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The $GABA_A$ Receptor RDL Acts in Peptidergic PDF Neurons to Promote Sleep in *Drosophila*

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SUMMARY

Sleep is regulated by a circadian clock that largely times sleep and wake to occur at specific times of day and a sleep homeostat that drives sleep as a function of duration of prior wakefulness[1]. To better understand the role of the circadian clock in sleep regulation, we have been using the fruit fly *Drosophila melanogaster*[2]. Fruit flies display all of the core behavioral features of sleep including relative immobility, elevated arousal thresholds and homeostatic regulation[2, 3]. We assessed sleep-wake modulation by a core set of 20 circadian pacemaker neurons that express the neuropeptide PDF. We find that PDF neuron ablation, loss of *pdf* or its receptor *pdfr* results in increased sleep during the late night in light:dark (LD) conditions and more prominent increases on the first subjective day of constant darkness (DD). Flies deploy similar genetic and neurotransmitter pathways to regulate sleep as their mammalian counterparts, including GABA[4]. We find that RNAi-mediated knockdown of the $GABA_A$ receptor gene, *Resistant to dieldrin* (*Rdl*), in PDF neurons, reduced sleep consistent with a role for GABA in inhibiting PDF neuron function. Patch clamp electrophysiology reveals GABA-activated picrotoxin-sensitive chloride currents on PDF+ neurons. In addition, RDL is detectable most strongly on the large subset of PDF+ pacemaker neurons. These results suggest that GABAergic inhibition of arousal promoting PDF neurons is an important mode of sleep-wake regulation *in vivo*.

RESULTS AND DISCUSSION

Loss of the Neuropeptide PDF, its receptor PDFR, or PDF Neurons Increases Sleep Amount

Expression of a bacterial sodium channel (NaChBac) in the PDF neurons reduces nocturnal sleep[5–8]. These arousal promoting effects map to the large subset of PDF neurons [6–8]. However, physiological analysis indicates that the net effect of *NaChBac* expression is complex, altering the time-of-day of peak neuronal activity, rendering the resting membrane potential more negative, and reducing the firing frequency but increasing action potential duration[6].

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To more fully examine the role of PDF neurons in sleep regulation, we examined the sleep phenotype of flies in which the PDF neurons had been selectively ablated by expression of the proapoptic gene *head involution defective*, or *hid* (*pdf*-GAL4/UAS-*hid*; PDF ablated), in flies bearing a null allele of *pdf* (pdf^{01}), and flies with a null allele of *pdfr* ($pdfr^{han5304}$) [9]. The PDF neuropeptide is thought to be critical to the function of PDF neurons. Prior studies had examined pdf^{01} mutants but the extent of control of genetic background is unclear[10]. We therefore re-examined both pdf^{01} and *pdfr* mutants by backcrossing for five generations into a *white* (*w*) isogenic strain, *iso31*, previously used for the DrosDel project[11] (see Methods). These flies were then compared to wild-type sibling controls. Similar backcrossing approaches have been employed by multiple groups for sleep analysis[12–15].

Under light:dark (LD) conditions, fruit flies display an increase in activity in advance of lights-on (morning anticipation) and lights-off (evening anticipation) reflecting circadian clock function. Loss of PDF[9], the PDF receptor[16–18], or PDF neurons[9, 19] result in a dramatic reduction in morning anticipation as well as a phase advance in evening behavior in LD. Under LD conditions, PDF ablated, pdf^{01}, and $pdfr^{han5304}$ flies display increases in sleep during the late night (ZT 24, $p<0.05$; Figure 1A–C). When wild-type flies were waking up in advance of lights-on, pdf^{01}, $pdfr^{han5304}$, and PDF ablation flies failed to wake up, consistent with the reported role of *pdf* in increasing activity in advance of lights-on[9, 19]. *pdfr* flies also display overall increases in sleep in LD, principally during the light phase (Supplemental Table 1). We principally report sleep in males but sleep during the light phase is sexually dimorphic [20]. However, we did not observe any consistent increases in light phase sleep in female pdf^{01} or *pdfr* mutant flies (Supplemental Table 2). Male PDF ablated flies display increases in sleep during the early day (ZT1–2, $p<0.05$) and early night (ZT13–14, $p<0.001$) as well as reduced sleep consolidation at night ($p<0.05$, Figure 1A, Supplemental Table 1). These additional phenotypes may reflect the function of other PDF neuron transmitters[5, 21].

Upon release into constant darkness (DD) conditions, flies lacking PDF display a reduced or absent morning peak, a slightly reduced period length, but largely retain rhythmicity early in DD[9]. During the first day of DD, we found significant increases in total sleep in pdf^{01}, $pdfr^{han5304}$, and PDF ablated flies during the subjective day ($p<0.01$). These effects were also observed in pdf^{01} flies transheterozygous for deficiency removing the *pdf* locus (data not shown). In flies lacking PDF/PDFR, these effects are accompanied by an increase in average sleep bout length ($p<0.01$; Supplemental Table 1). Similar effects were also observed in female *pdf* and *pdfr* mutant flies (Supplemental Table 2). These effects do not appear to be solely due to the loss of the morning activity peak as significant effects on sleep are sustained throughout the subjective day (Figure 1A–C). pdf^{01} and *pdfr* effects also persist into day 2 of DD (Supplemental Table 3). No significant effects on waking activity were observed indicating a primary sleep effect (Supplemental Table 1). As observed earlier, flies lacking PDF/PDFR still exhibit high amplitude changes in sleep and do not show a significant effect on behavioral phase during the first day of DD (Supplemental Table 4). The absence of large effects of sleep in LD (Supplemental Table 1) suggests that flies lacking PDF are not sick and that light can largely (but not completely) compensate for the lack of PDF.

In wild-type control flies, we noted a substantial reduction in total sleep levels between LD and the first day of DD of about 200–300 minutes. Similar observations have been made in a number of reports[10, 15, 22, 23] and suggest that while light can acutely stimulate wake/activity, light may also promote sleep at certain times of day, as it does in rodents[24, 25]. Thus, these sleep-promoting effects of light during LD may mask the increased sleep phenotype observed in flies lacking PDF.

To confirm that the increased sleep in flies lacking PDF is not simply due to quiet wakefulness, we assessed the behavioral responsiveness of pdf^{01} flies to a mechanical stimulus at CT3, a time of elevated sleep in these flies during the first subjective day. We observed that pdf^{01} flies were less likely to respond than wild-type flies, consistent with the notion that they are sleeping (Figure 1C). Taken together, these data suggest that PDF plays a principally wake promoting function. Indeed, the absence of significant effects on waking activity suggests that a major function of PDF neurons is to regulate the state transition from sleep to wake.

The $GABA_A$ Receptor RDL Functions in PDF Neurons to Promote Sleep

As part of an RNAi screen to identify genetic regulators of PDF neurons, we identified a potential role for the ionotropic $GABA_A$ receptor, *Resistant to Dieldrin* (*Rdl*) (data not shown). Most of the commonly prescribed hypnotics target $GABA_A$ receptors[26]. The GABAergic ventral lateral preoptic nucleus of the hypothalamus is important for promoting sleep and acts by directly inhibiting wake promoting circuits[27–29]. In *Drosophila*, mutants of the $GABA_A$ receptor that reduce desensitization, *Resistant to dieldrin* (*Rdl*), also display increased sleep and reduced sleep latency, indicating a conserved role for GABA[12]. However, the neural substrates of its actions were unclear.

To further elucidate the function of RDL in PDF neurons, we used tissue-specific RNAi in concert with expression of the RNAi component *Dicer2* (*dcr2*) to knockdown *Rdl* expression in PDF neurons[30]. Broad expression of *Rdl* RNAi using a pan-neuronal driver *elav*-GAL4 in combination with *dcr2* resulted in adult lethality, consistent with strong loss-of-function *Rdl* alleles [31]. Compared to the parental RNAi line alone (UAS-*Rdl*/+), *pdf*-GAL4/*dcr2* line alone (*pdf*-GAL4/+; UAS-*dcr2*/+) and an RNAi line from the same library that does not display significant sleep phenotypes (a negative genetic background control strain, *CG3380*), we observed a significant reduction in total sleep time in LD and in DD (Figure 2, Supplemental Table 3). Of note, *Rdl* knockdown in PDF neurons did not substantially alter waking activity (Supplemental Table 5), circadian period (only a 0.3 hour change) or rhythmicity under DD conditions (Supplemental Table 4), consistent with a primary effect on sleep regulation.

Expression and Function of RDL in PDF Neurons

We examined expression of RDL in the LNv using immunocytochemistry[32]. We found the lLNv somata are strongly labeled by anti-RDL staining, while the sLNv somata label either weakly or not at all (Figure 3A). Similarly, UAS-*GFP* expression (membrane-tethered or nuclear-localized) driven by any of five independent *Rdl*-GAL4 lines strongly labeled the lLNv (Supplemental Figure S1)[33]. Four out of the five lines showed no detectable expression in the sLNv (Supplemental Figure S1, data not shown). Outside the soma, we also found RDL puncta in close proximity to LNv terminals of the accessory medulla (Figure 3B). RDL receptors here and in the soma suggest GABA may regulate LNv excitability. To determine if these might represent synaptic inputs to LNv dendrites, we expressed a presynaptically localized synaptobrevin-GFP fusion protein using *GAD-GAL4* and colabeled with PDF to mark the LNv dendrites. Consistent with RDL staining, we found synaptobrevin-GFP puncta intermixed with LNv dendrites in the accessory medulla (Supplemental Figure S1). Interestingly we also found RDL puncta associated with the large PDF varicosities formed by lLNv in the optic lobe (Figure 3C). As these are putative PDF release sites, this may represent sites of local presynaptic regulation of PDF release. In houseflies, similar optic lobe varicosities show reduced PDF levels after lights on [34]. In *Drosophila* these terminals appeared to show a similar trend toward lower levels after lights on[35]. By contrast the lLNv soma showed no PDF oscillations. Perhaps GABAergic

inhibitory inputs to different cellular locations may act independently to sculpt different aspects of sleep-wake behavior.

To confirm the presence of $GABA_A$-activated currents in PDF neurons, we used a pico-spritzer to puff GABA (1 mM) on dissociated PDF neurons voltage-clamped at −90 mV [36]. Cells are selected in part based on their large size and thus, we believe that these are likely the large LNv. With E_{Cl} near 0 mV, we observed robust GABA-gated inward currents (Figure 4D, green trace, peaking at −99.3 ±15.7 pA, n=6). Consistent with being a chloride current, the amplitude was reduced approximately three-fold when the driving force was reduced threefold by shifting E_{Cl} to −60 mV (*i.e.*, reducing intracellular chloride, red trace, peaking at −30.5±13.8 pA, n=3). The current was blocked by 100 μM of the $GABA_A$ receptor blocker, picrotoxin (PTX; black trace, n=7). These results reveal the presence of functional GABAergic inputs to PDF neurons. Taken together, the physiological analysis coupled to the expression analyses suggest that the principal RDL effect is in the arousal promoting large LNv.

Here we have defined a key role for GABAergic input in vivo in regulating PDF function in sleep. We also provide important loss-of-function evidence, using *pdf* and *pdfr* mutants and PDF neuron ablation, that PDF is promoting wakefulness. Our extensive expression analyses suggest that the principal effect of GABA may be on the large subset of arousal promoting LNv. Work coincident with ours reaches similar conclusions regarding PDF/PDF neuron loss-of-function and *Rdl* function in PDF neurons and sleep[7, 8]. GABAergic inhibition of wake promoting circuits is a common theme in mammals[29] and our studies suggest that this mode of organization is preserved in *Drosophila*. These studies define wake-promoting circuits in *Drosophila* as well as the neurotransmitters that regulate the function of circadian pacemaker neurons in vivo.

We propose that GABA release inhibits large LNv output and PDF release to reduce wake, suggesting an important role for GABA inhibition. In this model, circadian clock times PDF neuron activation and PDF release during the late night and following day to promote waking behavior. Of note, a similar arousal promoting function for circadian pacemaker neurons has been described in mammals[37, 38]. It is also approximately the time when the large LNv have been shown to be more depolarized and have higher levels of spontaneous activity[39, 40]. RDL receptors on LNv soma and on fibers in the accessory medulla suggest GABA may regulate LNv excitability. It is interesting that GABA is also an important neurotransmitter in mammalian circadian pacemaker neurons, capable of reducing their spontaneous activity[41, 42]. In addition, RDL receptors on PDF varicosities in the optic lobe may function presynaptically to regulate PDF release. GABA may also act through metabotropic $GABA_B$ receptors, which have been described in the sLNv, but their function in circadian or sleep behavior is unknown[43]. GABAergic signaling may affect the function of the transcription factor ATF2 important for PDF neuron function in sleep[14]. Changes in PDF neuron function may in turn act by antagonizing sleep promoting circuits that exist within the mushroom bodies as well as the pars intercerebralis (PI) [22, 44–46]. Of note, the PI appears to express the PDF receptor[17, 18]. Identifying the anatomic targets of PDF as well as the neural sources of GABAergic inputs will be important to further define sleep/wake circuits in *Drosophila*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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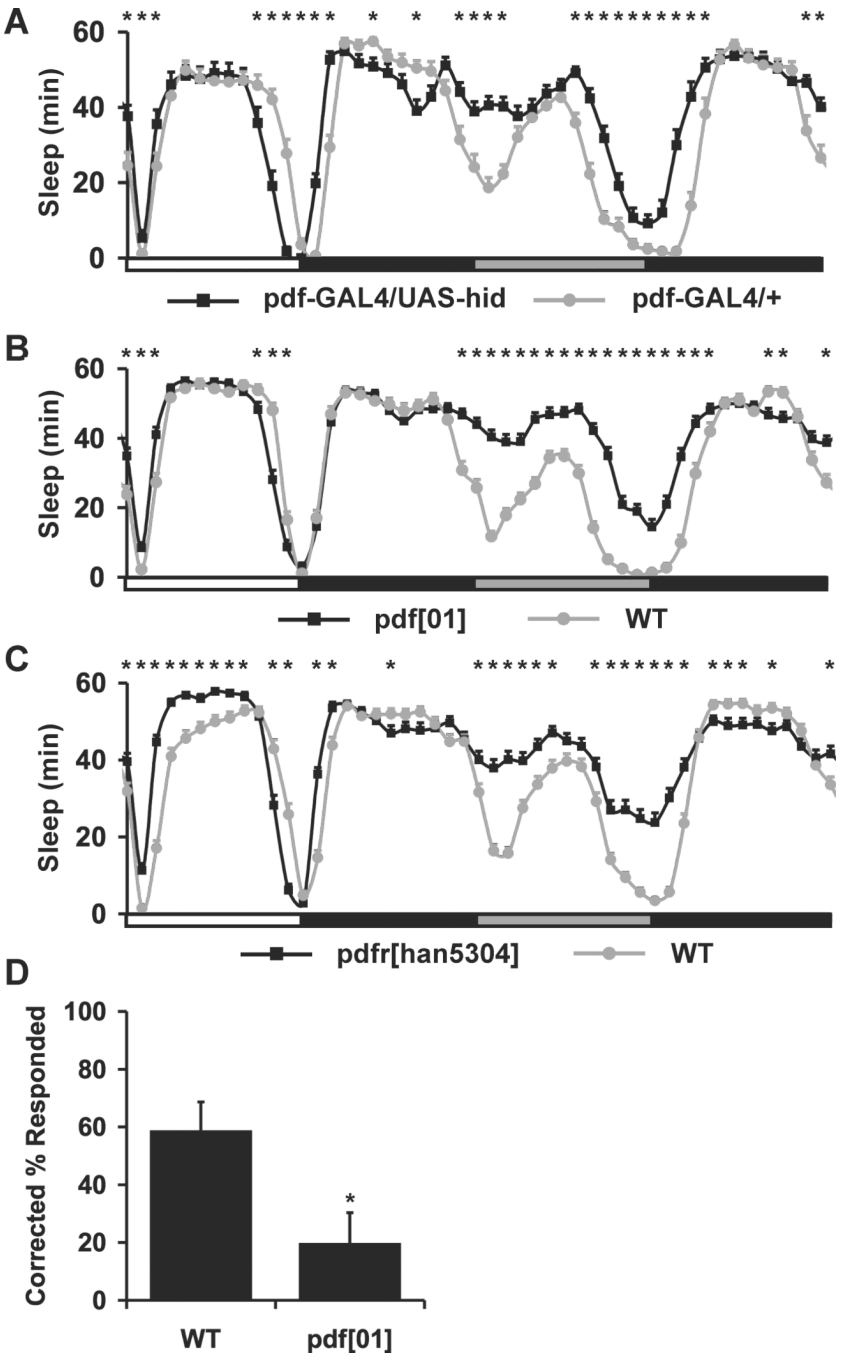

Figure 1. Loss of PDF results in increased sleep and reduced responsiveness to external stimuli

Hourly sleep amount plots for (A) *pdf*-GAL4/UAS-*hid* (black lines with square data points; N=35) and *pdf*-GAL4/+ (gray lines with circle data points; N=34), (B) pdf^{01} (black lines with square data points; N=72) and WT sibling controls (gray lines with circle data points; N=64), and (C) $pdfr^{han5304}$ (black lines with square data points; N=75) and WT sibling controls (gray lines with circle data points; N=70). Horizontal white and black boxes along the X-axis indicate light and dark periods of the last day of LD (LD5), respectively. Horizontal gray boxes indicate the subjective light period during the first day of DD (DD1). Data points represent mean ± SEM of three independent experiments. *p<0.05 as determined by Student's t-test. (D) Bar graph indicating the corrected % of flies that

responded to a mechanical stimulus administered at CT3 on DD1 (see methods for calculation). Data points represent mean ± SEM of four independent experiments. *p<0.05 as determined by Student's t-test.

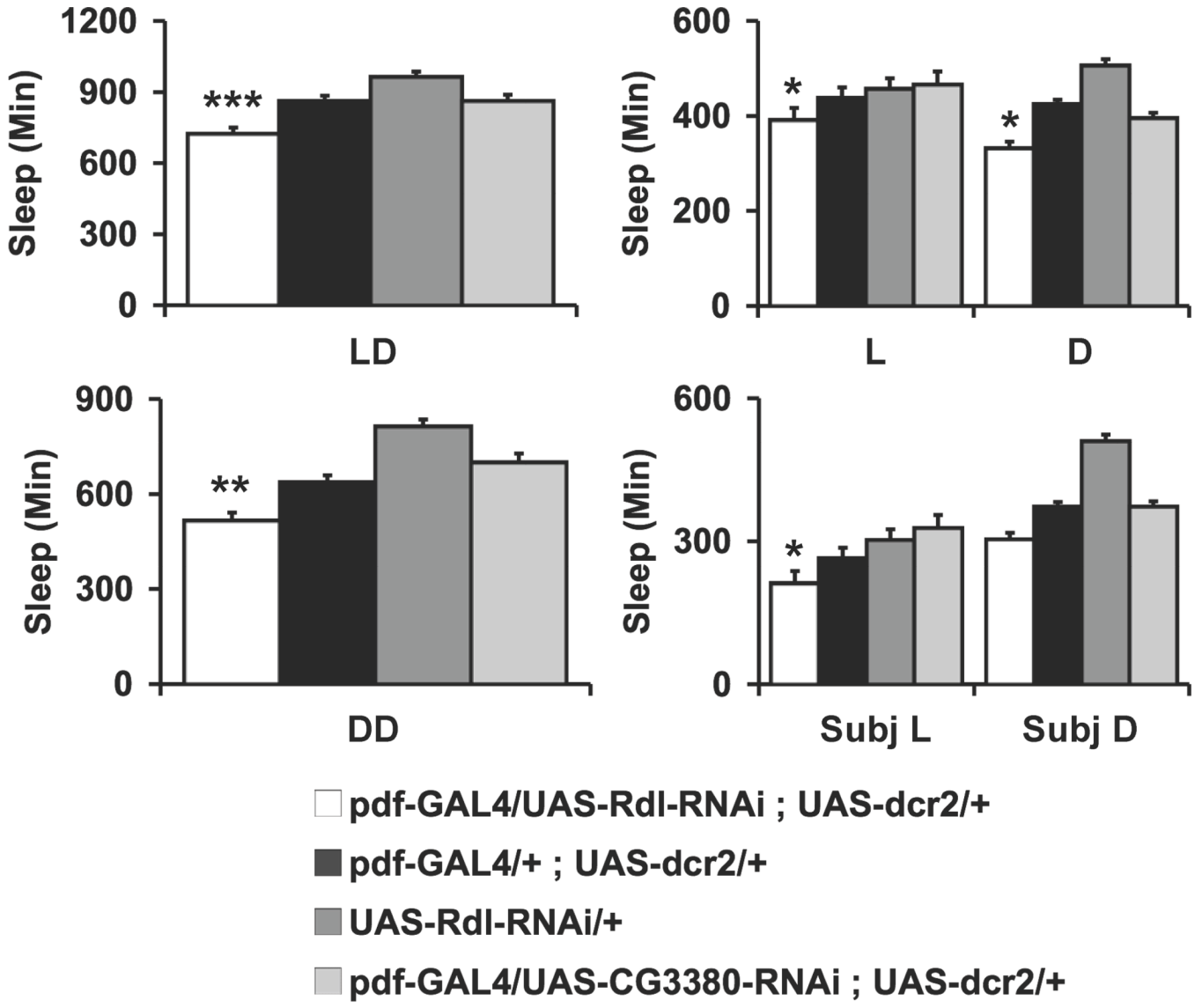

Figure 2. Knockdown of *Rdl* transcript in PDF cells results in decreased sleep

Averaged total sleep amount for LD days 2–5 (LD2–5) and DD days 1–5 (DD1–5). "L" indicates the light period of LD2–5, "D" indicates the dark period of LD2–5, "Subj L" indicates the subjective light period of DD1–5, and "Subj D" indicates the subjective dark period of DD1–5. All data bars represent mean ± SEM of three independent experiments. * at least $p<0.05$ to all controls, ** at least $p<0.01$ to all controls, and *** at least $p<0.001$ to all controls as determined by one-way ANOVA, Tukey HSD post hoc. Sample sizes for each genotype and for each condition can be found within Supplemental Table 5.

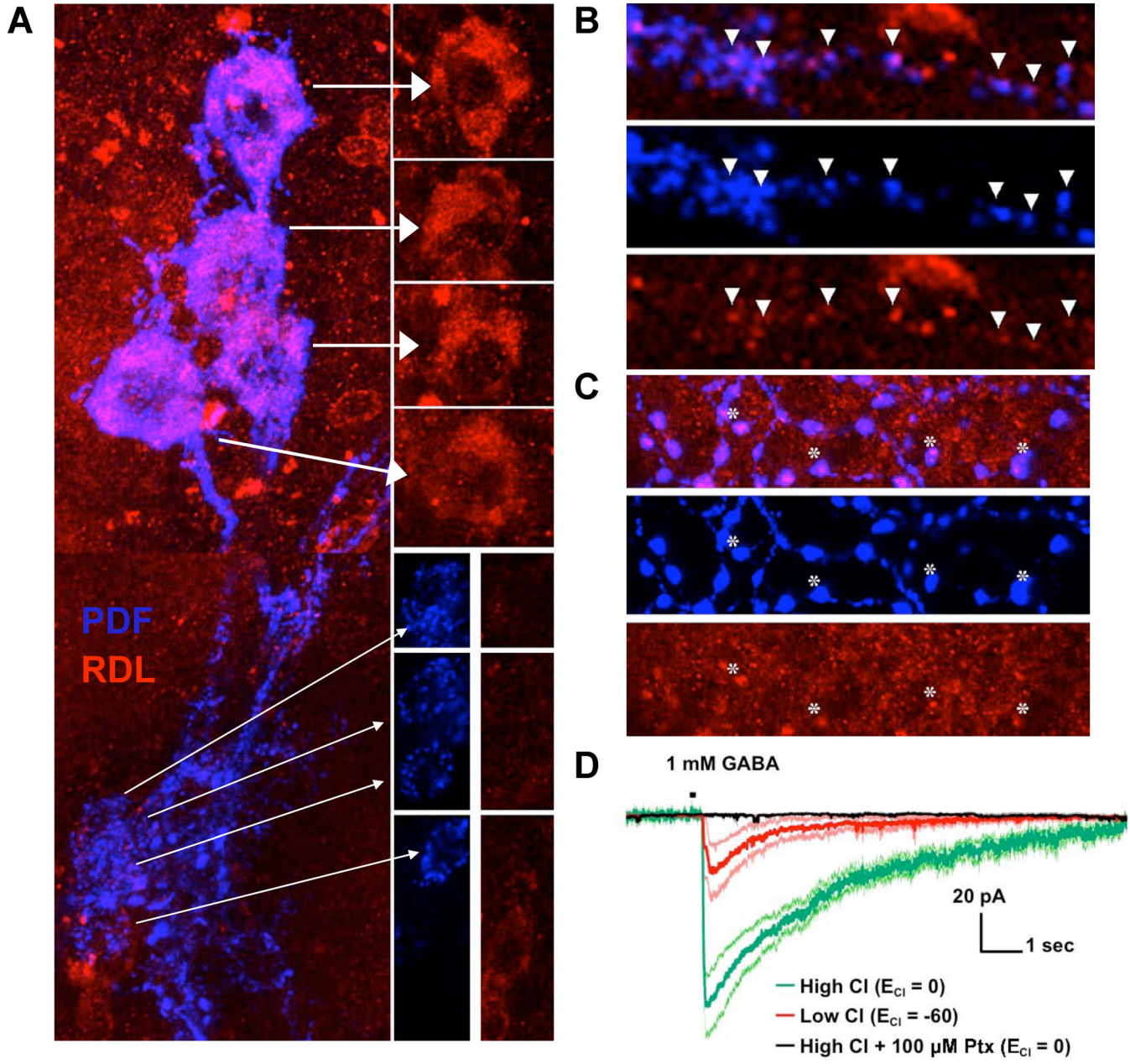

Figure 3. Inhibitory inputs to the lateral neurons

(A) $GABA_A$ receptor subunit RDL is expressed in lLNv and at much lower levels in the sLNv. Left panel, maximum projection; upper and lower halves comprise different regions of the same brain. Right panels show individual optical sections through the center of each cell soma, with large arrows pointing to each lLNv and small arrows to each sLNv. Blue = PDF, red = RDL. Note the RDL-stained somata below the sLNv in the final panel. (B) Individual PDF-containing fibers in the accessory medulla colocalize with RDL puncta (small arrows) in single sections. (C) PDF varicosities formed by lLNv neurites in the optic lobe also express RDL (asterisks). (D) The response of lateral neurons to puffer applications of 1 mM GABA in the presence and absence of 100 μM picrotoxin. Holding potential = −90mV. 100 ms puffs of 1 mM GABA were applied to the neurons as indicated. See methods for ionic conditions. Dark lines represent the average current of six cells in each

condition; light lines indicate the standard error. Green (gray) indicates control with high chloride (high Cl) concentration. Blue (dark) indicates picrotoxin(ptx) treated cells with high chloride (high Cl). Red indicates reduced chloride concentration (low Cl).