F, Beck Y, Ruiz C, Richards G (2000) Kruppel-homolog, a stage-specific modulator of the prepupal ecdysone response, is essential for *Drosophila* metamorphosis. Dev Biol 221:53–67.
Portera-Cailliau C, Weimer RM, De Paola V, Caroni P, Svoboda K (2005) Diverse modes of axon elaboration in the developing neocortex. PLoS Biol 3:e272.
Reynolds N, Cooke HJ (2005) Role of the DAZ genes in male fertility. Reprod Biomed Online 10:72–80.
Rorth P (1996) A modular misexpression screen in *Drosophila* detecting tissue-specific phenotypes. Proc Natl Acad Sci USA 93:12418–12422.
Roy B, Singh AP, Shetty C, Chaudhary V, North A, Landgraf M, Vijayraghavan K, Rodrigues V (2007) Metamorphosis of an identified serotonergic neuron in the *Drosophila* olfactory system. Neural Develop 2:20.
Schubiger M, Tomita S, Sung C, Robinow S, Truman JW (2003) Isoform specific control of gene activity in vivo by the *Drosophila* ecdysone receptor. Mech Dev 120:909–918.
Scott RC, Schuldiner O, Neufeld TP (2004) Role and regulation of starvation-induced autophagy in the *Drosophila* fat body. Dev Cell 7:167–178.
Shi L, Lin S, Grinberg Y, Beck Y, Grozinger CM, Robinson GE, Lee T (2007) Roles of *Drosophila* Kruppel-homolog 1 in neuronal morphogenesis. Dev Neurobiol 67:1614–1626.
Spletter ML, Liu J, Liu J, Su H, Giniger E, Komiyama T, Quake S, Luo L (2007) Lola regulates *Drosophila* olfactory projection neuron identity and targeting specificity. Neural Develop 2:14.
Thummel CS (2002) Ecdysone-regulated puff genes 2000. Insect Biochem Mol Biol 32:113–120.
Tomancak P, Berman BP, Beaton A, Weiszmann R, Kwan E, Hartenstein V, Celniker SE, Rubin GM (2007) Global analysis of patterns of gene expression during *Drosophila* embryogenesis. Genome Biol 8:R145.
Tsui S, Dai T, Warren ST, Salido EC, Yen PH (2000) Association of the mouse infertility factor DAZL1 with actively translating polyribosomes. Biol Reprod 62:1655–1660.
Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98:5116–5121.
Urano J, Fox MS, Reijo Pera RA (2005) Interaction of the conserved meiotic regulators, BOULE (BOL) and PUMILIO-2 (PUM2). Mol Reprod Dev 71:290–298.
Wan L, Kim JK, Pollard VW, Dreyfuss G (2001) Mutational definition of RNA-binding and protein-protein interaction domains of heterogeneous nuclear RNP C1. J Biol Chem 276:7681–7688.
Watts RJ, Hoopfer ED, Luo L (2003) Axon pruning during *Drosophila* metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. Neuron 38:871–885.
Watts RJ, Schuldiner O, Perrino J, Larsen C, Luo L (2004) Glia engulf degenerating axons during developmental axon pruning. Curr Biol 14:678–684.
White KP, Rifkin SA, Hurban P, Hogness DS (1999) Microarray analysis of *Drosophila* development during metamorphosis. Science 286:2179–2184.
White-Cooper H, Schafer MA, Alphey LS, Fuller MT (1998) Transcriptional and post-transcriptional control mechanisms coordinate the onset of spermatid differentiation with meiosis I in *Drosophila*. Development 125:125–134.
Williams DW, Truman JW (2005) Cellular mechanisms of dendrite pruning in *Drosophila*: insights from in vivo time-lapse of remodeling dendritic arborizing sensory neurons. Development 132:3631–3642.
Williams DW, Kondo S, Krzyzanowska A, Hiromi Y, Truman JW (2006) Local caspase activity directs engulfment of dendrites during pruning. Nat Neurosci 9:1234–1236.
Xu EY, Moore FL, Pera RA (2001) A gene family required for human germ cell development evolved from an ancient meiotic gene conserved in metazoans. Proc Natl Acad Sci USA 98:7414–7419.
Zhai Q, Wang J, Kim A, Liu Q, Watts R, Hoopfer E, Mitchison T, Luo L, He Z (2003) Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration. Neuron 39:217–225.
Zheng X, Wang J, Haerry TE, Wu AY, Martin J, O'Connor MB, Lee CH, Lee T (2003) TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. Cell 112:303–315.