Development/Plasticity/Repair

Pumilio Binds *para* mRNA and Requires Nanos and Brat to Regulate Sodium Current in *Drosophila* Motoneurons

Nara I. Muraro,[1] Andrew J. Weston,[2] Andre P. Gerber,[3] Stefan Luschnig,[4] Kevin G. Moffat,[2] and Richard A. Baines[1]

[1]Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, United Kingdom, [2]Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom, [3]Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, Eidgenössische Technische Hochschule Zurich, CH-8093 Zurich, Switzerland, and [4]Institute of Zoology, University of Zurich, CH-8057 Zurich, Switzerland

Homeostatic regulation of ionic currents is of paramount importance during periods of synaptic growth or remodeling. Our previous work has identified the translational repressor Pumilio (Pum) as a regulator of sodium current (I_{Na}) and excitability in *Drosophila* motoneurons. In this current study, we show that Pum is able to bind directly the mRNA encoding the *Drosophila* voltage-gated sodium channel *paralytic* (*para*). We identify a putative binding site for Pum in the 3′ end of the *para* open reading frame (ORF). Characterization of the mechanism of action of Pum, using whole-cell patch clamp and real-time reverse transcription-PCR, reveals that the full-length protein is required for translational repression of *para* mRNA. Additionally, the cofactor Nanos is essential for Pum-dependent *para* repression, whereas the requirement for Brain Tumor (Brat) is cell type specific. Thus, Pum-dependent regulation of I_{Na} in motoneurons requires both Nanos and Brat, whereas regulation in other neuronal types seemingly requires only Nanos but not Brat. We also show that Pum is able to reduce the level of *nanos* mRNA and as such identify a potential negative-feedback mechanism to protect neurons from overactivity of Pum. Finally, we show coupling between I_{Na} (*para*) and I_K (*Shal*) such that Pum-mediated change in *para* results in a compensatory change in *Shal*. The identification of *para* as a direct target of Pum represents the first ion channel to be translationally regulated by this repressor and the location of the binding motif is the first example in an ORF rather than in the canonical 3′-untranslated region of target transcripts.

Key words: **Pumilio; Nanos; Brat; *paralytic*; aCC; RP2**

Introduction

Neuronal activity is regulated by homeostatic mechanisms that serve to maintain membrane excitability within predefined limits. This is achieved, at least in part, by continual adjustment of both ligand- and voltage-gated ionic conductances to maintain stable action potential firing rates in response to changing synaptic excitation (Turrigiano and Nelson, 2000; Marder and Prinz, 2002; Davis, 2006). Such regulation is predicted to be particularly predominant when neural circuit synaptic activity is changing rapidly, for example during both neuronal circuit development and in the formation of memory (Turrigiano, 1999). However, although now well established, the molecular pathways that underlie homeostatic regulation remain mostly unknown.

Previous studies indicate that activity-dependent regulation of voltage-gated sodium channels is central to the control of membrane excitability in both mammalian and invertebrate neurons (Desai et al., 1999; Baines et al., 2001; Baines, 2003; Mee et al., 2004). Studies in *Drosophila* have shown that increased synaptic excitation of motoneurons is countered by a decrease in sodium current (I_{Na}) and membrane excitability in these cells (Baines, 2003). Similar, but opposite, changes in I_{Na} and excitability are observed in mutants that display decreased synaptic excitability (Baines et al., 2001). These changes require the known translational repressor Pumilio (Pum), which we have shown previously is both necessary and sufficient for activity-dependent changes of I_{Na} in *Drosophila* motoneurons (Mee et al., 2004). Our model predicts that prolonged change in exposure to synaptic excitation is countered by a reciprocal Pum-dependent regulation in translation of *paralytic* (*para*) mRNA and membrane excitability.

The role of Pum is well described from studies of early *Drosophila* embryogenesis (Barker et al., 1992; Murata and Wharton, 1995; Zamore et al., 1997; Wharton et al., 1998). Specification of the abdomen requires Pum-dependent repression of translation of *hunchback* (*hb*) mRNA. The first step begins with the recognition and binding of Pum to the Nanos response element (NRE)-motif located in the 3′-untranslated region (UTR) of *hb* mRNA (Zamore et al., 1997; Wharton et al., 1998). Once bound, Pum then recruits the cofactors Nanos (Sonoda and Wharton, 1999) and Brain Tumor (Brat) (Sonoda and Wharton, 2001) to form a repressor complex that results in the translational repression of *hb* mRNA. The mechanism of repression involves both deadenylation and poly(A)-independent silencing (Chagnovich and Leh-

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Correspondence should be addressed to either Nara I. Muraro or Richard A. Baines, Faculty of Life Sciences, Stopford 1.124, University of Manchester, Oxford Road, Manchester M13 9PT, UK. E-mail: nara.muraro@manchester.ac.uk or richard.baines@manchester.ac.uk.

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mann, 2001). In addition to its characterized roles in repression of *hb*, Pum has also been shown to bind, and repress translation of, mRNAs encoding the *eukaryotic initiation factor 4E* (*eIF4E*) (Menon et al., 2004) and *Cyclin B* (*CycB*) (Asaoka-Taguchi et al., 1999; Kadyrova et al., 2007). Indeed, these few mRNAs may represent just the tip of the iceberg because the actual list of targets is likely to be extensive based on a recent demonstration that Pum associates with >1000 different mRNAs in the ovaries of adult flies (Gerber et al., 2006). Pum proteins are evolutionarily conserved from yeast to mammals (Spassov and Jurecic, 2002; Wickens et al., 2002), and, moreover, Pum expression is activity dependent in mammalian neurons in culture (Vessey et al., 2006).

In this study, we report that Pum is able to directly bind *para* mRNA (encoding the *Drosophila* voltage-gated Na^+ channel). The mechanism of *para* translational repression shows similarities and differences to Pum-dependent repression of *hb* mRNA. We show that, unlike repression of *hb*, full-length Pum is necessary for *para* repression. As for most other Pum-dependent repressed transcripts described to date, *para* repression requires the presence of the cofactor Nanos. However, the requirement for the cofactor Brat is neuronal type specific. We also show that Pum is sufficient to downregulate *nanos* mRNA levels in the CNS, a property that may serve to protect neurons from the effects of overactivity of this translational repressor.

Materials and Methods

Fly stocks. Flies were maintained on apple juice agar plates supplemented with live yeast paste at 25°C. Wild type was Canton-S (CS). Tissue-specific expression of transgenes in the nervous system was achieved using the GAL4/UAS system (Brand and Perrimon, 1993). RN2-O-GAL4 (homozygous viable on the second chromosome) or RN2-E-GAL4 (homozygous viable on the third chromosome) were used to express UAS transgenes in aCC/RP2 motoneurons (Fujioka et al., 1999; Baines, 2003). These are identical transgenes inserted on different chromosomes. For real-time PCR experiments, 1407-GAL4 (homozygous viable on the second chromosome) was used to express transgenes in all CNS neurons. UAS-*pum* full-length (homozygous viable on the second chromosome) (Schweers et al., 2002), UAS-*pum*^{RBD} (homozygous viable on the second chromosome) (Menon et al., 2004), UAS-*pum*$^{RBD\text{-}V5}$ (homozygous viable on the third chromosome), UAS-Pum^{G1330D} (homozygous viable on the second chromosome) (Ye et al., 2004), and UAS-*nanos* (homozygous viable on second chromosome) (Ye et al., 2004) have been described previously. UAS-*brat* (on the second chromosome, rebalanced over *CyO*^{GFP} for this work) is described by Frank et al. (2002). *nanos*^{17} was provided by the Bloomington Stock Centre and rebalanced over TM3^{SerGFP}. *para* was removed using a small deficiency [Df(1) D34] (Baines and Bate, 1998) and rebalanced over FM7^{GFP}.

TAP-Pum^{RBD} pull-down assays. Five grams of elaV-GAL4; UAS-TAP-*pum*^{RBD} or elaV-GAL4; + mock control flies were collected 1–3 d after eclosure. Flies were frozen in liquid nitrogen and TAP-Pum^{RBD} was affinity-purified from extracts as previously described (Gerber et al., 2006). For reverse transcription (RT)-PCR, 1 μg and 100 ng of total RNA isolated from extracts and from tobacco etch virus (TEV) protease eluates, respectively, were mixed with oligo-dT [dNV$(T)_{22}$] and random nonamer primers (5 μg each) and made up to 15 μl with RNase-free water. The mix was incubated at 70°C for 10 min followed by incubation on ice. First-strand buffer (Invitrogen, Carlsbad, CA) (15 μl) was supplemented with 0.5 mM deoxyribonucleoside triphosphates (dNTPs) and 20 U of RNaseOUT (Invitrogen) was added to the mixture. A fraction, 10 μl, was transferred into a second tube (−RT control) and 1 μl (100 U) of Superscript RT II (Invitrogen) was added to the remaining 20 μl. Samples were incubated for 2 h at 42°C, 5 min at 95°C, and put on ice. PCR was conducted with 1.5 μl of the RT reaction with oligo pairs para-T7Fw1 (5′-TAATACGACTCACTATAGGGCACCCAGTACATACGC-TATG-3′, which bears sequences for the T7 promotor at the 5′ end) and para-Rev1 (5′-CAGACATCCGCCGTGCGCGACGTG-3′) for amplification of *para* transcripts, and gfat2F (5′-CTCCTCGCAGATTA-GGATCG-3′) and gfat2R (5′-AAGGCCTACACCTCCCAGTT-3′) to amplify glutamine-fructose-6-phosphate aminotransferase 2 (*gfat2*) transcripts. PCR was performed for 2 min at 94°C, 28 cycles at 94°C for 30 s, 53°C for 30 s, 72°C for 1 min, and 2 min at 72°C. To amplify *gfat2*, annealing was done at 58°C.

Pull-down experiments with biotinylated RNAs. Synthesis of biotinylated transcripts and pull-down assay were performed as described with minor modifications (Gerber et al., 2006). *para* was amplified by PCR from 100 ng of *Drosophila* genomic DNA with primer pairs para-T7Fw1 and para-Rev1. A total of 4 pmol of biotinylated RNA was mixed with 200 μl of extract (OD_{280}, 25) prepared from elaV-GAL4; UAS-TAP-*pum*^{RBD} adult flies.

Embryo and larvae dissection. Newly hatched larvae or late-stage 17 (19–21 h after egg laying at 25°C) embryos were dissected, and central neurons were accessed for electrophysiology as described by Baines and Bate (1998). Late-stage 17 embryos were first dechorionated using 50% bleach for 2 min, and the vitelline membrane was then manually removed using sharp tungsten wires. The larva/embryo was visualized using a water immersion lens (total magnification, 600×) combined with Normarski optics (BX51W1 microscope; Olympus Optical, Tokyo, Japan).

Electrophysiology. Recordings were performed in young first instar larvae, 1–4 h after hatching, or late-stage 17 embryos (in the case of nonviable genotype) at room temperature (22–24°C). Whole-cell voltage-clamp recordings were done using thick-walled borosilicate glass electrodes (GC100F-10; Harvard Apparatus, Edenbridge, UK), fire polished to resistances of between 15 and 20 MΩ. Cells were initially identified based on both size and dorsal position in the ventral nerve cord. Unequivocal identification was determined after recording by labeling with 0.1% Alexa Fluor 488 hydrazyde, sodium salt (Invitrogen), which was included in the patch saline. Recordings were made using a Multiclamp 700B amplifier controlled by pClamp 9.2 (Molecular Devices, Sunnyvale, CA). Only cells with input resistance >1 GΩ were accepted for analysis. To better resolve I_{Na}, an on-line leak subtraction protocol was used (P/4). Currents shown are the average of three trials for I_{Na} and five trials for I_K. Currents shown were normalized for cell capacitance. To determine the effect of gene expression on electrical properties, we analyzed the peak current for each ion (I_{Na} at −20 mV, I_{Kfast} and I_{Kslow} at +45 mV, and $I_{Ba(Ca)}$ at −10 mV).

Solutions. Saline for dissection consisted of the following (in mM): 135 NaCl, 5 KCl, 4 $MgCl_2 \cdot 6H_2O$, 2 $CaCl_2 \cdot 2H_2O$, 5 *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and 36 sucrose, pH 7.15. For isolation of I_{Na}, the following solution was used (in mM): 100 NaCl, 6 KCl, 2 $MgCl_2 \cdot 6H_2O$, 2 sucrose, 50 tetraethylammonium chloride (TEA), 10 4-aminopyridine (4-AP), and 10 HEPES, pH 7.1. For isolation of I_K, the following solution was used (in mM): 135 NaCl, 5 KCl, 4 $MgCl_2 \cdot 6H_2O$, 2 $CaCl_2 \cdot 2H_2O$, 5 TES, 36 sucrose, and 10^{-6} tetrodotoxin (TTX) (Alomone Labs, Jerusalem, Israel), pH 7.1. For isolation of $I_{Ba(Ca)}$, the following solution was used (in mM): 50 NaCl, 6 KCl, 50 BaCl, 10 $MgCl_2 \cdot 6H_2O$, 10 glucose, 50 TEA, 10 HEPES, and 10^{-6} TTX, pH 7.1. Internal patch solution was as follows (in mM): 140 K^+ methylsulfonate (KCH_3SO_3), 2 $MgCl_2 \cdot 6H_2O$, 2 EGTA, 2 KCl, and 20 HEPES, pH 7.4. When recording I_{Na} or $I_{Ba(Ca)}$, $CsCl_2$ was substituted for KCH_3SO_3.

Real-time PCR. RNA was extracted from whole late-stage 17 embryos or first instar larvae (*para*) or from isolated CNSs from these stages (*Shal*, *slo*, *nanos*, *DmCa1A*, and *pum* mRNA detection) using a Qiagen RNeasy Mini kit (Qiagen, Crawley, UK). Briefly, 35 late-stage 17 embryos or first instar larvae (or their isolated CNSs) were homogenized with a plastic mortar followed by repeated passage through a 20-gauge needle in 350 μl of lysis buffer containing 0.1 M β-mercaptoethanol. The lysate was then centrifuged, 1 vol of 70% ethanol added and passed through an RNeasy column. After washing in buffer, immobilized nucleic acids were then treated with ~190 U of DNase I for 15 min, washed again in stages according to manufacturer's protocol, and then eluted in ~35 μl of RNase-free water. Quantification of RNA concentration in eluates was made using a ND-1000 Nanodrop spectrophotometer (Nanodrop, Wilmington, DE).

Synthesis of cDNA was performed following the protocol in RevertAid

First Strand cDNA Synthesis kit (Fermentas, York, UK). RNA (concentration ≥1.25 ng/μl) was mixed with 0.2 μg (1 μl) random hexamer primers (Fermentas) and made up to 11 μl with RNase-free water. The mix was incubated at 65°C for 5 min to denature RNA followed by incubation on ice for 1 min. A total of 4 μl of reaction buffer (in mM: 250 Tris-HCl, 250 KCl, 20 $MgCl_2$, 50 DTT), 2 μl of 10 mM dNTPs, and 1 μl of Ribolock ribonuclease inhibitor (Fermentas) were added, and the mix was incubated at 25°C for 5 min. Then, after addition of 1 μl of RevertAid M-MuLV (monkey murine leukemia virus) reverse transcriptase (Fermentas), the reaction was subsequently incubated for 10 min at 25°C, 60 min at 42°C, and 15 min at 65°C. From the total reaction volume of 20 μl, 1 μl of cDNA was used for each PCR.

Clone Manager software (Sci-Ed, Cary, NC) was used to design primers for *para*, *pum*, *nanos*, *slo*, *Shal*, *DmCa1A*, and *ribosomal protein 49* (*rp49*), a housekeeping gene. All primers are shown in 5′ to 3′ orientation: *rp49* forward and reverse primers, CACCGGAAACTCAATGGATACTG and TTCTTCACGATCTTGGGCC; *para* forward and reverse primers, GATCTATATGGGCGTGCTCACGCAGAAGTG and TGCAGGCACACGTAATCGTCGTCGCATTG; *pum* forward and reverse primers, CGGCCCAACAGAATCTCTACTC and GCGGCGACCCGTCAA; *nanos* forward and reverse primers, CAATGGCGGCAACTTAATG and CCACACGTTGTTCAGATG; *slo* forward and reverse primers, CTTAACACACAAGGAAAAATTTCGTGG and GTGTTCGTTCTTTTGAATTTGAATTGG; *Shal* forward and reverse primers, ATGGCCAACGTGGTGGAGACGGTGCCGTGTGG and TTCGCTGGCGCAGGACTTGAGCGTGTAGCC; *DmCa1A* forward and reverse primers, TGTACTGCCATCTCCAGTTC and GTGCGTATCTTGGTGTTGTC; respectively.

A Roche Lightcycler 1.5 was used to undertake relative quantification of target mRNAs. Reactions contained 5 μl of Mastermix (3 mM $MgCl_2$, *Taq* polymerase, dNTPs; Biogene, Kimbolton, UK), 0.5 μl of each forward and reverse primer (both 10 mM), 2 μl of water, and 1 μl of 1:1000 dilution SYBR Green (Invitrogen) and 1 μl of cDNA. Cycling was as follows: initial denaturation of 10 s at 94°C, and then 35 cycles of 5 s annealing at 54°C for *para*, 65°C for *Shal*, 60°C for *slo*, and 57°C for all the other primer pairs used (determination of *rp49* was performed at either temperature), extension at 72°C for 10 or 20 s (for amplicons of <250 or >250 nt, respectively), and denaturation at 94°C. Reactions were performed in triplicate. Fluorescence was acquired at the end of each elongation step using the F1 detection channel with a gain of 1. Authenticity of PCR products was verified by melting-curve analysis and comparison with melting curves for a nontemplate control for each primer pair used. mRNA levels are expressed as relative fold change normalized against *rp49* mRNA. The comparative cycle threshold (Ct) method (User Bulletin 2, 1997; Applied Biosystems, Foster City, CA) was used to analyze the data by generating relative values of the amount of target cDNA (Mee et al., 2004).

Statistics. Statistical significance between control and experimental groups was calculated using a nonpaired *t* test with a confidence interval of $^{*}p \leq 0.05$ or $^{**}p \leq 0.01$.

Results

Pumilio binds *para* mRNA

Our previous work has shown that increased expression of Pum is able to downregulate the mRNA of *para* and reduce the peak amplitude voltage-gated I_{Na} in identified motoneurons aCC/RP2 (Mee et al., 2004). However, no evidence for binding between Pum and *para* mRNA has been demonstrated. To directly test whether Pum associates with *para* mRNA in the *Drosophila* nervous system, we generated transgenic flies that express a tandem-affinity purification (TAP)-tagged RNA binding domain (RBD) of Pum (Gerber et al., 2006) specifically in neuronal cells using the GAL4/UAS system. Tagged Pum^{RBD} was then recovered from adult fly extracts by affinity selection on IgG beads and subsequent cleavage with TEV protease as previously described (Gerber et al., 2006). As a control, the same procedure was performed in parallel with flies not expressing the tagged Pum^{RBD} construct

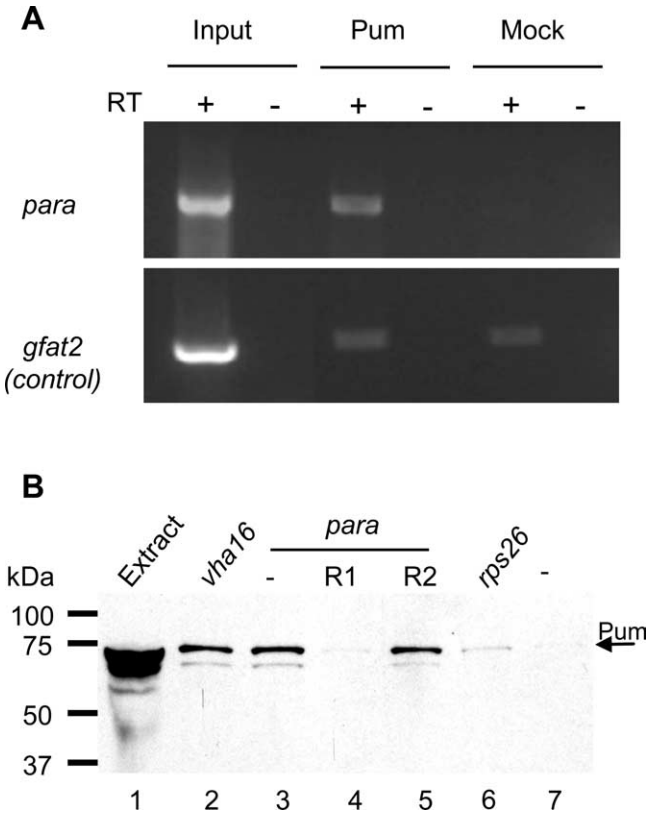

Figure 1. TAP-Pum^{RBD} specifically associates with *para* transcripts. ***A***, *para* transcript was detected by RT-PCR in total RNA isolated from fly extracts (input) and from elaV:TAP-Pum^{RBD} affinity isolations (Pum). In comparison, no detectable *para* was amplified from the mock control. Loading control was *gfat2*. Control reactions without RT (−) are shown next to samples performed with RT (+). ***B***, RNA–protein complexes of biotin-labeled RNA and *Drosophila* extracts expressing UAS-TAP-*pum*^{RBD} monitored for the presence of TAP-Pum^{RBD} by immunoblot analysis. Lane 1, Input (*Drosophila* extract); lane 2, 3′-UTR sequence of *Vha16*; lanes 3–5, a fragment of *para* encompassing parts of the ORF (lane 3) combined with 100-fold excess of competitor RNA (R1; AUUGUAAAUA; lane 4) or control RNA (R2; AUACAAAAUA; lane 5). *rps26* 3′-UTR is the negative control probe RNA (lane 6). No RNA was added in lane 7.

(mock control). Microarray analysis of the associated RNAs revealed significant association of *para* mRNA in elaV:Pum^{RBD} versus mock controls (A. Gerber and S. Luschnig, unpublished data). To further substantiate these results, we performed RT-PCR on RNA from the affinity isolates. The *para* transcript was detected in RNA isolated from affinity-purified material of TAP Pum^{RBD} expressing flies but was not detectable in RNA isolated from mock controls (Fig. 1*A*). In contrast, no particular enrichment was seen for the messages coding for *gfat2* (*glutamine-fructose-6-phosphate aminotransferase 2*) or *actin* (*act5*), both of which are abundant messages that are not predicted to be Pum targets (Fig. 1*A*) (data not shown). Thus, together, the microarray and RT-PCR data show that *para* mRNA associates with Pum in neurons.

To determine the location of potential Pum-binding sites in the *para* transcript, a bioinformatic search was undertaken using a consensus core 8 nt motif (UGUAAAUA) previously identified from an analysis of Pum-bound transcripts from ovaries (Gerber et al., 2006). This search revealed one exact match in the open reading frame (ORF) of *para* (chromosome coordinates X: 16358030, 16358037). To test whether this sequence was sufficient to bind Pum, a region encompassing this motif (chromosome coordinates X: 16357822, 16358541) was used in RNA pull-down experiments using synthetic biotinylated transcripts added

to *Drosophila* extracts expressing TAP-Pum^{RBD} (Fig. 1*B*). Similar to a positive control RNA encoding a fragment of *Vha16* 3′-UTR, previously shown to bind to Pum^{RBD} (Gerber et al., 2006), this region of *para* mRNA was able to bind to Pum^{RBD} (Fig. 1*B*, lanes 2 and 3). Moreover, this binding was specifically competed by the addition of excess of a 10 nt RNA fragment comprising the Pum-binding consensus sequence (Fig. 1*B*, lane 4) but not with a control RNA where the conserved core UGU was mutated to ACA (Fig. 1*B*, lane 5). Finally, mutation of the Pum-binding consensus sequence in the *para* transcript (cGUcAAUA) is sufficient to abolish binding of Pum (data not shown). These results not only corroborate our previous binding observations gained from microarray and PCR but, additionally, show that a region within the *para* ORF is sufficient to bind Pum.

Full-length Pumilio is necessary for repression of *para*

The Pum^{RBD} consists of eight imperfect repeats that mediate the binding of the target mRNA and the cofactor Nanos to produce a translation repressor complex (Sonoda and Wharton, 1999). A high degree of conservation of the RBD has been described in proteins of the Pum family from yeast to humans (Zamore et al., 1997). Expression of only the Pum^{RBD} has been reported to be sufficient for a partial rescue of the *pum* mutant embryonic abdominal segmentation phenotype resulting from the lack of Pum-mediated *hb* mRNA repression (Wharton et al., 1998). However, the portion of Pum protein relevant to CNS-related processes remains controversial. On the one hand, Pum^{RBD} is sufficient to mimic the dendrite branching phenotype resulting from full-length Pum expression in dendritic arborization neurons (Ye et al., 2004). In contrast, full-length Pum is required to rescue the neuromuscular junction defects seen in *pum* mutants (Menon et al., 2004). Given this controversy, we investigated whether overexpression of the Pum^{RBD} on its own was sufficient to repress *para* mRNA and I_{Na} in central neurons. To do this, we overexpressed UAS-*pum*^{RBD}, a construct containing only the RBD of Pum. We also tested UAS-*pum*$^{RBD\text{-}V5}$ and UAS-TAP-*pum*^{RBD}, independent constructs also bearing only the *pum*^{RBD}. Overexpression of these constructs was tested for their ability to downregulate both I_{Na} in identified motoneurons and *para* mRNA in whole CNS. Figure 2, *A–C*, shows that only full-length Pum is able to repress I_{Na} in

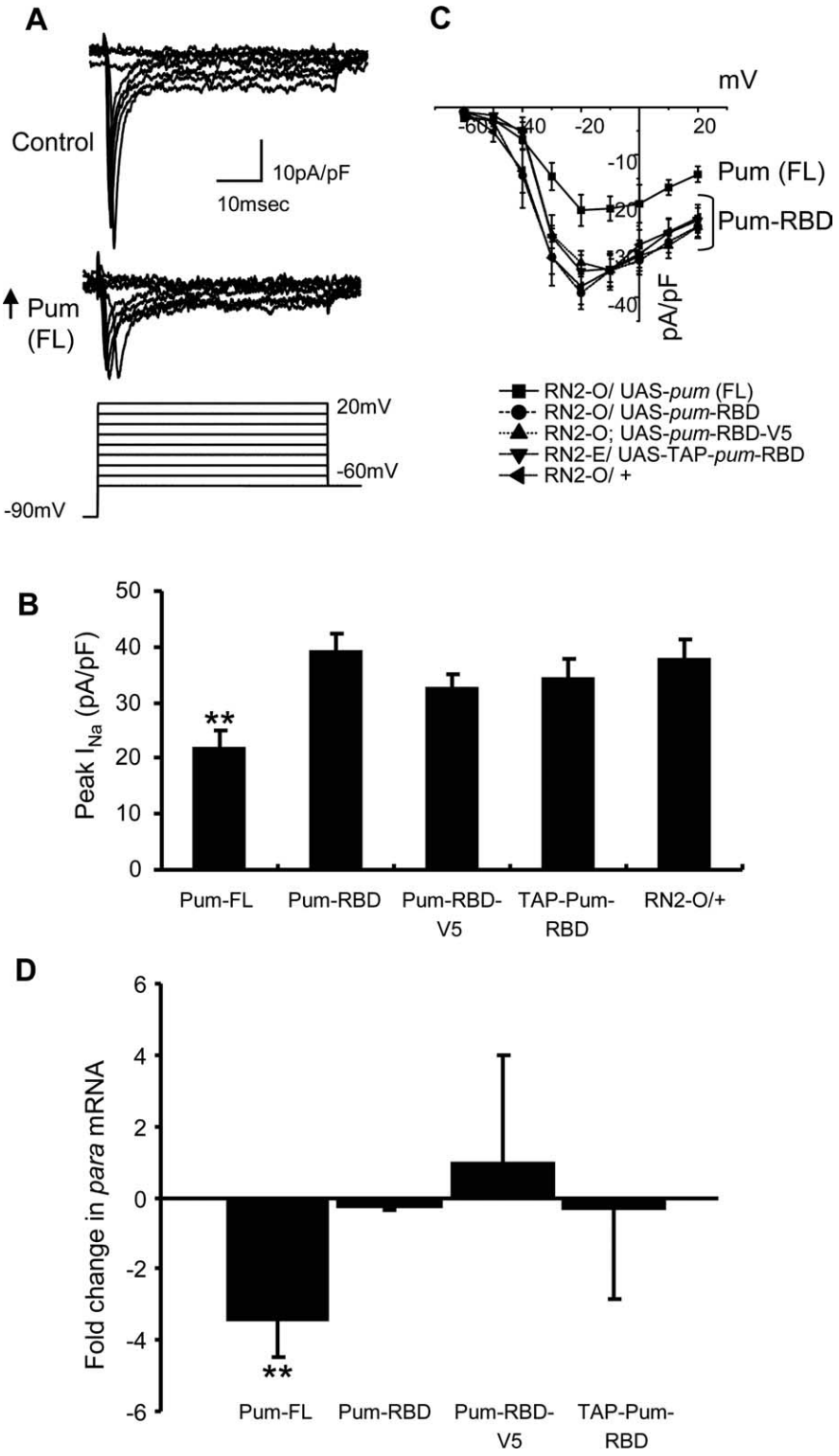

Figure 2. Full-length Pumilio is required for repression of voltage-gated I_{Na} and *para* mRNA. ***A***, Representative voltage-clamp recordings of voltage-gated I_{Na} in aCC/RP2 motoneurons of control (RN2-O-GAL4/+; top trace) and increased *pum* expression (UAS-*pum*/RN2-O-GAL4; middle trace). The voltage protocol used is shown (bottom trace). ***B***, Peak current amplitudes (measured at −20 mV) show that only Pum (FL) is sufficient to significantly decrease in I_{Na} ($p = 0.004$) compared with RN2-O-GAL4/+ control. Values shown are as follows: 21.7 ± 3.3, 39.1 ± 3.4, 32.7 ± 2.4, 34.4 ± 3.3, and 37.7 ± 3.7, respectively. ***C***, Current–voltage plots for I_{Na} measured after overexpression of UAS-*pum* FL or *pum*-RBD constructs (UAS-*pum*^{RBD}, UAS-*pum*$^{RBD\text{-}V5}$, UAS-TAP-*pum*^{RBD}). ***D***, Real time RT-PCR quantification of *para* mRNA shows that Pum^{RBD} is not sufficient to downregulate *para* mRNA. Levels of *para* mRNA were compared with the driver control (1407-GAL4). Only Pum (FL) produces a significant reduction ($p = 0.003$) of *para* mRNA. Values shown are as follows: −3.47 ± 0.98, −0.22 ± 0.14, 0.93 ± 3.1, and −0.22 ± 2.56, respectively. For ***B*** and ***C***, values given are means ± SE ($n \geq 8$). For ***D***, values given are means ± SE ($n \geq 4$). **$p \leq 0.001$.

aCC/RP2. Real-time RT-PCR quantification of *para* mRNA similarly shows that only full-length Pum is able to downregulate *para* mRNA in whole CNS (Fig. 2*D*). These results suggest that the translational repression mechanism of *para* mRNA requires the participation of parts of the Pum protein outside the RBD. This represents a clear difference between the mechanism of repression of the *para* transcript compared with the known mechanism of repression of *hb* mRNA.

Nanos is necessary for Pumilio-dependent *para* repression

The mechanism for translational repression of a majority of mRNAs by Pum requires the participation of the cofactor Nanos (Sonoda and Wharton, 1999; Kadyrova et al., 2007). Therefore, we tested whether this cofactor is also necessary for Pum to downregulate *para* mRNA and I_{Na}. We overexpressed full-length Pum in a zygotic *nanos*^{17} homozygous mutant background. This mutation contains a single amino acid change in the C-terminal region of Nanos that is sufficient to influence embryonic segmentation presumably through disrupted translational repression of *hb* mRNA (Curtis et al., 1997). *nanos*^{17} is a weak allele that produces embryos with variable numbers of abdominal segments and, although stronger *nanos* alleles have been described, *nanos*^{17} was chosen for this study because it produces viable first instar larvae. Whole-cell recordings from aCC/RP2 motoneurons in larvae overexpressing full-length Pum in a *nanos*^{17} mutant background failed to show a reduction in I_{Na} (Fig. 3*A*,*B*), indicative that Nanos is necessary for Pum-dependent repression of *para* mRNA. One copy of wild-type *nanos* in a heterozygous *nanos*^{17} mutant (*nanos*^{17}/+) rescues the ability of Pum to downregulate I_{Na}. The extent of downregulation in the heterozygote is equivalent to that observed when two normal copies of *nanos* are present (i.e., wild type), suggesting that, although necessary, the dose of *nanos* does not determine the level of *para* repression (see also Fig. 5*A*). This represents another key difference between *para* and *hb* repression in which Nanos is the principal factor limiting the translational repression of *hb* mRNA by means of a posterior-to-anterior concentration gradient (Barker et al., 1992). The *nanos*^{17} mutation alone does not show any differences in I_{Na} presumably because enough functional Nanos protein is present to allow endogenous Pum (but not increased Pum expression) to function normally (Fig. 3*A*,*B*). Quantification of *para* mRNA after pan-neuronal overexpression of Pum in the *nanos*^{17} mutant background also shows a clear necessity of Nanos for the downregulation of *para* mRNA (Fig. 3*C*). Thus, it would seem that, as for most Pum mRNA targets examined so far, translational repression of *para* requires the cofactor Nanos. However, it should be noted that this conclusion is based on the use of *nanos*^{17}, which is a point mutation and not a genetic null.

A requirement of Brain Tumor for *para* repression is cell type specific

Brat is a second cofactor that is required for the translational repression of *hb* mRNA (Sonoda and Wharton, 2001). To analyze the requirement of Brat in the translational regulation of *para*, we took advantage of UAS-*pum*^{G1330D}, a single amino acid substitution that renders Pum unable to recruit Brat to the repression complex and, as such, unable to translationally repress *hb* mRNA (Wharton et al., 1998). Patch-clamp analysis, after overexpression of UAS-*pum*^{G1330D} in aCC/RP2 motoneurons, shows normal I_{Na} (Fig. 4*A*), implicating that Brat binding is a necessary step for the downregulation of *para* in these neurons. However, *para* mRNA quantification from whole CNS after overexpression of UAS-*pum*^{G1330D} pan-neuronally suggests otherwise. This is because *para* mRNA levels are still downregulated when UAS-*pum*^{G1330D} is overexpressed in all neurons (Fig. 4*B*). This apparent dichotomy in requirement for Brat is consistent with the

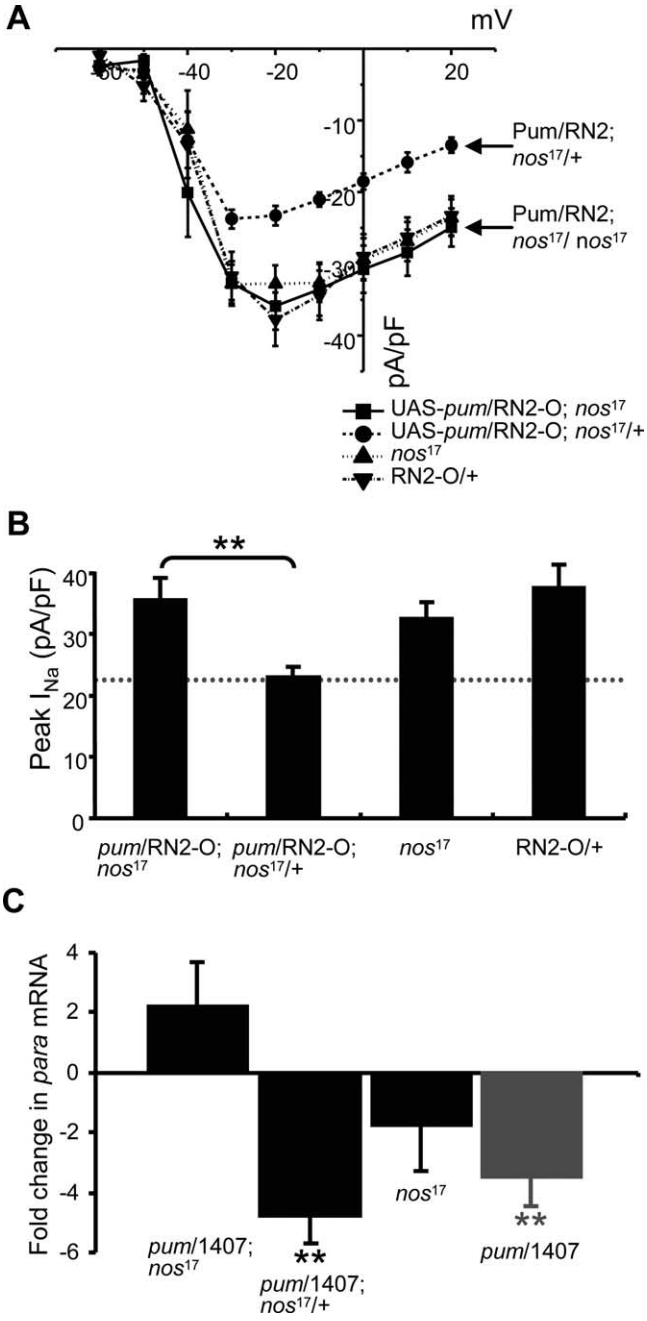

Figure 3. Nanos is necessary for Pumilio-dependent *para* repression. ***A***, Current–voltage plots of I_{Na} in aCC/RP2 comparing the following genotypes are shown: [UAS-*pum*/RN2-O; *nanos*^{17}/*nanos*^{17}], [UAS-*pum*/RN2-O; *nanos*^{17}/+], [*nanos*^{17}/*nanos*^{17}], and [RN2-O/+]. ***B***, Peak current amplitudes (measured at −20 mV) show that overexpression of UAS-*pum* (FL) is unable to repress I_{Na} in a *nanos*^{17} homozygous background. Overexpression of UAS-*pum* in a *nanos*^{17} heterozygote is sufficient to repress I_{Na} ($p = 0.003$ compared with UAS-*pum*/RN2-O; *nanos*^{17}). The *nanos*^{17} mutation alone does not affect I_{Na}. Peak I_{Na} after overexpression of *pum* in a wild-type background is shown by the continuously dotted horizontal line. Values shown are as follows: 35.9 ± 3.3, 23.3 ± 1.4, 32.8 ± 2.6, and 37.7 ± 3.7, respectively. ***C***, Real-time RT-PCR quantification of *para* mRNA for the same genotypes shown in part ***B***. Overexpression of *pum* in the absence of *nanos* fails to repress *para* mRNA levels (compared with 1407-GAL4 control). Values shown are as follows: 2.23 ± 1.47, −4.80 ± 0.87, −1.76 ± 1.50, and −3.47 ± 0.98, respectively. For ***A*** and ***B***, values given are means ± SE ($n \geq 8$). For ***C***, values given are means ± SE ($n \geq 4$). $^{**}p \leq 0.01$.

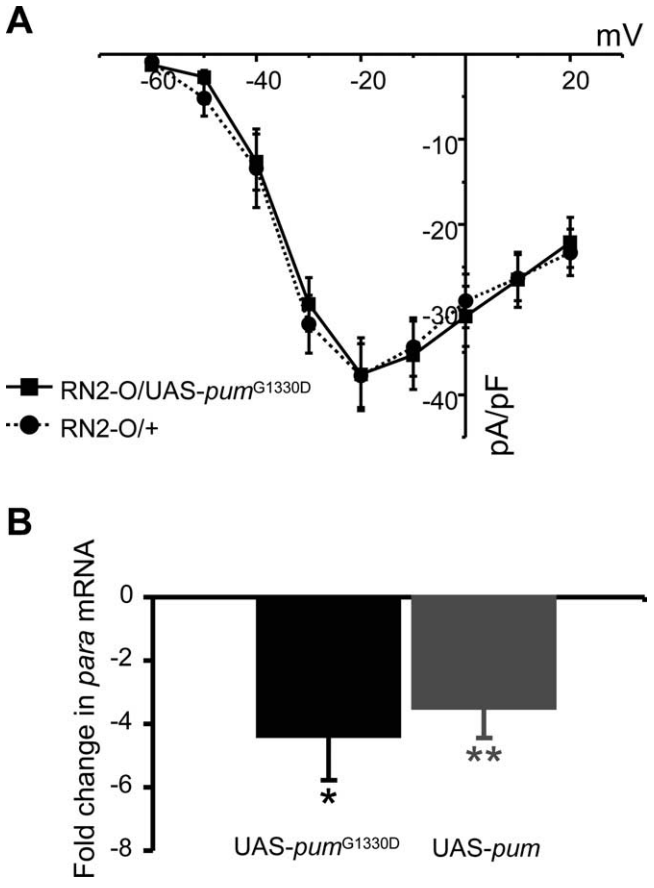

Figure 4. The requirement of Brat for *para* repression is cell type specific. ***A***, Overexpression of UAS-*pum* ^{G1330D} is unable to reduce I_{Na} in aCC/RP2 motoneurons. ***B***, Real-time RT-PCR quantification of *para* mRNA shows a significant reduction after pan-neuronal overexpression of UAS-*pum* ^{G1330D} using 1407-GAL4 ($p \leq 0.05$). No significant differences exist between the overexpression of UAS-*pum* ^{G1330D} and UAS-*pum* (gray; shown for comparative purposes). Values shown are as follows: -4.39 ± 1.34 and -3.47 ± 0.98, respectively. For ***A***, values given are means $\pm$ SE ($n \geq 8$). For ***B***, values given are means $\pm$ SE ($n \geq 4$). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

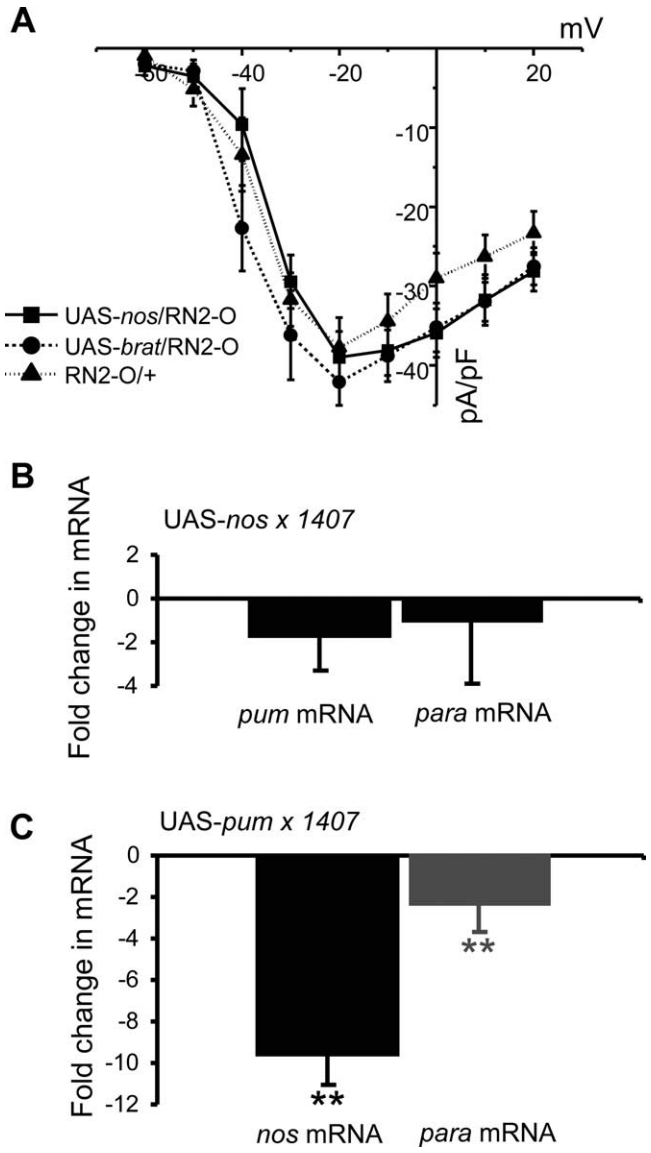

Figure 5. Pumilio is the limiting factor in *para* repression. ***A***, Overexpression of either UAS-*nanos* or UAS-*brat* in aCC/RP2 motoneurons (using RN2-O-GAL4) does not affect I_{Na}. ***B***, Pan-neuronal overexpression of UAS-*nanos* (1407-GAL4) is unable to significantly change levels of *pum* or *para* mRNA ($p > 0.05$ for both). Values shown are as follows: -1.67 ± 1.64 and -1.02 ± 2.89, respectively. ***C***, Pan-neuronal overexpression of UAS-*pum* (FL) is sufficient to greatly reduce *nanos* mRNA ($p < 0.00001$) in addition to reducing *para* mRNA (gray; shown for comparative purposes). Values shown are as follows: -9.56 ± 1.53 and -3.47 ± 0.98, respectively. For ***A***, values given are means $\pm$ SE ($n \geq 8$). For ***B*** and ***C***, values given are means $\pm$ SE ($n \geq 4$). $^{**}p \leq 0.01$.

majority of cells in the CNS not requiring Brat for the translational repression of *para*, whereas Pum-dependent repression of *para* mRNA in motoneurons is Brat dependent. Pum repression without Brat involvement has been already reported. *CycB* mRNA, a gene important for germ cell proliferation, is regulated by Pum and Nanos but does not require Brat (Kadyrova et al., 2007). In conclusion, our results are suggestive of Brat being necessary for Pum function in some cell types (i.e., aCC/RP2 motoneurons), but not in other types of neurons.

Pumilio is the limiting factor in *para* repression

A key factor for Pum-mediated repression of *hb* mRNA in the *Drosophila* embryo is the spatial gradient of the cofactor Nanos. The posterior-to-anterior Nanos gradient defines the precise spatial zone of *hb* mRNA translational repression. Pum, however, is distributed homogeneously throughout the embryo (Barker et al., 1992). To test whether Nanos was also limiting for repression of *para* mRNA, we overexpressed UAS-*nanos* in aCC/RP2 motoneurons and recorded I_{Na}. No changes in I_{Na} were observed (Fig. 5*A*). Consistent with this observation, real-time RT-PCR quantification of *para* mRNA, after overexpression of *nanos* pan-neuronally, also showed no significant difference (Fig. 5*B*). Because our previous experiments demonstrate that the Pum protein is constitutively active [a mutation in *pum* results in an increase in *para* mRNA (Mee et al., 2004)], our observation that overexpression of *nanos* is without effect is unlikely to be attributable to lack of functional Pum. These results, together with the fact that the *nanos*^{17} mutation alone does not affect the level of I_{Na} (Fig. 3), suggest that Nanos is not a limiting factor in *para* repression. Similarly, overexpression of *brat* does not produce a change in I_{Na} in aCC/RP2 (Fig. 5*A*). Therefore, given that I_{Na} in aCC/RP2 motoneurons is sensitive to Pum dosage (Fig. 2) (Mee et al., 2004), our data are consistent with Pum being a limiting factor for *para* mRNA translational repression in these neurons.

Pumilio downregulates *nanos*

To investigate further the Pum-dependent repression mechanism of *para* in the CNS, we examined whether Pum and Nanos

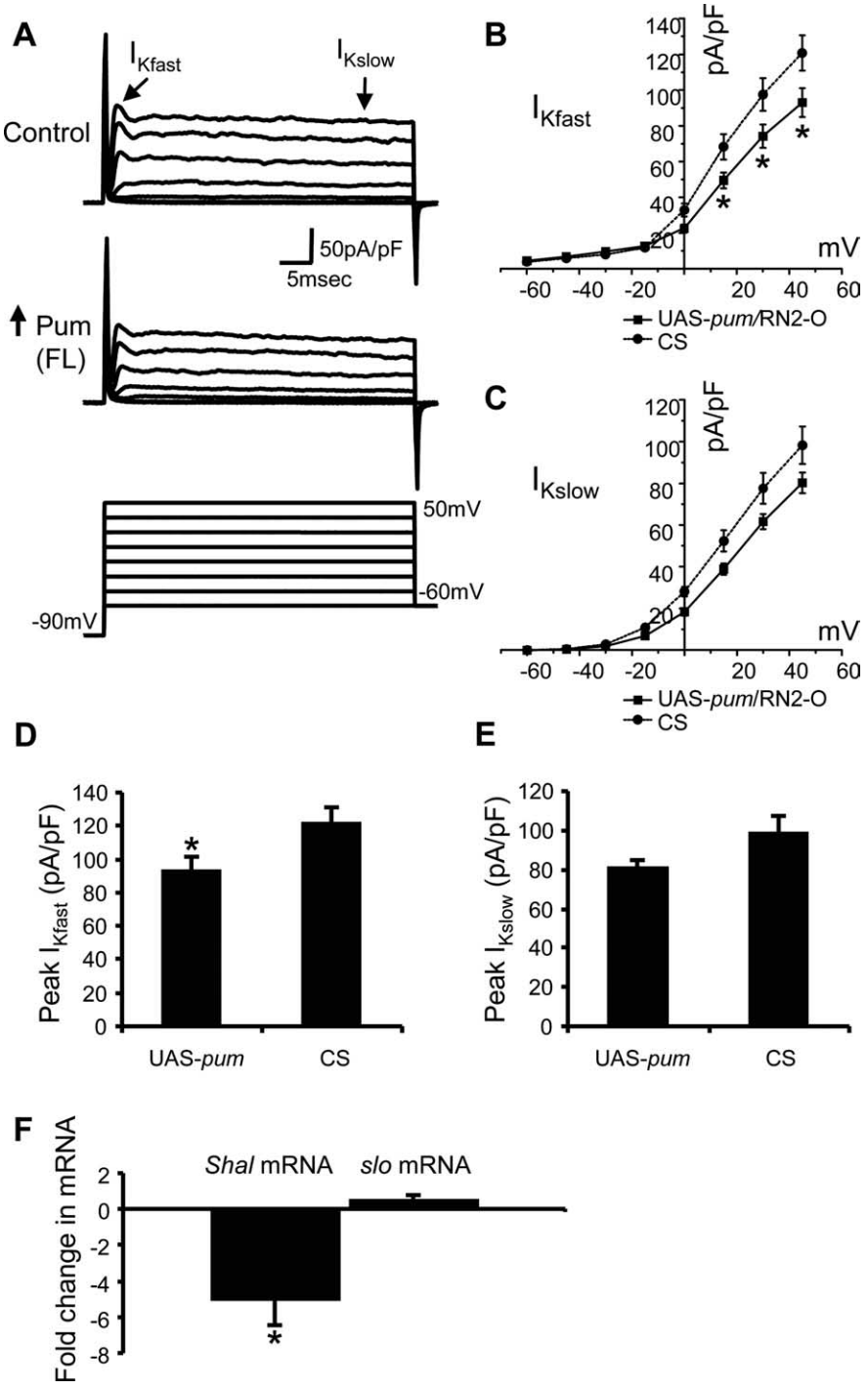

Figure 6. Pumilio negatively regulates I_{Kfast} and *Shal* mRNA. ***A***, Representative voltage– clamp recordings of voltage-gated I_K in aCC/RP2 motoneurons in control larvae (CS) (top trace) and after overexpression of *pum* (FL) (middle trace). The fast and slow components of I_K in these motoneurons are indicated (arrows). Voltage protocol used to evoke I_K is shown (bottom trace). ***B***, ***C***, Current–voltage plots for I_{Kfast} and I_{Kslow} measured after overexpression of UAS-*pum* and in control (CS). ***D***, ***E***, Peak current amplitudes (measured at +45 mV) show that overexpression of UAS-*pum* results in a significant decrease in I_{Kfast} ($p = 0.03$) but not I_{Kslow}. Values shown are as follows: 93.1 ± 8.0, and 120.8 ± 9.9 (***D***), and 80.2 ± 4.9, and 98.3 ± 8.9 (***E***), respectively. ***F***, Pan-neuronal overexpression of UAS-*pum* negatively regulates *Shal* mRNA ($p = 0.027$), but not *slo* mRNA. Values shown are as follows: −4.94 ± 1.52 and 0.41 ± 0.31, respectively. For ***B–E***, values given are means ± SE ($n \geq 8$). For ***F***, values given are means ± SE ($n \geq 5$). *$p \leq 0.05$.

could regulate one another. Such an interaction, if it exists, might be ideally suited to act as a control mechanism to safeguard neurons from excessive repression of *para* mRNA. Overexpression of UAS-*nanos* pan-neuronally does not produce any changes in *pum* mRNA levels (Fig. 5*B*). In contrast, overexpression of UAS-*pum* pan-neuronally resulted in a very large and significant decrease in *nanos* mRNA (Fig. 5*C*). The relative efficiency of Pum to downregulate *nanos* mRNA is approximately four times that of its ability to repress *para* mRNA. This regulation is possibly direct because binding of Pum to *nanos* mRNA has been reported (Gerber et al., 2006).

Pumilio indirectly regulates I_{Kfast} and *Shal* mRNA

In the motoneurons under study, activity-dependent homeostasis likely requires the coregulation of several proteins, including Pum, Para, and one or more K^+ channel proteins (Baines et al., 2001; Mee et al., 2004). Here, we showed that changes in I_{Na} current density are mediated by direct binding of Pum to *para* transcripts and subsequent translational repression. We therefore asked whether Pum can directly regulate transcript levels and ionic current densities of outward K^+ channels.

In the first instance, we looked at the effects of increased expression of Pum on potassium currents (I_K). In aCC/RP2 motoneurons I_K exhibits characteristic fast (I_{Kfast}) and slow (I_{Kslow}) inactivating phases (Fig. 6*A*) (Baines and Bate, 1998). Overexpression of full-length Pum, selectively in aCC/RP2 motoneurons, is sufficient to produce a significant decrease in the fast component of I_K in these neurons (Fig. 6*B*,*D*). In contrast, overexpression of Pum does not affect significantly the slow component (Fig. 6*C*,*E*). In *Drosophila* neurons, the fast component of I_K has been associated with the voltage-dependent potassium channel gene *Shal* (*Shaker cognate I*) (Tsunoda and Salkoff, 1995) and the voltage-dependent and calcium-activated potassium channel gene *slowpoke* (*slo*) (Pym et al., 2006). Therefore, we analyzed the effect of overexpression of Pum on both *Shal* and *slo* mRNA abundance. Real-time RT-PCR quantification shows significant diminution of *Shal* mRNA when Pum is overexpressed pan-neuronally, whereas *slo* mRNA levels are not affected (Fig. 6*F*).

Although we demonstrated that Pum can affect I_{Kfast} in addition to I_{Na} in aCC/RP2, we were unable to identify a consensus Pum binding sequence in the *Shal* transcript. Furthermore, our microarray analysis did not identify *Shal* mRNA as a direct target of Pum binding (A. Gerber and S. Luschnig, unpublished data). Because of this, it is conceivable that the effect of Pum on I_K may be an indirect consequence of a Pum-related reduction in I_{Na}. This kind of compensatory mechanism has been

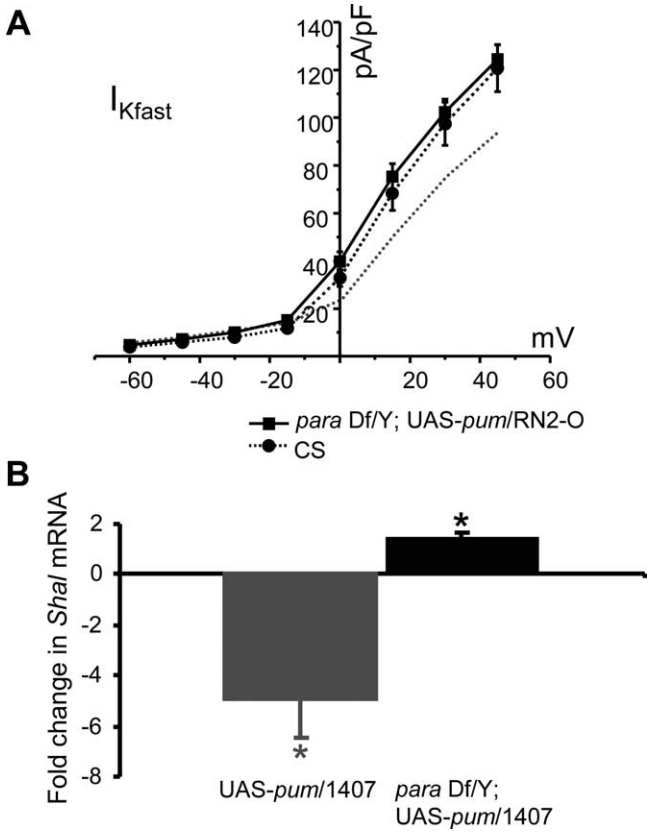

Figure 7. The effect of Pumilio on I_{Kfast} is indirect. ***A***, Overexpression of UAS-*pum* (RN2-O-GAL4) in a *para* Df background fails to reduce I_{Kfast}, compared with overexpression in a wild-type background (continuously dotted line). ***B***, Pan-neuronal overexpression of UAS-*pum* in a *para* Df background also fails to reduce *Shal* mRNA compared with overexpression in a wild-type background (gray box). The baseline corresponds to 1407-GAL4 control. Values shown are as follows: -4.94 ± 1.52 and 1.40 ± 0.23, respectively. For ***A***, values given are means $\pm$ SE ($n \geq 8$). For ***B***, values given are means $\pm$ SE ($n \geq 4$). *$p \leq 0.05$.

described previously (Baines et al., 2001). To test this, we overexpressed Pum in aCC/RP2 motoneurons in a genetic background bearing a deficiency chromosome for the *para* locus. The *para* deficiency causes late embryonic lethality, and, although embryos do not hatch, aCC/RP2 motoneuron recordings can still be performed (Baines and Bate, 1998). Overexpression of *pum*, in the complete absence of *para*, results in an I_K that is not significantly different than wild-type controls (Fig. 7*A*). Real-time RT-PCR quantification of *Shal* mRNA confirms our physiology. Thus, overexpression of *pum* pan-neuronally, in the absence of *para*, results in no downregulation of *Shal* mRNA. On the contrary, there is a small, but significant, increase in *Shal* mRNA under these conditions (Fig. 7*B*). We conclude, therefore, that the reduction in I_{Kfast} and *Shal* observed when *pum* is overexpressed in wild-type backgrounds is most likely to be an indirect effect: Pum directly represses translation of *para* mRNA and this in turn produces a compensatory reduction of *Shal* mRNA abundance and I_{Kfast}.

Discussion

Identification of the molecular components that underlie homeostasis of membrane excitability in neurons remains a key challenge. Here, we show that the known translational repressor Pum binds *para* mRNA, which encodes the *Drosophila* voltage-gated Na^+ channel. This observation provides a mechanistic understanding for the previously documented ability of Pum to regulate I_{Na} and membrane excitability in *Drosophila* motoneurons (Mee et al., 2004). Thus, alteration in activity of Pum, in response to changing exposure to synaptic excitation, enables neurons to continually reset membrane excitability through the translational control of a voltage-gated Na^+ channel.

Previous studies report several mRNAs subject to direct Pum regulation including *hb* (Murata and Wharton, 1995), *bicoid* (*bcd*) (Gamberi et al., 2002), *CycB* (Asaoka-Taguchi et al., 1999), *eIF4E* (Menon et al., 2004), and possibly the transcript destabilization factor *smaug* (*smg*) (Tadros et al., 2007). The majority of these identified transcripts concentrate the roles of Pum to the establishment of the embryonic anterior-posterior axis (*hb* and *bcd*) and germ-line function/oogenesis (*CycB*). However, in the last few years, new findings have expanded the role of Pum to encompass predicted roles in memory formation, neuron dendrite morphology, and glutamate receptor expression in muscle (Dubnau et al., 2003; Menon et al., 2004; Ye et al., 2004). Indeed, the role of Pum is likely to be very much more widespread given that Pum pull-down assays followed by microarray analysis of bound mRNAs have now identified a plethora of possible additional targets of translational regulation (Gerber et al., 2006). The ~1000 or so genes identified are implicated to be involved in various cellular functions, suggesting that Pum-dependent translational repression might be a mechanism used in different stages of development and in diverse tissue function. To date, *para* is the first confirmed Pum target encoding a voltage-gated ion channel.

Pum-binding motifs have been identified in the 3′-UTRs of many mRNAs known to bind to this protein. Analysis of 113 such genes expressed in adult *Drosophila* ovaries has identified a consensus 8 nt binding motif [UGUAHAUA (Gerber et al., 2006)]. This sequence contains the UGUA tetranucleotide that is a defining characteristic of the NRE-like motif described in the 3′-UTR of *hb* mRNA (Zamore et al., 1997). We identified such an 8 nt motif within the ORF of *para* at the 3′ end of the transcript. Our biochemical binding data support the notion that this motif is indeed sufficient to bind Pum and as such represents the first such site to be localized to an ORF of any transcript. However, to translationally repress *para* mRNA, our data also show a requirement for regions of Pum in addition to the RBD. Interestingly, this kind of requirement has also been shown for another Pum target, *eIF4E* (Menon et al., 2004). The translational silencing of mRNAs is a complex mechanism on which only little information is available. It could involve deadenylation and degradation of the mRNA and/or the circularization of the mRNA and the recruitment of factors that would preclude translation (Chagnovich and Lehmann, 2001). The fact that different Pum targets may require only the RBD (*hb*) or the full-length protein (*eIF4E* and *para*) suggests that Pum-mediated translational repression may follow complex target mRNA-specific mechanisms, most probably involving the interaction of other domains of Pum with additional, so far unknown, factors. In this regard, it is interesting to note that the N terminus of Pum has regions of low complexity including prion-like domains rich in Q/R. These domains may provide a platform for other proteins that influence the fate of Pum targets.

The putative Pum binding motif that we identify lies within an exon that is common to all *para* splice variants identified (at least in the embryo) but is possibly subject to editing by adenosine deamination. Thus, in an analysis of splicing of *para*, a number of individual cDNA clones were sequenced and one splice variant was recovered that shows A-to-I editing in this motif (D. E. Wright and R. A. Baines, unpublished data). Together with a differential requirement for specific cofactors (see below), editing

of this motif might serve to influence how *para* is affected by Pum and, as such, further increase diversity in level of expression of I_{Na} in differing neurons or disease states (Song et al., 2004).

The known mechanism of action of Pum-dependent translational repression is absolutely dependent on additional cofactors. The most studied example, that of *hb* mRNA during early embryogenesis, requires the presence of both Nanos (Sonoda and Wharton, 1999) and Brat (Sonoda and Wharton, 2001). However, the requirement for these two cofactors is seemingly transcript dependent. Thus, Pum-mediated repression of *CycB* mRNA requires Nanos but not Brat (Sonoda and Wharton, 2001). However, Pum-dependent repression of *bcd* is apparently Nanos independent, because levels of Nanos in the anterior of the early embryo are undetectable (Gamberi et al., 2002). Although we clearly show that Pum-dependent repression of *para* mRNA in the *Drosophila* CNS requires Nanos, the requirement for Brat is less clear and seems to be neuronal cell type specific. A requirement for a different combination of cofactors for Pum-dependent translational regulation of a single gene transcript has not been reported previously, but clearly might represent an additional level of regulation. Such differential regulation might be required to spatially restrict the effect of Pum to certain cell types within the CNS. Voltage-gated Na^+ currents are responsible for the initiation and propagation of the action potential and determine, together with other voltage-gated ion conductances, the membrane excitability of a neuron. Despite *para* being the sole voltage-gated sodium channel gene in *Drosophila* [compared with at least nine different genes in mammals (Catterall et al., 2005)], neuronal subpopulations nevertheless exhibit distinctive I_{Na} characteristics (O'Dowd et al., 1995) (N. Muraro and R. Baines, unpublished observations). To achieve this, *para* is known to undergo extensive alternative splicing (Thackeray and Ganetzky, 1994; Thackeray and Ganetzky, 1995) and, additionally, RNA editing (Hanrahan et al., 2000). It is highly likely that both alternative splicing and RNA editing generate mRNAs that encode channels with differing electrophysiological properties (Song et al., 2004). It is also conceivable that these mechanisms might yield *para* transcripts that contain differing arrangements of Pum/Nanos binding sites, which may, or may not, recruit Brat. Indeed, it has been proposed that variations of the NRE consensus sequence may result in Pum–NRE–Nanos complexes with different topographies, resulting in altered recruitment abilities for additional cofactors such as Brat (Kadyrova et al., 2007). Additional work is necessary to clarify where, in *para* mRNA, the binding sites for the Pum/Nanos complex are localized and how the recruitment of Brat is facilitated in only some neurons. In the *hb* repression complex, Brat has been shown to interact with the cap-binding protein d4EHP (Cho et al., 2006). Therefore, additional cofactors might be necessary for Pum-dependent *para* repression in the Brat-independent neuronal cell subtypes that we propose here.

In contrast to translational repression of *hb*, our data show that Nanos is unlikely to be a limiting factor of Pum-dependent repression of *para* translation. Consistent with this finding is our observation that overexpression of *pum* is sufficient to downregulate (and probably translationally repress) *nanos* mRNA. However, the opposite is not true; overexpression of *nanos* does not affect levels of *pum* mRNA. These data suggest that Pum is at least a principal orchestrating factor (if not the prime factor) in regulation of *para* translation. Moreover, our demonstration that overexpression of *pum* is sufficient to greatly downregulate *nanos* mRNA (relative to *para* mRNA), together with a requirement of Nanos for Pum-dependent *para* mRNA repression, implicates the existence of a protective negative-feedback mechanism that prevents overrepression of *para* mRNA. In the absence of such feedback, it is conceivable that excessive overrepression of *para* mRNA might lead to neurons falling silent as their membrane excitability drops below a critical threshold. Were this to happen, then signaling in the affected neuronal circuit would be severely compromised.

We show that overexpression of full-length Pum in aCC/RP2 motoneurons not only causes a decrease in I_{Na} but also a significant decrease in I_{Kfast}. Additionally, pan-neuronal overexpression of Pum causes a significant decrease in *Shal* mRNA, a gene encoding a potassium channel known to contribute to I_{Kfast} (Tsunoda and Salkoff, 1995). This result was surprising given that we did not identify *Shal* as a Pum target from our microarray analysis (A. P. Gerber and S. Luschnig, unpublished results). That this mechanism might, therefore, be indirect is corroborated by our finding that I_{Kfast} and *Shal* mRNA remain at wild-type levels when Pum is overexpressed in a *para*-null background. It is, perhaps, counterintuitive that a reduction in I_{Na}, to achieve a reduction in membrane excitability, should be accompanied by a similar decrease in outward I_{Kfast}. However, changes in ionic conductances should not be considered in isolation and such a relationship might serve to maintain action potential kinetics within physiological constraints (Baines, 2003). Covariation of I_{Na} and I_K as a mechanism for changing neuronal excitability has been described in these motoneurons previously (Baines et al., 2001). Moreover, there is precedent for coupling between transcripts: injection of *Shal* mRNA into lobster PD (pyloric dilator) neurons results in an expected increase in I_A but also an unexpected linearly correlated increase in I_h, an effect that acts to preserve membrane excitability. Injection of a mutated, nonfunctional, *Shal* mRNA is also sufficient to increase I_h indicative that this coregulation is activity independent (MacLean et al., 2003). It remains to be shown whether genetic manipulation of *para* mRNA levels in *Drosophila* motoneurons will similarly evoke compensatory changes in *Shal* expression.

In a previous study, it was shown that blockade of synaptic release, through pan-neuronal expression of tetanus toxin light chain, was sufficient to evoke a compensatory increase in membrane excitability in aCC/RP2 that was accompanied by increases in I_{Na}, I_{Kfast}, and also I_{Kslow} (Baines et al., 2001). In contrast, we show here that overexpression of *pum* is sufficient to decrease I_{Na} and I_{Kfast} but does not significantly affect I_{Kslow} (although there is a small nonsignificant reduction in this current). Clearly, the complete absence of synaptic input is a more severe change that likely elicits a greater compensatory change in these neurons than when Pum is overexpressed. However, whether removal of synaptic excitation also invokes additional compensatory mechanisms that act preferentially on I_{Kslow} remains to be determined. What is consistent, however, is that change in synaptic excitation of these motoneurons is countered by Pum-dependent regulation of both *para* mRNA translation and magnitude of I_{Na}.

A key question remains as to what the mechanism is that transduces changes in synaptic excitation to altered Pum activity. Perhaps the most parsimonious mechanism will be one linked to influx of extracellular Ca^{2+}. Indeed, experimental evidence supports a role for Ca^{2+}, because blocking its entry can preclude changes in neuronal excitability observed as a result of activity manipulation (Offord and Catterall, 1989; Desarmenien and Spitzer, 1991; Golowasch et al., 1999). In addition, changes of gene expression resulting from activity-mediated Ca^{2+} entry have been described both *in vitro* (Xiang et al., 2007) and *in vivo* after plasticity changes such as long-term potentiation (Miy-

amoto, 2006). Whether Ca^{2+} influx influences translation and/or transcription of Pum remains to be shown. Stimulation of mammalian neurons in culture with glutamate, after a preconditioning period of forced quiescence, results in an increase of Pum2 protein levels after just 10 min (Vessey et al., 2006). The rapidity of this response suggests that it is mediated by a posttranscriptional mechanism. We examined the role of Pum on Ca^{2+} channel activity. We find that neither $I_{Ba(Ca)}$ nor levels of the voltage-gated calcium channel coded by *Dmca1A* (*cacophony, Calcium channel α1 subunit, type A*) (Peng and Wu, 2007) are affected in aCC/RP2 motoneurons in which *pum* [full length (FL)] is overexpressed (data not shown). The fact that Pum does not affect Ca^{2+} channel activity directly could reinforce the idea of its serving as a primary sensor of activity changes.

In summary, we show that Pum is able to bind to *para* mRNA, an effect that we previously showed to be sufficient to regulate both I_{Na} and membrane excitability in *Drosophila* motoneurons (Mee et al., 2004). This mechanism requires the cofactor Nanos but does not obligatorily require Brat. Given that mammals express two Pum genes, *Pum1* and *Pum2* (Spassov and Jurecic, 2002), it will be of importance to determine whether this protein is also able to regulate sodium channel translation in the mammalian CNS.

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