Expression and Function of Variants of Slob, Slowpoke Channel Binding Protein, in Drosophila

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The electrical behavior of neurons is flexible and subject to modulation. This plasticity is attributed in part to the modulation of membrane ion channels (Birch et al. 2004), by a variety of molecular mechanisms. For example, auxiliary subunits, protein kinases, and phosphoprotein phosphatases associate with ion channels and either directly or indirectly alter their function (Levitan 1994; Wang et al. 1999; Weiger et al. 2000). One such modulatable ion channel is the Drosophila calcium-dependent potassium channel, Slowpoke (dSlo). It is found in neurons as well as other cells and plays a critical role in the regulation of neuronal firing and neurotransmitter release (Becker et al. 1995; Gho and Ganetzky 1992). We previously showed that Slob participates in the modulation of membrane excitability. The recent discovery of at least two RNA editing sites in the slopoke gene increases the potential for functional diversity (Hoopengardner et al. 2003). We report here the characterization of multiple slob mRNA transcripts and Slob proteins in Drosophila. Previously we showed that Slob participates in circadian rhythms (Jaramillo et al. 2004) and now we extend this finding to include other Slob variants, the transcripts of which also cycle in fly heads. Our results also reveal differential binding of the different Slobs to dSlo and 14-3-3. Together these observations raise the possibility that the Slob variants offer the ion-channel complex more functional options as it participates in the modulation of membrane excitability.

M E T H O D S

Fly strains and maintenance

Drosophila melanogaster strain wv was raised at 25°C on standard Drosophila medium.

PCR and reverse-transcription PCR

Total RNA was extracted from wv flies using the Ultraspex RNA Isolation System (Biotecx Laboratories, Houston, TX). Genomic DNA was extracted from wv flies using phenol:chloroform. Single-stranded cDNA was generated using oligo-dT primers from the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Primers specific for slob transcripts were designed using Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). The following primers were used to amplify slob transcripts: 1) 5’ end of slob51-A and 57-A: (P1) 5’-GGC GTG CAG CGA GTG AAC TAC TTTG and (P2) 5’-CTG GTG TAG GTG GGA AAA AGC; 2) 5’ end of slob65-B and 71-B: (P3) 5’-TTA CAG CTA ACC AAC TGC TAT TTG; 3) 5’ end of slob57-C1 and C2: (P4) 5’-TTA CAC CCA AGT TTT ACA; 3’ end of all slob transcripts: (P5) 5’-GGG GGA GTA CTG ATA CTT GATG and (P6) 5’-ACA TGG TGA AGG ACT TCT TGG CGGT. PCR amplification of the target cDNAs was carried out using Taq DNA Polymerase (Promega, Madison, WI). Amplified products were separated on a 1% agarose electrophoresis gel.

In situ hybridizations

The RNA antisense and sense probes were synthesized using the DIG RNA Labeling Mix (Roche Applied Science, Indianapolis, IN). The sequence used for the all slob RNA probe was made from base pairs 1142–1441 of slob57-A. The sequence used for recognition of multiple transcripts and splicing capabilities of the Drosophila slopoke gene are spatially regulated by their tissue-specific promoters (Adelman et al. 1992; Becker et al. 1995; Brenner and Atkinson 1996; Chang et al. 2000; Lagrutta et al. 1994). The recent discovery of at least two RNA editing sites in the slopoke gene increases the potential for functional diversity (Hoopengardner et al. 2003). We report here the characterization of multiple slob mRNA transcripts and Slob proteins in Drosophila. Previously we showed that Slob participates in circadian rhythms (Jaramillo et al. 2004) and now we extend this finding to include other Slob variants, the transcripts of which also cycle in fly heads. Our results also reveal differential binding of the different Slobs to dSlo and 14-3-3. Together these observations raise the possibility that the Slob variants offer the ion-channel complex more functional options as it participates in the modulation of membrane excitability.
the smaller slob transcripts constituted base pairs 123–356 of slob57-A. The sequence unique to the slob71/65 probe was base pairs 198–486 and the sequence used for the alternative exon 168 (ae168) probe was base pairs 1222–1476. Both of these latter sequences are found in slob71-B. In situ hybridization on adult 12 μm head sections was done according to the protocols found at http://www.rockefeller.edu/labheads/vosshall/protocols.php with slight modifications. All hybridizations and washes were done at 60°C. Sections were developed in the dark for 3 days.

Quantitative PCR

Flies were entrained to a light/dark (LD) cycle for 3 days and collected at 4 h intervals as described previously (Jaramillo et al. 2004). RNA was extracted from a minimum of 50 yw heads per time point with the Ultraspec RNA Isolation System. Total RNA was treated with RNase-free DNAse, RQ1 (Promega) for 30 min. Single-stranded cDNA was generated using oligo-dT primers from the Superscript First-Strand Synthesis System (Invitrogen). Primers for slob57-A/51-A and slob71-B/65-B were designed using Primer Express software (Applied Biosystems, Foster City, CA). The following primer sequences were used: 1) slob57-A/51-A: forward primer: 5′-CTG GTC TAG GGT GGA AAA AGCC, reverse primer: 5′-CTG CAA GTG TCC TGC TTT CG; 2) slob71-B/65-B: forward primer: 5′-TTA CAG CTA ACC AAC TGC GACG, reverse primer: 5′-GCA TCG CAC GTT CCT TTC AT; 3) actin: forward primer: 5′-GCG CGG TTA CTC TTT CAC CA, reverse primer: 5′-ATG TCA CGG ACG ATT TCA CG. cDNAs from all time points were pooled and diluted four times to generate a standard curve for each primer set. SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of the appropriate primers were used for each 25 μl reaction. Experiments, performed in triplicate, were repeated at least three times, using the Applied Biosystems 7000 Sequence Detection System ABI Prism. Transcripts were normalized to the quantity of actin for each time point. Data analysis was done with ABI Prism 7000 SDS software and Origin software.

Antibodies

A GST-N-terminus Slob71/65 fusion protein (consisting of amino acids 2–91 of Slob71/65; see Fig. 1D) was used to immunize rabbits as described previously (Schooperle et al. 1998). Polyclonal antibodies specific to the N-terminus of Slob71/65 were generated by purifying the serum using a combination of CNBr-conjugated GST and CNBr-conjugated GST-N-Terminus Slob71/65 columns. Polyclonal antibodies that recognize all Slob57s and dSlob were prepared as described previously (Jaramillo et al. 2004; Wang et al. 1999). Anti-PER antibody was generously provided by A. Sehgal (University of Pennsylvania) and anti-HA antibody by D. Oprian (Brandeis University). Anti-FLAG M2 antibody was purchased from Sigma (St. Louis, MO).

Immunohistochemistry

For brain wholemount staining, fly brains were dissected and kept in cold phosphate-buffered saline (PBS), after which they were fixed with 4% paraformaldehyde (PFA) in PBS. Brains were blocked with 1% normal donkey serum in PBS/0.3% Triton X-100 (PBST) for 1 h. For presorption experiments, 10 μg/ml of GST-N-terminus Slob71/65 fusion protein was preincubated with anti-Slob71/65 antibody for 1 h at room temperature. Samples were incubated with primary antibody (at a dilution of 1:500 for anti-Slob71/65, 1:300 for anti-Slob, and 1:200 for anti-PER) overnight at 4°C. After being washed in PBST.
three times for 30 min each at room temperature, samples were incubated with the appropriate secondary antibody (Fluorescein (FITC)-conjugated AffiniPure donkey anti-rabbit IgG, FITC-conjugated AffiniPure donkey anti-rat IgG, and Texas Red dye–conjugated AffiniPure Donkey anti-rabbit IgG from Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:500 in 10% normal donkey serum in PBST for 1 h at room temperature, and washed in PBST three times for 30 min each. Brains were mounted onto slides with Fluoro- mount-G (Southern Biotechnology Associates, Birmingham, AL). Wholemounts were visualized using fluorescence microscopy on a Leica DMIRE2 microscope.

DNA constructs

An EST clone for CG6772-RB (GH25872) was obtained from Invitrogen. For biochemistry, the Slob cDNAs were subcloned into the mammalian expression vector pcDNA3 (BD Biosciences, Palo Alto, CA) using a PCR strategy to introduce an EcoRI/KpnI site. The 14-3-3 and dSlo cDNA contracts were subcloned into the mammalian expression vector pCMV-HA (BD Biosciences, Palo Alto, CA) using a PCR strategy to introduce an EcoRI site. The predicted molecular weights: Slob51, Slob57, Slob65, and Slob71 protein.

Transfection and immunoprecipitation

tsA201 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The CalPhos Mammalian Transfection Kit (BD Biosciences) was used to introduce cDNAs into the cells. Cells were lysed in 1% CHAPS, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 120 mM NaCl, 50 mM KCl, 2 mM DTT, and protease inhibitors (1 mM PMSF, 1 μg/ml each aprotonin, leupeptin, and pepstatin A [Sigma]). After centrifugation, the supernatant was pre- cipitated with anti-FLAG M2 antibody (Sigma, 2 μg/ml) and the supernatant was washed three times for 30 min each. The ratio of immunoprecipitate to lysate was averaged in each lane. The ratio of protein for each lysate and immuno- precipitate was calculated as the protein signal minus the background for Western blotting. An enhanced chemiluminescence detection system (ECL-Amersham Biosciences, Piscataway, NJ) was used to visualize proteins. Film exposures of Western blots were scanned using BioRad Molecular Analyst. The level of protein for each lysate and immuno- precipitate was calculated as the protein signal minus the background in each lane. The ratio of immunoprecipitate to lysate was averaged from at least three Western blots.

Western blotting and quantitation

Cell lysates and immunoprecipitates were loaded on polyacryl- amide gradient gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk in TBST (0.1% Tween 20 in Tris-buffered saline), the blots were probed with the appropriate primary and secondary antibodies (horseradish peroxidase–conju- gated donkey anti-rabbit or sheep anti-mouse IgG [Amersham Biosciences, Piscataway, NJ]). An enhanced chemiluminescence detection system (ECL-Amersham Biosciences) was used to visualize the proteins. Film exposures of Western blots were scanned using BioRad Molecular Analyst. The level of protein for each lysate and immuno- precipitate was calculated as the protein signal minus the background in each lane. The ratio of immunoprecipitate to lysate was averaged from at least three Western blots.

RESULTS

There are multiple slob transcripts in Drosophila heads

Release 3.1 Annotated Drosophila Genome (www.fruitfly.org) predicts three slob transcripts, CG6772-RA and RC that both encode a 57 kDa protein, and CG6772-RB that encodes a 71 kDa protein. To test these predictions, we performed PCR and reverse-transcription PCR on total RNA and genomic DNA from yw flies, using primers designed to amplify specific predicted transcripts. Figure 1A, lane 2, shows amplification of a region of mRNA predicted to be in both CG6772-RA and -RC, whereas lane 3 shows amplification of the mRNA se- quence unique to CG6772-RB. Thus at least two of these predicted transcripts are indeed expressed in flies. Much larger bands are amplified with the same primers when genomic DNA is used as template (Fig. 1A, lanes 4 and 5), confirming that the bands seen in lanes 2 and 3 arise from mRNA and not contamination of genomic DNA

To analyze these transcripts further, we used 5′-primers that allowed us to distinguish among them. As shown in Fig. 1B, two bands are amplified by each primer pair (specific for CG6772-A [lane 2], -B [lane3], and -C [lane 4], respectively), suggesting that in fact six transcripts are present in flies. Sequencing of the PCR products confirmed this suggestion and indicated that there are alternative splice variants of all three predicted transcripts (Fig. 1C). We designated the transcripts according to their predicted protein molecular weights: slob51-A and slob57-A (CG6772-RB), slob57-B and slob57-C (CG6772-RB), slob57-C and slob57-C2 (CG6772-RC) (Fig. 1C). slob51-A and slob57-B lack the same coding 168-bp exon (Fig. 1C). Interestingly, the region encoded by this exon includes 76RSAS79, one of two motifs that participate in the binding of 14-3-3 to Slob (Zhou et al. 1999).

slob71-B/65-B transcripts cycle in Drosophila heads

Microarray analyses (Ceriani et al. 2002; Claridge-Chang et al. 2001; Lin et al. 2002; McDonald and Rosbash 2001; Ueda et al. 2002) of cycling transcripts in Drosophila heads show that a slob transcript cycles. However, based on the probe used, the regulation of individual slob isoforms could not be dis- cerned. The Affymetrix probe was designed to recognize the 3′ end of the slob transcript (http://www.affymetrix.com/ products/arrayexpress/specific/Fly_affx), which corresponds to base pairs 1817–2278 of slob57-A, 2089–2550 of slob71-B, and 1884–2345 of slob57-C1.

We used quantitative PCR to determine whether slob57-A/ 51-A and slob71-B/65-B transcripts cycle in Drosophila heads. We entrained yw flies for 3 days to a 12:12 h light/dark cycle (LD) and total RNA was collected from heads at six time points throughout the fourth day in LD. Quantitative PCR was done on single-stranded cDNA using primers specific for the UTR regions of either slob57-A/51-A or slob71-B/65-B. We found that both transcripts cycle (Fig. 2) in phase with each other, peaking at Zeitgeber Time (ZT) 18. To confirm these quantitative PCR results, we used slob57-A,C/51-A and slob71- B/65-B in situ hybridization probes on Drosophila frontal head sections taken at ZT 6 and ZT 18. We found a difference in the intensity of the hybridizations of both probes (data not shown), suggesting that there is more mRNA present at ZT 18.

There are differences in mRNA expression patterns among the slob

To determine whether there are any differences in the expression pattern of the various slob transcripts, we per-
A regional probe was designed to a region that is common to all sense RNA probes to discriminate between the yw heads. We designed digoxigenin-labeled antisense and formed in situ hybridization on frontal sections of *Drosophila* yw heads. We designed digoxigenin-labeled antisense and sense RNA probes to discriminate between the slob probes. The all slob probe was designed to be a region that is common to all slob and therefore can recognize all transcripts. A second probe, alternative exon 168 (ae168) probe, was designed to recognize the 168 nucleotide alternative exon that is found in transcripts encoding Slob57 and Slob71. The third and fourth probes, slob57-A,C/51-A and slob71-B/65-B probes, are unique for the 5′-UTR regions of the smaller (51 and 57 kDa) or larger (65 and 71 kDa) slob mRNA. The right panels of Fig. 3, A–D show adult head frontal sections treated with the sense probes, illustrating no significant background signal. Figure 3A (left) shows all slob mRNA in the photoreceptors, optic lobe, and brain cortex. The head sections treated with ae168 probe exhibit a pattern similar to that of the all slob probe (Fig. 3B). slob71-B/65-B is found preferentially in the optic lobes, particularly in the medulla (Fig. 3C). There is also staining in the brain cortex, but little in the photoreceptors. In contrast, slob57-A,C/51-A mRNA is seen in the eye and, to a lesser extent, in the optic lobe and brain cortex (Fig. 3D).

**Slob71/65 is in the optic lobe and large ventral lateral neurons**

Previously, using an antibody raised against most of Slob57 that is predicted to recognize all Slobs, we demonstrated that Slob protein is present at the neuromuscular junction of *Drosophila* larvae (Zhou et al. 1999) and in the adult eye and brain (Jaramillo et al. 2004). Immunohistochemical staining of brain wholemounts using anti-Slob71/65 antibody reveals bright staining in the optic lobe (Fig. 4A) and large ventral lateral neurons (1-LN,s) (Fig. 4, A–D), and less intense staining in the surrounding brain cortex (Fig. 4, A and C). Punctate staining of Slob71/65 is seen in the medulla (Fig. 4, C and D), reminiscent of the punctate staining of PDF-positive projections from the large lateral neurons (Helfrich-Forster 1997). No staining is observed when the antibody is preabsorbed with antigen at concentrations ranging from 1 to 100 μg/ml (Fig. 4E) or when preimmune serum is used (data not shown).

Figure 5A shows a *Drosophila* brain wholemount treated with the original anti-Slob antibody staining the pars intercerebralis (PI) neurons (see Jaramillo et al. 2004) and the dorsal giant interneurons (DGIs) that are located dorsally on both hemispheres (Helfrich-Forster et al. 2000). This may be contrasted with Slob71/65 staining, which is absent from the PI and DGI neurons (Fig. 5B). The circadian protein PER (Fig. 5, C–F) is not present in the PI and DGI neurons (Fig. 5, C and E), but PER and Slob71/65 are expressed together in the 1-LN,s. Note that Slob71/65 staining appears cytoplasmic and surrounds the PER-enriched nuclei of the 1-LN,s (Fig. 5, D and F). These brain wholemounts were dissected at ZT 23, a time when PER protein accumulates in the nucleus.

**Binding interactions with Slowpoke differ among the Slobs**

Previously we showed that Slob57 binds dSlo (Schopperle et al. 1998). A region of 42 amino acids, residues 191–233 in Slob57, is necessary for it to interact with dSlo (Zhou et al. 2003). This region is present in all four Slobs. To determine whether all Slobs bind dSlo, we transfected HA-tagged Slobs and untagged dSlo in tsA201 cells, and anti-HA antibody was used to immunoprecipitate HA-tagged Slobs from cell lysates. The immunoprecipitates and lysates were

![Image](http://jn.physiology.org/)

**FIG. 3.** Four different slob transcripts are expressed differentially. Frontal sections of *Drosophila* heads were probed with digoxigenin-labeled RNA slob probes. Left: antisense probes. Right: sense probes. A: all slob probe hybridizes to the photoreceptors, optic lobe, and the surrounding brain cortex. B: sections labeled with ae168 probe reveal a similar pattern of distribution. C: slob71-B/65-B probe, which hybridizes to the slob71-B and slob65-B mRNAs, reveals preferential staining in the optic lobes. There is also staining in the brain cortex and significantly less in the photoreceptors. D: slob57-A,C/51-A probe hybridizes strongly to the photoreceptors. There is also some staining in the brain cortex and optic lobe.
probed with a polyclonal anti-dSlo antibody and an anti-HA antibody on Western blots. We find that all Slobs bind dSlo, but the strengths of interaction vary (Fig. 6, A and B). Two trends are seen: the first is that the larger the Slob protein, the weaker is its binding to dSlo (Fig. 6B); a second and unexpected trend occurs within Slob groups. The full-length Slobs (Slob57 and Slob71) bind to the channel less well than their smaller counterparts (Slob51 and Slob65) that lack the region encoded by the alternatively spliced exon (Fig. 6B). Two additional Slob proteins, Slob53 and Slob47, can be produced in heterologous cells as a result of a downstream translational start site (Zeng et al. 2005). As shown in Fig. 6C, these two Slobs also bind dSlo, but we do not know whether this downstream translational start site is used by the fly in vivo.

**Slob51 binds least well to 14-3-3**

Slob57 binds 14-3-3 using its two phosphoserine 14-3-3-binding motifs, RSNS<sup>54</sup> and RSAS<sup>79</sup> (Zhou et al. 1999). Because of these previous findings, we suspected that the 51- and 65-kDa Slobs would display a weaker interaction with 14-3-3 because the region encoded by the missing exon includes RSAS<sup>79</sup>. To determine the relative binding strengths of the Slobs to 14-3-3, we transfected HA-tagged Slob and FLAG-tagged 14-3-3 in tsA201 cells. Anti-FLAG-antibody was used to immunoprecipitate FLAG-tagged 14-3-3 from cell lysates, and the immunoprecipitates and lysates were probed with anti-Slob57/51 antibody or anti-FLAG antibody on a Western blot. We find that all Slobs bind 14-3-3, but Slob51 binds 14-3-3 least well (Fig. 7, A and B). The other Slobs all bind 14-3-3 to approximately the same extent.

**DISCUSSION**

The abundance and importance of multiple gene transcripts and alternative splice variants are becoming increasingly evident. The preferential expression of a transcript can result in fine-tuning of the dynamic relationships between sets of proteins, such as ion channels and their modulators. We report here that there are multiple slob transcripts expressed in Drosophila heads, two of which arise from each of the database-predicted transcripts, CG6772-RA, CG6772-RB, and CG6772-RC. We named these transcripts according to the molecular weights of the proteins they encode.
Innovative Methodology

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P to dSlo than does Slob51 (minimum of three repetitions per transfection condition. Slob57 binds less well in the lysate is plotted for the 4 Slobs tested. Shown are means ± SE for a minimum of three repetitions per transfection condition. Slob57 binds less well to dSlo than does Slob51 (\( P = 0.05 \)) and Slob71 binds less well to dSlo than does Slob65 (\( P = 0.00125 \)). C: 2 additional Slobs, Slob53 and Slob47, produced in this heterologous expression system, also coinmunoprecipitate with dSlo. dSlo was expressed either alone or with 1 of 3 slobs. Slob immunoprecipitates were blotted for dSlo (top). Lysates were probed for dSlo (middle) and the Slobs (bottom).

The Drosophila genome (www.fruitfly.org) predicts eight dsls proteins and Philip et al. (2001) described two 14-3-3 proteins (although the genome predicts only one). Thus the number of possible regulatory complexes becomes quite staggering when one considers combinations between dSlo (keeping in mind that there are four subunits to each channel), Slob and 14-3-3. The impressive potential functional diversity of this complex evokes the image of a control dial for cell excitability. Preferential expression of certain variants might regulate excitability over a wide range. The behavioral importance of splicing has been demonstrated for another circadian gene, per. In fact, the preferential expression of one of the per splice variants is regulated by temperature, light, and the circadian clock (Collins et al. 2004; Majercak et al. 1999, 2004) and is presumed to promote adaptive circadian behavior. A handful of other circadian genes that have splice variants, such as cryptochrome (Eun and Kang 2003), bmal2 (Schoenhard et al. 2002), and pac1 (Ajpru et al. 2002), also have been characterized.

Because the microarray analyses of cycling transcriptions (Ceriana et al. 2002; Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Ueda et al. 2002) did not distinguish between the various slob transcripts, we asked more specifically which slob transcript cycles in Drosophila heads. Using quantitative PCR and choosing amplicons unique to slob57-A/S1-A and slob71-B/65-B, we found that, in fact, both of these predicted transcripts do cycle (Fig. 2). We were not able to design an amplicon to differentiate the alternatively spliced variants. From the microarray analyses the average peak for the slob transcripts is at ZT 15, similar to our peak of ZT 18 (Fig. 2).

The six slob transcripts differ in two major ways. The first is in the 5′-region including the UTR and translational start site, which causes the chief difference between slob57-A/C/51-A and slob71-B/65-B. The second is the alternatively spliced exon that encodes a potential binding domain for 14-3-3. We took advantage of these sequence differences and designed in situ RNA probes to these regions, to distinguish between slob57-A/C/51-A and the slob71-B/65-B transcripts. We could also differentiate between the slobs that do and do not have the alternatively spliced exon, but in situ probes could not be designed to be sufficiently specific for each individual transcript. We find that slob transcripts are expressed widely in fly brain and eye, with different patterns of expression of different transcripts. For example, slob71-B/65-B appears to be especially prominent in the medulla of the optic lobes, whereas slob57-A/C/51-A transcripts are also expressed in photoreceptors and brain cortex. A pattern of transcript distribution in photoreceptors, optic lobes, and brain cortex is also seen for the circadian transcripts timeless, clock, and cycle (So et al. 2000).

Previously we reported Slob immunohistochemistry on Drosophila brain wholemounts (Jaramillo et al. 2004) based on an antibody raised against Slob57. Anti-Slob antibody recognizes six to eight PI neurons of subgroup 3 (Jaramillo et al. 2004) in the dorsal protocerebrum. These are large neurosecretory neurons (Rajashekhar and Singh 1994), and we have demonstrated that within these PI neurons Slob protein is regulated in a circadian manner (Jaramillo et al. 2004). We now also observe a set of Slob-positive dorsal giant interneurons in each hemisphere close to the lateral nucleus dorsal (LNds) neurons. It is possible that these DGIs are also neurosecretory cells because they have been shown to be associated with the PI neurons (Helfrich-Forster et al. 2000; Ito et al. 1997). The LNds consist of about six small neurons that are found where the anterior optic tract enters the brain. It is interesting to note that both the DGIs and the LNds have projections that run along the surface of the lateral horn and extend to the dorsal protocerebrum, close to if not onto the PI region (Helfrich-Forster 2003; Ito et al. 1997).
We now have used an antibody specific for the unique N-terminal region of Slob71/65 to ask where Slob71/65 protein is expressed in the fly head. Using anti-Slob71/65 antibody we saw no immunoreactivity in either the PI neurons or the DGIs. Instead we saw intense staining in the large lateral nucleus ventral (LNv) neurons. The large LNvs are clock gene–expressing neurons that participate in rhythmic activity patterns of the fly (Stanewsky 2002). These four neurons have large somata, about 8–12 μm in diameter, and they release the neuropeptide pigment-dispersing factor (PDF). In the absence of this peptide, flies become arrhythmic under conditions of constant darkness (Park et al. 2000; Renn et al. 1999; Stanewsky 2002). The LNvs project onto the optic lobe with wide-field tangential arborizations on the surface of the medulla. The large LNvs also connect the two brain hemispheres by fibers in the posterior tract (Helfrich-Forster 1996). Using different Slob antibodies, it is evident that in the PI neurons, DGIs, or the large LNvs, Slobs are characteristically cytoplasmic in the large cell bodies. It is somewhat surprising that the anti-Slob antibody does not recognize all forms of Slob in all brain regions. For example, the anti-Slob antibody does not stain the LNvs, perhaps because the epitope recognized by anti-Slob is masked in the larger Slob proteins.

It is not surprising that all the Slobs bind the dSlo channel because the dSlo binding domain (Zhou et al. 2003a) is conserved in all the proteins. However, there are several differences among Slob variants in their binding to dSlo. First, the removal of the region of Slob encoded by the alternatively spliced exon contributes to a tighter interaction between Slob and dSlo. Second, the addition of 123 amino acids to the N-terminal of the protein destabilizes the interaction between Slob and dSlo, with or without the exon-encoded region. The weakest interaction between Slob and dSlo is when Slob has both the exon-encoded region and the additional amino terminal 123 amino acids, as in Slob71. The strongest interaction between Slob and dSlo is with Slob51, which lacks both of these protein domains. We have found that Slob57 and Slob51 produce very different modulatory effects on dSlo than do the other Slobs (Zeng et al. 2005).

The 14-3-3 proteins are dimeric, cytosolic proteins involved in multiple biological processes ranging from apoptosis and cell cycle control to synaptic transmission (Broadie et al. 1997; Fu et al. 2000). A phosphoserine-recognition motif for 14-3-3, RSXpSXP, has been characterized extensively (Yaffe et al. 1997). Most binding partners of 14-3-3 have one binding motif, but some like Slob have two, 50RSNS54 and 76RSAS79 (Zhou et al. 1999). In the case of Raf-1, its two 14-3-3 recognition sites are believed to contribute to a regulatory conformational change (Morrison and Cutler 1997). Both motifs in Slob have been shown to participate in the binding interaction between Slob and dSlo. The functional
implications of these differential binding interactions remain to be explored.

In summary, our findings show that multiple slob transcripts are expressed differentially in the fly head and antibody staining suggests that different Slob proteins are expressed in distinct neuronal populations. The preferential expression of Slob71/65 in the clock cells and the rhythmicity of its transcript suggest a potential role in circadian rhythms that is deserving of further testing. As Ceriani et al. (2002) demonstrated for dSlo and we for Slob (Jaramillo et al. 2004), ion channels and their modulators can be under direct or indirect control of circadian clock genes. Furthermore, the importance of electrical activity for circadian oscillations is becoming increasingly evident (Cloues and Sather 2003; Nitabach et al. 2002; Pennartz et al. 2002). It will be interesting to determine the mechanism by which the different Slob proteins modulate dSlo differently (Zeng et al. 2005) and to explore how the modulation by different Slob is influenced by 14-3-3. It is possible that the variety of Slob offers greater flexibility in the regulation of circadian rhythms or other Slob-related behaviors.

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