Dopaminergic Ric GTPase activity impacts amphetamine sensitivity and sleep quality in a dopamine transporter-dependent manner in *Drosophila melanogaster*

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Abstract

Dopamine (DA) is required for movement, sleep, and reward, and DA signaling is tightly controlled by the presynaptic DA transporter (DAT). Therapeutic and addictive psychostimulants, including methylphenidate (Ritalin; MPH), cocaine, and amphetamine (AMPH), markedly elevate extracellular DA via their actions as competitive DAT inhibitors (MPH, cocaine) and substrates (AMPH). DAT silencing in mice and invertebrates results in hyperactivity, reduced sleep, and blunted psychostimulant responses, highlighting DAT's essential role in DA-dependent behaviors. DAT surface expression is not static; rather it is dynamically regulated by endocytic trafficking. PKC-stimulated DAT endocytosis requires the neuronal GTPase, Rit2, and Rit2 silencing in mouse DA neurons impacts psychostimulant sensitivity. However, it is unknown whether or not Rit2-mediated changes in psychostimulant sensitivity are DAT-dependent. Here, we leveraged *Drosophila melanogaster* to test whether the *Drosophila* Rit2 ortholog, Ric, impacts dDAT function, trafficking, and DA-dependent behaviors. Orthologous to hDAT and Rit2, dDAT and Ric directly interact, and the constitutively active Ric mutant Q117L increased dDAT surface levels and function in cell lines and *ex vivo Drosophila* brains. Moreover, DAergic RicQ117L expression caused sleep fragmentation in a DAT-dependent manner, but had no effect on total sleep and daily locomotor activity. Importantly, we found that Rit2 is required for AMPH-stimulated DAT internalization in mouse striatum, and that DAergic RicQ117L expression significantly increased *Drosophila* AMPH sensitivity in a DAT-dependent manner, suggesting a conserved impact of Ric-dependent DAT trafficking on AMPH sensitivity. These studies support that the DAT/Rit2 interaction impacts both baseline behaviors and AMPH sensitivity, potentially by regulating DAT trafficking.

Introduction

Dopamine (DA) is critical for a variety of behaviors, including motor function, sleep, learning, and reward[1, 2]. Multiple neuropsychiatric and neurologic disorders are linked to DAergic dysfunction, including addiction, attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), schizophrenia, and Parkinson's disease (PD)[3-7]. Extracellular DA levels are tightly restricted by the presynaptic DA transporter (DAT). Following evoked DA release, DAT takes up released DA back into DAergic presynaptic terminals, thereby limiting the spatial and temporal DA signal[8]. DAT not only limits extracellular DA levels, but is also critical to maintain homeostatic DA levels and synaptic DAergic tone across multiple species[9]. DAT's pivotal role in regulating DAergic signaling is best illustrated by observing the consequences that follow from either pharmacological DAT inhibition or genetic DAT perturbation. For example, addictive and therapeutic psychostimulants, such as cocaine, AMPH, methamphetamine (METH), and methylphenidate (Ritalin, MPH), are competitive DAT antagonists (cocaine, MPH) and substrates (AMPH, METH) that increase extracellular DA levels and drive hyperlocomotion, sleep perturbation, and rewarding behaviors in both vertebrates and invertebrates[10, 11]. Moreover, genetic DAT ablation in both vertebrates and invertebrates 1) blocks psychostimulant-induced hyperactivity and reward, and 2) causes hyperactivity and sleep loss[12-15]. DAT knock-out (*DAT*$^{-/-}$) mice display alterations in their sleep and waking patterns in which their waking episodes (or bouts) are three times longer than wildtype controls[13]. *Drosophila melanogaster* DAT (dDAT)-null fruit flies ("fumin", *fmn*) also sleep significantly less than wildtype flies, with shorter and fewer resting bouts[14]. In addition to mouse and *Drosophila* DAT manipulations, numerous DAT coding variants have been reported in ADHD, ASD, and PD patients[16-21],

underscoring that DAT availability and function play an indispensable role in basal DAergic function.

Given DAT's fundamental role in DAergic signaling, the cellular mechanisms that modify DAT function and/or expression at the plasma membrane are ideally poised to impact DAergic neurotransmission and DA-dependent behaviors. Decades of research support that DAT surface expression is acutely modulated by endocytic trafficking[22-24]. Both PKC activation and AMPH exposure acutely increase DAT internalization, resulting in a rapid net loss of DAT from the plasma membrane, as demonstrated both *in vitro* and in *ex vivo* mouse striatal slices[8, 22, 24-27]. However, it remains unknown whether or not regulated DAT trafficking impacts DA-dependent behaviors.

Rit2 is a neuronal-specific[28-30] GTPase that is enriched in DA neurons[31], and is highly homologous to Rit1, which is ubiquitously expressed[32, 33]. Multiple genome-wide association studies (GWAS) have identified Rit2 as a risk allele selectively in DA-related neuropsychiatric disorders, including PD, ASD, schizophrenia, and speech delay[34-42]. We previously reported that Rit2 binds directly to DAT[43, 44], and that Rit2 is required for PKC-stimulated DAT endocytosis in both cell lines and striatal DAergic terminals[43, 44]. PKC activation drives DAT and Rit2 to dissociate at the cell surface, and failure of DAT and Rit2 to dissociate correlates with increased DAT stability at the plasma membrane, and inability to undergo PKC-stimulated endocytosis[43]. Importantly, we also found that conditional Rit2 knockdown (Rit2-KD) in DA neurons significantly increases acute cocaine sensitivity *in vivo* in male mice[45]. These data raise the possibility that Rit2-mediated changes in psychostimulant sensitivity are due to inability of DAT to undergo regulated trafficking. However, it is not known whether Rit2-dependent behavior phenotypes in

mice are DAT-dependent or DAT-independent. In order to address this question, we leveraged the model organism, *Drosophila melanogaster*, which expresses a single Rit2 homolog, Ric. Ric shares ~71% homology with Rit2, but is expressed ubiquitously in the fruit fly, similar to Rit1. Given that the DA system is highly conserved between flies and mammals, *Drosophila* provide a highly tractable and simplified model to study the impact of regulated DAT trafficking on DA-dependent behaviors[46]. Similar to *DAT*$^{(-/-)}$ mice, DAT null flies (*fmn)* are hyperactive, lack psychostimulant response, and numerous reports indicate that hDAT suffices to rescue hyperactivity and psychostimulant response in *fmn* flies[15, 21, 47, 48]. Moreover, expressing hDAT ASD and ADHD coding variants on the *Drosophila fmn* background leads to hyperactivity and disrupted psychostimulant response[21, 49]. In the present study, we found that Ric GTPase binds to and regulates dDAT function and expression, similar to its mammalian homolog, Rit2. Moreover, DAergic expression of the constitutively active RicQ117L leads to reduced sleep consolidation and increased AMPH sensitivity in a DAT-dependent manner.

MATERIALS AND METHODS

Materials

Nisoxetine was from Tocris-Cookson. Rabbit anti-HA antibody (clone C29F4) was from Cell Signaling Technology and rat anti-DAT antibody (MAB369) was from Millipore. HRP-conjugated goat anti-rabbit (Cat#111-035-003) and goat anti-rat (Cat#112-035-062) secondary antibodies were from Jackson ImmunoResearch. All other reagents were from either Sigma-Aldrich or Fisher Scientific, and were of the highest possible grade.

cDNA plasmids

dDAT and WT-Ric plasmids were from Addgene and Dr. Douglas Harrison (University of Kentucky), respectively, were subcloned into pcDNA3.1(+), and HA tags were inserted at their N-termini by PCR. Ric S73N and Q117L mutants were generated using the QuikChange II mutangenesis kit (Stratagene). YFP- and CFP-dDAT and Ric constructs were generated by subcloning into pEYFP-C1 and pECFP-C1 vectors, respectively, at the HindIII and XbaI sites. All sequences were verified by Sanger sequencing (GeneWiz).

Fly stocks

All fly stocks were maintained on low yeast medium as previously described[50, 51]. Transgenic strains *TH-GAL4* and *fmn* were gifts from Dr. Heinrich Matthies (University of Alabama School of Medicine). *UAS-HA-Ric*, *UAS-HA-RicQ117L*, and *UAS-HA-RicS73N* lines were generated by subcloning into the 5xUAS, w+ vector[52, 53] (gift of Dr. Marc Freeman, Vollum Institute), and were inserted into the genome via phiC31-mediated integration (BestGene). All fly strains were backcrossed to the isogenic w^{1118} wildtype (+/+; VDRC60000) strain for at least six generations, and balanced prior to behavioral studies. Transgenes were verified by eye color or PCR.

Drosophila circadian behavior and AMPH feeding

Sleep and locomotor behavior were assessed in male progeny 1-3dpe using the *Drosophila* Activity Monitor system, as previously described[50, 51, 54]. 1-3 days old flies were loaded in Trikinetics DAM monitors (Waltham, MA) and exposed to 6 days of LD cycles. The last three days were used to measure sleep. At least 3 independent experiments, with multiple flies/experiment, were performed, and data from 3 days of LD were averaged for each

experiment. Data were analyzed using the sleep and circadian analysis MATLAB program (SCAMP)[55]. A sleep bout was defined as 5 consecutive minutes of inactivity. For AMPH feeding studies, flies were starved and then fed either base fly behavior food, or food supplemented with the indicated AMPH doses, as previously reported[48, 49]. Locomotor activity was recorded for 24 hrs after introducing AMPH.

Cell culture and transfections

HEK293T and HEK293 (FRET studies) cells were maintained at 37°C, 5% CO_2, and transiently transfected with Lipofectamine or JetPrime (FRET studies), as previously described[43]. Cells were seeded one day prior to transfection onto poly-D-lysine coated plates at a density of $5x10^5$ cells/well for biochemical studies (6-well), or $1.67x10^4$ cells/well for [^{3}H]DA uptake studies (96-well white opaque/clear bottom plates), and were transfected using 2µg plasmid DNA/well for 6 well dishes, and 0.067µg plasmid DNA/well for 96 well plates, at a lipid:DNA ratio of 2:1. For co-transfection studies, cDNA was transfected at a co-expressor:DAT ratio of 4:1. For FRET microscopy, $2x10^4$ cells/chamber were seeded in an ibidi 8-well chambered coverslips and were transfected using 0.25µg plasmid DNA/chamber. All assays were conducted 24-48 hrs post-transfection.

Live cell FRET microscopy

FRET was measured and at the plasma membrane and analyzed as previously described[43]. Normalized FRET (NFRET) was calculated as follows:

$$\mathrm{NFRET} = \frac{I_{FRET} - (BT_{Donor} \times I_{Donor}) - (BT_{Acceptor} \times I_{Acceptor})}{\sqrt{I_{Donor} \times I_{Acceptor}}}\ x\ 100$$

A fused CFP-YFP construct (CYFP) was included as an internal instrument control in each experiment, and non-fused donor and acceptor fluorophores were included as a negative control.

[^{3}H]DA uptake

Transfected cells: [^{3}H]DA uptake was measured in transfected cells essentially as previously described[27, 56, 57]. Non-specific uptake was defined using 10μM nisoxetine, a highly potent and selective dDAT inhibitor[58]. HA-Ric expression was confirmed in each experiment via immunoblotting.

Ex vivo Drosophila brains: Whole brain uptake assays were performed as described previously, with slight modifications[49]. Brains from male progeny 0-3dpe were dissected in hemolymph-like solution (HL3; 70mM NaCl, 5mM KCl, 1.5mM Ca^{2+} acetate, 10mM $MgSO_4$, 5mM HEPES, supplemented daily with fresh 115mM sucrose, 5mM trehalose, 10mM $NaHCO_3$, 10μM sodium ascorbate, 10μM pargyline), and 4 brains/condition were used for a single measurement in each independent experiment. Brains were pre-incubated in HL3 ±10μM nisoxetine for 20min at 25°C in cell culture inserts, and DA uptake was initiated by adding 1μM [^{3}H]DA. Uptake proceeded for 7 min, RT, and was terminated by rapidly washing brains 3x1mL ice cold HL3. Brains were transferred to 96-well white/clear plates, solubilized in scintillation fluid, and radioactivity was measured using a Wallac MicroBeta scintillation plate counter.

Cell surface biotinylation

Surface dDAT was measured by biotinylation in transfected HEK293T cells as previously described for hDAT[27, 43, 57]. Biotinylated proteins were quantitatively recovered by streptavidin batch chromatography from equivalent amounts of cellular protein, and were eluted in 2X Laemmli sample buffer, 30 min, room temperature with rotation. Eluted proteins and input lysates were resolved by SDS-PAGE and proteins were detected and quantified by immunoblotting. Immunoreactive bands were detected by chemiluminescence (SuperSignal™ West Dura Extended Duration Substrate, ThermoFisher) and were captured with a CCD-camera (VersaDoc, Biorad). Bands in the linear range of detection were quantified using Quantity One software (Bio-rad), and the %surface DAT was determined by normalizing surface DAT to its corresponding DAT input signal.

Vertebrate Animals

All studies were conducted in accordance with UMASS Medical School IACUC Protocol A-1506 (H.E.M). *Pitx3*$^{IRES2\text{-}tTA}$ mice on the *C57Bl/6* background were the generous gift of Dr. Huaibin Cai (National Institute on Aging) and were continually backcrossed to *C57Bl/6J* mice. Mice were maintained in 12hr light/dark cycle at constant temperature and humidity and food and water were available ad libitum.

AAV production and stereotaxic viral delivery

AAV9 particles expressing TRE-eGFP and TRE-shRit2-eGFP were produced by the UMASS Medical School Viral Vector Core, as previously described[43, 45]. P21-25 male *Pitx3*$^{IRES2\text{-}tTA}$ mouse VTA were bilaterally injected with 1µl AAV particles (Bregma: anterior/posterior: -3.08mm,

medial/lateral: ±0.5mm, dorsal/ventral: -4.5mm) at a rate of 0.2μL/min as previously described[45]. Studies were performed 4-6 weeks post-injection, and viral expression was confirmed by visualizing GFP expression in midbrain slices.

Ex vivo slice biotinylation

Mouse striatal slices were prepared and hemisected along the midline as previously described[27, 43, 45], and were treated ±10μM AMPH in ACSF, 30min, 37°C with constant oxygenation, using paired contralateral hemi-slices as a vehicle and drug-treated samples. Following drug treatment, surface proteins were labeled by biotinylation, hemi-slices were subdissected to enrich for dorsal striatum, and tissue was solubilized as previously described[43, 45]. Biotinylated proteins were isolated by batch streptavidin chromatography from equivalent amounts of protein, and DAT was detected by immunoblot. %surface DAT was calculated by normalizing surface signals to their corresponding total DAT input signals, detected in parallel. Note that slice biotinylation data in the current study were acquired during the course of a previous study, in which we first reported the effect of DAergic Rit2 KD on DAT basal surface levesls[45]. To avoid duplicate reporting basal DAT surface levels from the previous study, we used those vehicle-treated DAT surface signals to determine the magnitude of AMPH's effect on DAT surface expression in their contralateral hemislices. Therefore, data were reported as %change in DAT surface levels, normalized to vehicle-treated hemi-slices, rather than as raw surface levels. There were no statistically significant differences between male and female DAT surface levels, in either eGFP- or shRit2-injected mice. Therefore, male and female data were pooled.

Statistical analysis

All data were analyzed using GraphPad Prism software. Statistical outliers were identified with Grubb's or Rout's outlier tests (α- and Q-value set to 0.05 and 5%, respectively), and were excluded from further analysis. Student's *t* test was used to compare between two groups, and Welch's correction was applied in instances where standard deviations significantly differed from each other (as determined using Bartlett's test). One- or two-way ANOVA were used to compare among more than two experimental groups, and appropriate post-hoc multiple comparisons tests were applied to determine significant differences among experimental groups, as described within each figure legend.

RESULTS

Ric interacts with dDAT and impacts dDAT activity

Although hDAT and Rit2 interact at the plasma membrane, it is unknown whether dDAT and Ric interact in an orthologous fashion. Therefore, we first used live-cell FRET to directly test this possibility in HEK293 cells co-transfected with CFP-dDAT and YFP-Ric. As previously reported, CFP-hDAT/YFP-Rit2 elicited a significant FRET signal over control (Fig. 1A). CFP-dDAT and YFP-Ric likewise elicited a significant FRET signal, which did not significantly differ from the CFP-hDAT/YFP-Rit2 FRET signal ($p=0.53$, Fig. 1A). Given our previous results that indicate an integral role of the DAT-Rit2 interaction in regulating DAT endocytosis, these data strongly suggested that *Drosophila* Ric may similarly regulate dDAT.

Constitutively active Ric increases dDAT function and surface levels

We next used Ric GTPase mutants to test whether Ric activity impacts dDAT-mediated DA uptake kinetics in HEK293T cells co-transfected with dDAT and either vector, wildtype Ric (Ric-WT) constitutively active (CA) Ric (RicQ117L), or dominant negative (DN) Ric (RicS73N) (Fig. 1B). Neither WT ric, nor any of the mutant Rics, significantly affected dDAT K_m values, as compared to vector co-transfected controls (Fig. 1C *top*). Similarly, neither Ric-WT nor RicS73N significantly affected dDAT V_{max} as compared to vector co-transfected cells (Fig. 1C *bottom)*. However, RicQ117L significantly increased dDAT V_{max} (Fig. 1C *bottom*). To test whether increased dDAT V_{max} was due to increased dDAT surface expression, or enhanced substrate turnover rates, we measured dDAT plasma membrane expression by surface biotinylation. dDAT surface levels were significantly increased when co-expressed with RicQ117L, as compared with cells co-transfected with Ric-WT (Fig. 1D), indicating that RicQ117L-mediated increased dDAT V_{max} was likely due to enhanced dDAT surface expression. We further tested whether RicQ117L similarly increases DA uptake *in situ,* as measured by *ex vivo Drosophila* whole brain [^{3}H]DA uptake[49]. *TH-GAL4* flies were crossed to either wildtype (+/+) or *UAS-HA-RicQ117L* flies to drive RicQ117L expression selectively in DA neurons[59], and DA uptake was measured in male progeny (*TH-GAL/UAS-RicQ117L*). RicQ117L overexpression significantly increased DA uptake as compared to control *TH-GAL4/+* flies (Fig. 1E). Together, these data demonstrate that Ric GTPase activity regulates dDAT function in intact fly brains, likely by regulating dDAT surface expression, and suggests that the dDAT/Ric interaction is orthologous to the hDAT/Rit2 interaction.

Manipulating DAergic Ric activity does not impact locomotor activity or total sleep

Given that DA plays a central role in mammalian and *Drosophila* locomotion and sleep[60, 61], and that Ric activity impacts DAT function, we asked whether DAergic Ric activity impacts *Drosophila* basal locomotion and sleep. Using the *TH-GAL4* driver, we overexpressed Ric-WT (*TH-GAL4/UAS-Ric-WT*), DN-RicS73N (*TH-GAL4/UAS-RicS73N*), or CA-RicQ117L (*TH-GAL4/UAS-RicQ117L*) in DA neurons and monitored male fly circadian behavior. Total locomotor activity was not significantly affected in *TH-GAL4/UAS-Ric-WT*, *TH-GAL4/UAS-RicS73N*, or *TH-GAL4/UAS-RicQ117L* flies, compared to *TH-Gal4/+* controls, assessed over either 24 hrs, or specifically during the day or night (Fig. S1). Total sleep time was likewise unaffected, with the exception of Ric-WT overexpression which resulted in a small, but significant, decrease in daytime sleep and increase in nighttime sleep (Fig. S2).

Increasing DAergic Ric activity decreases sleep bout consolidation in a DAT-dependent manner

Sleep and activity bout fragmentation is indicative of reduced sleep consolidation and quality, and can negatively impact health and cognitive function[62, 63]. Therefore, we also analyzed our locomotor data to ask whether DAergic Ric activity impacts the number of sleep episodes. DAergic Ric-WT and DN RicS73N overexpression had no significant impact on overall (24-hr) sleep bout number, although a subtle reduction was observed during the day with WT and at night with RicS73N (Fig. 2). On the other hand, DAergic RicQ117L overexpression led to a significant increase in overall sleep episode number, which was entirely caused by increased daytime sleep fragmentation (Fig. 2). This indicates a specific defect in sleep consolidation caused by DAergic RicQ117L overexpression. RicQ117L-mediated decreased sleep

consolidation may be a result of the increased DA reuptake caused by overexpression, or may be due to a Ric-mediated, DAT-independent mechanism. To discern between these possibilities, we performed epistasis studies in which both driver (*TH-GAL4*) and responder (*UAS-HA-RicQ117L*) transgenes were introduced into the dDAT null background (*fmn; TH-GAL4/UAS-RicQ117L*). We predicted that if any of the locomotor phenotypes caused by RicQ117L expression were DAT-dependent, they would be lost in the DAT null background. In the absence of dDAT, RicQ117L had no effect on sleep bout number as compared to control flies (Fig. 2), indicating that the RicQ117L-mediated sleep phenotype is DAT-dependent, and may be due to the enhanced dDAT function that we observed in intact fly brains (Fig. 1E).

Ric activity impacts amphetamine sensitivity in a DAT-dependent manner

We previously reported that DAergic Rit2 knockdown significantly increases acute cocaine sensitivity in male mice[45]. However, it is not known whether Rit2-mediated changes in psychostimulant sensitivity are mediated by a DAT-dependent, or -independent, mechanism. Given that the dDAT/Ric interaction is similar to hDAT/Rit2 , we leveraged *Drosophila* to test whether 1) Ric activity similarly impacts psychostimulant sensitivity, and 2) Ric-dependent psychostimulant sensitivity is DAT-dependent. Given the inherent challenges in administering cocaine to flies, which must be volatized, we instead assessed AMPH-induced hyperlocomotion via a feeding assay[64]. AMPH elicited significant, dose-dependent, hyperlocomotion in control (*TH-GAL4/+*) flies (Fig. 3A), consistent with previous studies[64]. Surprisingly, RicQ117L expression in DAergic neurons significantly increased fly sensitivity to AMPH, resulting in significant hyperactivity at lower doses than of that required for TH-Gal4/+ controls (Fig. 3B). The increased fly AMPH sensitivity is likely due to RicQ117L effects on DAT surface stability

and function. Alternatively, the RicQ117L mutant could be influencing AMPH sensitivity in a DAT-independent manner. To discern between these possibilities, we again performed an epistasis study, and expressed RicQ117L in the DAT null (*fmn)* background, to test whether the enhanced AMPH sensitivity we observed in the *TH-Gal4/UAS-RicQ117L* flies is DAT-dependent. AMPH did not significantly increase locomotion in *fmn* flies (Fig. 4A), consistent with the known requirement for DAT in AMPH-stimulated locomotion. Moreover, AMPH significantly decreased locomotion in *fmn* flies at the highest AMPH doses tested (Fig. 4A). When RicQ117L was expressed on the *fmn* background, AMPH failed to significantly increase locomotion at any of the doses tested (Fig. 4B), consistent with a DAT-dependent mechanism. The enhanced AMPH sensitivity that RicQ117L imparts may be due to disrupted DAT trafficking, or due to an altered AMPH affinity for dDAT. To discern between these possibilities, we measured the ability of AMPH to inhibit DA uptake in HEK293T cells co-transfected with dDAT and either WT-Ric or RicQ117L. RicQ117L had no effect on the ability of AMPH to inhibit DA uptake (AMPH IC_{50}: WT-Ric: 3.04±1.5μM vs RicQ117L: 4.1±2.3μM, p=0.70, two-tailed Student's t test, n=6-7), suggesting that RicQ117L-enhanced AMPH sensitivity is likely due to Ric-dependent DAT trafficking, and not due to intrinsic changes in the AMPH/DAT interaction.

Rit2 is required for AMPH-mediated DAT internalization in DAergic terminals

In addition to its effects on DA uptake and efflux, AMPH also stimulates robust DAT internalization in cell lines[65], midbrain neurons[66, 67], and striatal DAergic terminals[24]. However, it is unknown whether Rit2 is required for AMPH-stimulated DAT internalization. Given that both Ric and Rit2 impact DAT surface expression and psychostimulant response, we conditionally knocked down Rit2 in mouse DAergic neurons[43, 45], to ask whether Rit2 is required for AMPH-

stimulated DAT endocytosis in dorsal striatum. DAergic Rit2-KD significantly attenuated AMPH-mediated DAT internalization, as compared to striata from mice injected with control AAV particles (Fig. 5). Thus, Rit2 is required for AMPH-stimulated DAT internalization.

DISCUSSION

The present study aimed to investigate how Rit2-dependent DAT trafficking impacts DA-dependent behaviors, using *Drosophila* as a model system. Given our current findings that dDAT and Ric interact, and that DAergic Ric activity impacts DAT activity, surface expression, baseline DA-dependent behaviors, and AMPH sensitivity, it is likely that *Drosophila* is an orthologous model for studying the DAT/Rit2 interaction. Indeed, *Drosophila* has proven a powerful system to investigate AMPH-stimulated DAT efflux mechanisms[15, 47, 48, 64], as well as hDAT coding variants identified in ADHD and ASD[21, 49, 68-70]. In the current study, *Drosophila* genetics enabled us to determine that Ric-mediated changes in behavior are, indeed, DAT-dependent, and not due to another Ric-mediated, DAT-independent mechanism. For instance, Ric activity was also demonstrated to stimulate neurite outgrowth in PC6 cells[71]. Ric binds to calmodulin[29], and *in vivo* RicQ117L overexpression induced ectopic wing vein growth, which was exacerbated by concurrent null mutations in calmodulin[71]. Furthermore, genetic Ric ablation reduced fly viability in response to environmental stress[72]. Interestingly, a pan-neuronal RNAi screen identified Ric as a suppressor of olfactory-associated memory formation[73], which requires DA signaling in the mushroom body[61, 74]. Thus, our observations that DAergic Ric regulates other DA-dependent behaviors via its interaction with DAT are consistent with these reports.

What is the mechanism by which RicQ117L increases dDAT function and surface expression? We previously reported that DAT and Rit2 dissociate at the plasma membrane during PKC-stimulated DAT endocytosis[43, 44], and that the CA Rit2 mutant appeared to lack dissociation from DAT following PKC activation[44]. Moreover, a SERT/DAT chimera, in which the DAT N-terminus was replaced with that of SERT, could not dissociate from Rit2, was resistant to PKC-stimulated endocytosis, and exhibited significantly decreased basal endocytosis[44]. Together these data raise the possibility that RicQ117L may inhibit dDAT/Ric dissociation, likewise stabilizing dDAT at the plasma membrane and increasing dDAT surface levels over time. However, it remains to be determined whether dDAT is subject to either PKC- or AMPH-stimulated endocytosis. Unfortunately, the excessive overexpression inherent in transient transfection is a significant obstacle to testing regulated dDAT trafficking *in vitro*, as we previously reported that regulated DAT trafficking is highly saturable and exquisitely sensitive to DAT expression level[75]. Moreover, the lack of dDAT-specific antibodies is prohibitive to studying dDAT trafficking *in situ.* Thus, additional reagents, and/or tagged fly lines, will be required to test whether dDAT undergoes regulated trafficking, and/or whether Ric activity plays a role in that process. However, the increased DAT surface expression *in vitro* and increased DAT activity *in situ* (Fig. 1) are consistent with a trafficking-mediated effect on DAT surface expression.

How might increased dDAT activity decrease sleep consolidation? Increases in DAT activity would be predicted to decrease extracellular DA half-life, leading to a shorter DA signal length. In addition, enhanced DA reuptake could increase the overall DAergic tissue content. Consistent with this possibility, extracellular DA concentrations are reduced by ~50% in a DAT overexpression transgenic mouse, which expresses ~2.5 times more total DAT protein[76]. This

hypothesis is also consistent with the fact that *DAT*$^{-/-}$ animals display 1) increased extracellular DA[9, 77], and 2) decreased sleep bout number (or increased wake episode length)[13, 14], as well as significantly reduced DAergic tone[9]. However, whether or not DA release and/or overall DA concentrations in the fly brain are altered by RicQ117L expression remain to be tested. dDAT is also required for sleep rebound characterized by increased sleep after mechanical sleep deprivation, indicating a role for dDAT in homeostatic sleep regulation[14]. Further, DAergic RicQ117L expression increased sleep bout frequency during the light phase of activity, but not at night (Fig. 2), suggesting a possible influence of circadian regulation. Extracellular DA concentrations and rates of DA uptake vary over the light/dark cycle, and DAT is required for circadian oscillations in DA release[78]. However, whether DAT is differentially sensitive to Ric-mediated regulation throughout the day remains unknown.

We previously reported that conditionally silencing Rit2 in mouse DA neurons decreased baseline anxiety, but had no effect on overall locomotion, consistent with our current results testing the role of DAergic Ric in *Drosophila* (Fig. S1 and S2). Notably, DAergic Rit2-KD altered acute cocaine responses, increasing cocaine sensitivity in male mice, and decreasing cocaine sensitivity in females[45]. Although cocaine binding to DAT is required for cocaine-induced reward[79], it is unclear whether Rit2-KD alters the cocaine response though DAT. *Drosophila* also exhibit psychostimulant-mediated hyperactivity and preference[64, 80, 81], and dDAT is required for AMPH-dependent hyperlocomotion[15]. Moreover, our current results indicate that DAergic CA Ric increases *Drosophila* sensitivity to AMPH DAT-dependently (Fig. 4), suggesting that DAT surface regulation impacts psychostimulant-induced hyperactivity. Interestingly, Rit2-KD and RicQ117L expression both increased psychostimulant sensitivity, but had opposite effects on

DAT surface expression. These data raise the possibility that disrupting Rit2/Ric GTPase by knockdown and mutant expression may lead to changes in DAT regulation that disrupt DA homeostasis, thereby increasing psychostimulant sensitivity, regardless of the absolute amount of surface DAT. Importantly, AMPH also stimulates DAT internalization in a Rho-dependent manner[67]. However, we do not know whether Rit2 acts with Rho to facilitate AMPH-induced DAT internalization, nor is it clear whether RIt2's role in PKC- or AMPH-induced internalization are at play in the observed enhanced psychostimulant sensitivity. Nevertheless, future studies investigating the *in vivo* impact of regulated DAT trafficking, via leveraging DAT trafficking-dysregulated mutants, will more directly examine the impact of regulated DAT endocytosis on psychostimulant sensitivity and other DA-dependent behaviors.

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References

1. Wise RA. Dopamine, learning and motivation. *Nat Rev Neurosci* 2004; **5**(6)**:** 483-494.

2. Schultz W. Multiple dopamine functions at different time courses. *Annu Rev Neurosci* 2007; **30:** 259-288.

3. Sharma A, Couture J. A review of the pathophysiology, etiology, and treatment of attention-deficit hyperactivity disorder (ADHD). *Ann Pharmacother* 2014; **48**(2)**:** 209-225.

4. Howes OD, McCutcheon R, Owen MJ, Murray RM. The Role of Genes, Stress, and Dopamine in the Development of Schizophrenia. *Biol Psychiatry* 2017; **81**(1)**:** 9-20.

5. Eissa N, Al-Houqani M, Sadeq A, Ojha SK, Sasse A, Sadek B. Current Enlightenment About Etiology and Pharmacological Treatment of Autism Spectrum Disorder. *Front Neurosci* 2018; **12:** 304.

6. Geibl FF, Henrich MT, Oertel WH. Mesencephalic and extramesencephalic dopaminergic systems in Parkinson's disease. *J Neural Transm (Vienna)* 2019.

7. Iversen SD, Iversen LL. Dopamine: 50 years in perspective. *Trends Neurosci* 2007; **30**(5)**:** 188-193.

8. Kristensen AS, Andersen J, Jorgensen TN, Sorensen L, Eriksen J, Loland CJ *et al.* SLC6 neurotransmitter transporters: structure, function, and regulation. *Pharmacol Rev* 2011; **63**(3)**:** 585-640.

9. Gainetdinov RR, Jones SR, Fumagalli F, Wightman RM, Caron MG. Re-evaluation of the role of the dopamine transporter in dopamine system homeostasis. *Brain Res Brain Res Rev* 1998; **26**(2-3)**:** 148-153.

10. Sitte HH, Freissmuth M. Amphetamines, new psychoactive drugs and the monoamine transporter cycle. *Trends Pharmacol Sci* 2015; **36**(1)**:** 41-50.

11. Amara SG, Sonders MS. Neurotransmitter transporters as molecular targets for addictive drugs. *Drug Alcohol Depend* 1998; **51**(1-2)**:** 87-96.

12. Giros B, Jaber M, Jones SR, Wightman RM, Caron MG. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* 1996; **379**(6566)**:** 606-612.

13. Wisor JP, Nishino S, Sora I, Uhl GH, Mignot E, Edgar DM. Dopaminergic role in stimulant-induced wakefulness. *J Neurosci* 2001; **21**(5)**:** 1787-1794.

14. Kume K, Kume S, Park SK, Hirsh J, Jackson FR. Dopamine is a regulator of arousal in the fruit fly. *J Neurosci* 2005; **25**(32)**:** 7377-7384.

15. Pizzo AB, Karam CS, Zhang Y, Yano H, Freyberg RJ, Karam DS *et al.* The membrane raft protein Flotillin-1 is essential in dopamine neurons for amphetamine-induced behavior in Drosophila. *Mol Psychiatry* 2013; **18**(7)**:** 824-833.

16. Grünhage F, Schulze TG, Müller DJ, Lanczik M, Franzek E, Albus M *et al.* Systematic screening for DNA sequence variation in the coding region of the human dopamine transporter gene (DAT1). *Molecular Psychiatry* 2000; **5**(3)**:** 275-282.

17. Mazei-Robison MS, Blakely RD. Expression studies of naturally occurring human dopamine transporter variants identifies a novel state of transporter inactivation associated with Val382Ala. *Neuropharmacology* 2005; **49**(6)**:** 737-749.

18. Sakrikar D, Mazei-Robison MS, Mergy MA, Richtand NW, Han Q, Hamilton PJ *et al.* Attention deficit/hyperactivity disorder-derived coding variation in the dopamine

transporter disrupts microdomain targeting and trafficking regulation. *J Neurosci* 2012; **32**(16)**:** 5385-5397.

19. Bowton E, Saunders C, Reddy IA, Campbell NG, Hamilton PJ, Henry LK *et al.* SLC6A3 coding variant Ala559Val found in two autism probands alters dopamine transporter function and trafficking. *Transl Psychiatry* 2014; **4:** e464.

20. Kurian MA, Zhen J, Cheng SY, Li Y, Mordekar SR, Jardine P *et al.* Homozygous loss-of-function mutations in the gene encoding the dopamine transporter are associated with infantile parkinsonism-dystonia. *J Clin Invest* 2009; **119**(6)**:** 1595-1603.

21. Hamilton PJ, Campbell NG, Sharma S, Erreger K, Herborg Hansen F, Saunders C *et al.* De novo mutation in the dopamine transporter gene associates dopamine dysfunction with autism spectrum disorder. *Mol Psychiatry* 2013; **18**(12)**:** 1315-1323.

22. Bermingham DP, Blakely RD. Kinase-dependent Regulation of Monoamine Neurotransmitter Transporters. *Pharmacol Rev* 2016; **68**(4)**:** 888-953.

23. Vaughan RA, Foster JD. Mechanisms of dopamine transporter regulation in normal and disease states. *Trends Pharmacol Sci* 2013; **34**(9)**:** 489-496.

24. Fagan RR, Kearney PJ, Melikian HE. In Situ Regulated Dopamine Transporter Trafficking: There's No Place Like Home. *Neurochem Res* 2020; **45**(6)**:** 1335-1343.

25. Melikian HE, Buckley KM. Membrane trafficking regulates the activity of the human dopamine transporter. *J Neurosci* 1999; **19**(18)**:** 7699-7710.

26. Daniels GM, Amara SG. Regulated trafficking of the human dopamine transporter. Clathrin-mediated internalization and lysosomal degradation in response to phorbol esters. *J Biol Chem* 1999; **274**(50)**:** 35794-35801.

27. Gabriel LR, Wu S, Kearney P, Bellve KD, Standley C, Fogarty KE *et al.* Dopamine transporter endocytic trafficking in striatal dopaminergic neurons: differential dependence on dynamin and the actin cytoskeleton. *J Neurosci* 2013; **33**(45)**:** 17836-17846.

28. Lee CH, Della NG, Chew CE, Zack DJ. Rin, a neuron-specific and calmodulin-binding small G-protein, and Rit define a novel subfamily of ras proteins. *J Neurosci* 1996; **16**(21)**:** 6784-6794.

29. Wes PD, Yu M, Montell C. RIC, a calmodulin-binding Ras-like GTPase. *EMBO J* 1996; **15**(21)**:** 5839-5848.

30. Spencer ML, Shao H, Tucker HM, Andres DA. Nerve growth factor-dependent activation of the small GTPase Rin. *J Biol Chem* 2002; **277**(20)**:** 17605-17615.

31. Zhou Q, Li J, Wang H, Yin Y, Zhou J. Identification of nigral dopaminergic neuron-enriched genes in adult rats. *Neurobiol Aging* 2011; **32**(2)**:** 313-326.

32. Cai W, Rudolph JL, Sengoku T, Andres DA. Rit GTPase regulates a p38 MAPK-dependent neuronal survival pathway. *Neurosci Lett* 2012; **531**(2)**:** 125-130.

33. Shi GX, Cai W, Andres DA. Rit subfamily small GTPases: regulators in neuronal differentiation and survival. *Cell Signal* 2013; **25**(10)**:** 2060-2068.

34. Bossers K, Meerhoff G, Balesar R, van Dongen JW, Kruse CG, Swaab DF *et al.* Analysis of gene expression in Parkinson's disease: possible involvement of neurotrophic support and axon guidance in dopaminergic cell death. *Brain Pathol* 2009; **19**(1)**:** 91-107.

35. Pankratz N, Beecham GW, DeStefano AL, Dawson TM, Doheny KF, Factor SA *et al.* Meta-analysis of Parkinson's disease: identification of a novel locus, RIT2. *Ann Neurol* 2012; **71**(3)**:** 370-384.

36. Glessner JT, Reilly MP, Kim CE, Takahashi N, Albano A, Hou C *et al.* Strong synaptic transmission impact by copy number variations in schizophrenia. *Proc Natl Acad Sci U S A* 2010; **107**(23)**:** 10584-10589.

37. Bouquillon S, Andrieux J, Landais E, Duban-Bedu B, Boidein F, Lenne B *et al.* A 5.3Mb deletion in chromosome 18q12.3 as the smallest region of overlap in two patients with expressive speech delay. *Eur J Med Genet* 2011; **54**(2)**:** 194-197.

38. Latourelle JC, Dumitriu A, Hadzi TC, Beach TG, Myers RH. Evaluation of Parkinson disease risk variants as expression-QTLs. *PLoS One* 2012; **7**(10)**:** e46199.

39. Emamalizadeh B, Movafagh A, Akbari M, Kazeminasab S, Fazeli A, Motallebi M *et al.* RIT2, a susceptibility gene for Parkinson's disease in Iranian population. *Neurobiol Aging* 2014; **35**(12)**:** e27-e28.

40. Liu X, Shimada T, Otowa T, Wu YY, Kawamura Y, Tochigi M *et al.* Genome-wide Association Study of Autism Spectrum Disorder in the East Asian Populations. *Autism Res* 2016; **9**(3)**:** 340-349.

41. Emamalizadeh B, Jamshidi J, Movafagh A, Ohadi M, Khaniani MS, Kazeminasab S *et al.* RIT2 Polymorphisms: Is There a Differential Association? *Mol Neurobiol* 2017; **54**(3)**:** 2234-2240.

42. Hamedani SY, Gharesouran J, Noroozi R, Sayad A, Omrani MD, Mir A *et al.* Ras-like without CAAX 2 (RIT2): a susceptibility gene for autism spectrum disorder. *Metab Brain Dis* 2017; **32**(3)**:** 751-755.

43. Fagan RR, Kearney PJ, Sweeney CG, Luethi D, Uiterkamp FES, Schicker K *et al.* Dopamine transporter trafficking and Rit2 GTPase: Mechanism of action and in vivo impact. *Journal of Biological Chemistry* 2020.

44. Navaroli DM, Stevens ZH, Uzelac Z, Gabriel L, King MJ, Lifshitz LM *et al.* The plasma membrane-associated GTPase Rin interacts with the dopamine transporter and is required for protein kinase C-regulated dopamine transporter trafficking. *J Neurosci* 2011; **31**(39)**:** 13758-13770.

45. Sweeney CG, Kearney PJ, Fagan RR, Smith LA, Bolden NC, Zhao-Shea R *et al.* Conditional, inducible gene silencing in dopamine neurons reveals a sex-specific role for Rit2 GTPase in acute cocaine response and striatal function. *Neuropsychopharmacology* 2020; **45**(2)**:** 384-393.

46. Martin CA, Krantz DE. Drosophila melanogaster as a genetic model system to study neurotransmitter transporters. *Neurochem Int* 2014; **73:** 71-88.

47. Pizzo AB, Karam CS, Zhang Y, Ma CL, McCabe BD, Javitch JA. Amphetamine-induced behavior requires CaMKII-dependent dopamine transporter phosphorylation. *Mol Psychiatry* 2014; **19**(3)**:** 279-281.

48. Hamilton PJ, Belovich AN, Khelashvili G, Saunders C, Erreger K, Javitch JA *et al.* PIP2 regulates psychostimulant behaviors through its interaction with a membrane protein. *Nat Chem Biol* 2014; **10**(7)**:** 582-589.

49. Cartier E, Hamilton PJ, Belovich AN, Shekar A, Campbell NG, Saunders C *et al.* Rare autism-associated variants implicate syntaxin 1 (STX1 R26Q) phosphorylation and the dopamine transporter (hDAT R51W) in dopamine neurotransmission and behaviors. *EBioMedicine* 2015; **2**(2)**:** 135-146.

50. Foley LE, Ling J, Joshi R, Evantal N, Kadener S, Emery P. Drosophila PSI controls circadian period and the phase of circadian behavior under temperature cycle via tim splicing. *Elife* 2019; **8**.

51. Zhang Y, Lamba P, Guo P, Emery P. miR-124 Regulates the Phase of Drosophila Circadian Locomotor Behavior. *J Neurosci* 2016; **36**(6)**:** 2007-2013.

52. Farley JE, Burdett TC, Barria R, Neukomm LJ, Kenna KP, Landers JE *et al.* Transcription factor Pebbled/RREB1 regulates injury-induced axon degeneration. *Proc Natl Acad Sci U S A* 2018; **115**(6)**:** 1358-1363.

53. Smith GA, Lin TH, Sheehan AE, Van der Goes van Naters W, Neukomm LJ, Graves HK *et al.* Glutathione S-Transferase Regulates Mitochondrial Populations in Axons through Increased Glutathione Oxidation. *Neuron* 2019; **103**(1)**:** 52-65 e56.

54. Parisky KM, Agosto Rivera JL, Donelson NC, Kotecha S, Griffith LC. Reorganization of Sleep by Temperature in Drosophila Requires Light, the Homeostat, and the Circadian Clock. *Curr Biol* 2016; **26**(7)**:** 882-892.

55. Donelson NC, Kim EZ, Slawson JB, Vecsey CG, Huber R, Griffith LC. High-resolution positional tracking for long-term analysis of Drosophila sleep and locomotion using the "tracker" program. *PLoS One* 2012; **7**(5)**:** e37250.

56. Sweeney CG, Tremblay BP, Stockner T, Sitte HH, Melikian HE. Dopamine Transporter Amino and Carboxyl Termini Synergistically Contribute to Substrate and Inhibitor Affinities. *J Biol Chem* 2017; **292**(4)**:** 1302-1309.

57. Wu S, Bellve KD, Fogarty KE, Melikian HE. Ack1 is a dopamine transporter endocytic brake that rescues a trafficking-dysregulated ADHD coding variant. *Proc Natl Acad Sci U S A* 2015; **112**(50)**:** 15480-15485.

58. Porzgen P, Park SK, Hirsh J, Sonders MS, Amara SG. The antidepressant-sensitive dopamine transporter in Drosophila melanogaster: a primordial carrier for catecholamines. *Mol Pharmacol* 2001; **59**(1)**:** 83-95.

59. Friggi-Grelin F, Coulom H, Meller M, Gomez D, Hirsh J, Birman S. Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J Neurobiol* 2003; **54**(4)**:** 618-627.

60. Ueno T, Tomita J, Tanimoto H, Endo K, Ito K, Kume S *et al.* Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. *Nat Neurosci* 2012; **15**(11)**:** 1516-1523.

61. Riemensperger T, Isabel G, Coulom H, Neuser K, Seugnet L, Kume K *et al.* Behavioral consequences of dopamine deficiency in the Drosophila central nervous system. *Proc Natl Acad Sci U S A* 2011; **108**(2)**:** 834-839.

62. Koh K, Evans JM, Hendricks JC, Sehgal A. A Drosophila model for age-associated changes in sleep:wake cycles. *Proc Natl Acad Sci U S A* 2006; **103**(37)**:** 13843-13847.

63. Medic G, Wille M, Hemels ME. Short- and long-term health consequences of sleep disruption. *Nat Sci Sleep* 2017; **9:** 151-161.

64. Belovich AN, Aguilar JI, Mabry SJ, Cheng MH, Zanella D, Hamilton PJ *et al.* A network of phosphatidylinositol (4,5)-bisphosphate (PIP2) binding sites on the dopamine transporter regulates amphetamine behavior in Drosophila Melanogaster. *Mol Psychiatry* 2019.

65. Saunders C, Ferrer JV, Shi L, Chen J, Merrill G, Lamb ME *et al.* Amphetamine-induced loss of human dopamine transporter activity: an internalization-dependent and cocaine-sensitive mechanism. *Proc Natl Acad Sci U S A* 2000; **97**(12)**:** 6850-6855.

66. Hong WC, Amara SG. Differential targeting of the dopamine transporter to recycling or degradative pathways during amphetamine- or PKC-regulated endocytosis in dopamine neurons. *FASEB J* 2013; **27**(8)**:** 2995-3007.

67. Wheeler DS, Underhill SM, Stolz DB, Murdoch GH, Thiels E, Romero G *et al.* Amphetamine activates Rho GTPase signaling to mediate dopamine transporter internalization and acute behavioral effects of amphetamine. *Proc Natl Acad Sci U S A* 2015; **112**(51)**:** E7138-7147.

68. Campbell NG, Shekar A, Aguilar JI, Peng D, Navratna V, Yang D *et al.* Structural, functional, and behavioral insights of dopamine dysfunction revealed by a deletion in SLC6A3. *Proc Natl Acad Sci U S A* 2019; **116**(9)**:** 3853-3862.

69. DiCarlo GE, Aguilar JI, Matthies HJ, Harrison FE, Bundschuh KE, West A *et al.* Autism-linked dopamine transporter mutation alters striatal dopamine neurotransmission and dopamine-dependent behaviors. *J Clin Invest* 2019; **129**(8)**:** 3407-3419.

70. Hamilton PJ, Campbell NG, Sharma S, Erreger K, Hansen FH, Saunders C *et al.* Drosophila melanogaster: a novel animal model for the behavioral characterization of autism-associated mutations in the dopamine transporter gene. *Mol Psychiatry* 2013; **18**(12)**:** 1235.

71. Harrison SM, Rudolph JL, Spencer ML, Wes PD, Montell C, Andres DA *et al.* Activated RIC, a small GTPase, genetically interacts with the Ras pathway and calmodulin during Drosophila development. *Dev Dyn* 2005; **232**(3)**:** 817-826.

72. Cai W, Rudolph JL, Harrison SM, Jin L, Frantz AL, Harrison DA *et al.* An evolutionarily conserved Rit GTPase-p38 MAPK signaling pathway mediates oxidative stress resistance. *Mol Biol Cell* 2011; **22**(17)**:** 3231-3241.

73. Walkinshaw E, Gai Y, Farkas C, Richter D, Nicholas E, Keleman K *et al.* Identification of genes that promote or inhibit olfactory memory formation in Drosophila. *Genetics* 2015; **199**(4)**:** 1173-1182.

74. Jacob PF, Waddell S. Spaced Training Forms Complementary Long-Term Memories of Opposite Valence in Drosophila. *Neuron* 2020; **106**(6)**:** 977-991 e974.

75. Loder MK, Melikian HE. The dopamine transporter constitutively internalizes and recycles in a protein kinase C-regulated manner in stably transfected PC12 cell lines. *J Biol Chem* 2003; **278**(24)**:** 22168-22174.

76. Salahpour A, Ramsey AJ, Medvedev IO, Kile B, Sotnikova TD, Holmstrand E *et al.* Increased amphetamine-induced hyperactivity and reward in mice overexpressing the dopamine transporter. *Proc Natl Acad Sci U S A* 2008; **105**(11)**:** 4405-4410.

77. Makos MA, Kim YC, Han KA, Heien ML, Ewing AG. In vivo electrochemical measurements of exogenously applied dopamine in Drosophila melanogaster. *Anal Chem* 2009; **81**(5)**:** 1848-1854.

78. Ferris MJ, Espana RA, Locke JL, Konstantopoulos JK, Rose JH, Chen R *et al.* Dopamine transporters govern diurnal variation in extracellular dopamine tone. *Proc Natl Acad Sci U S A* 2014; **111**(26)**:** E2751-2759.

79. Chen R, Tilley MR, Wei H, Zhou F, Zhou FM, Ching S *et al.* Abolished cocaine reward in mice with a cocaine-insensitive dopamine transporter. *Proc Natl Acad Sci U S A* 2006; **103**(24)**:** 9333-9338.

80. Andretic R, van Swinderen B, Greenspan RJ. Dopaminergic modulation of arousal in Drosophila. *Curr Biol* 2005; **15**(13)**:** 1165-1175.

81. Bainton RJ, Tsai LT, Singh CM, Moore MS, Neckameyer WS, Heberlein U. Dopamine modulates acute responses to cocaine, nicotine and ethanol in Drosophila. *Curr Biol* 2000; **10**(4)**:** 187-194.

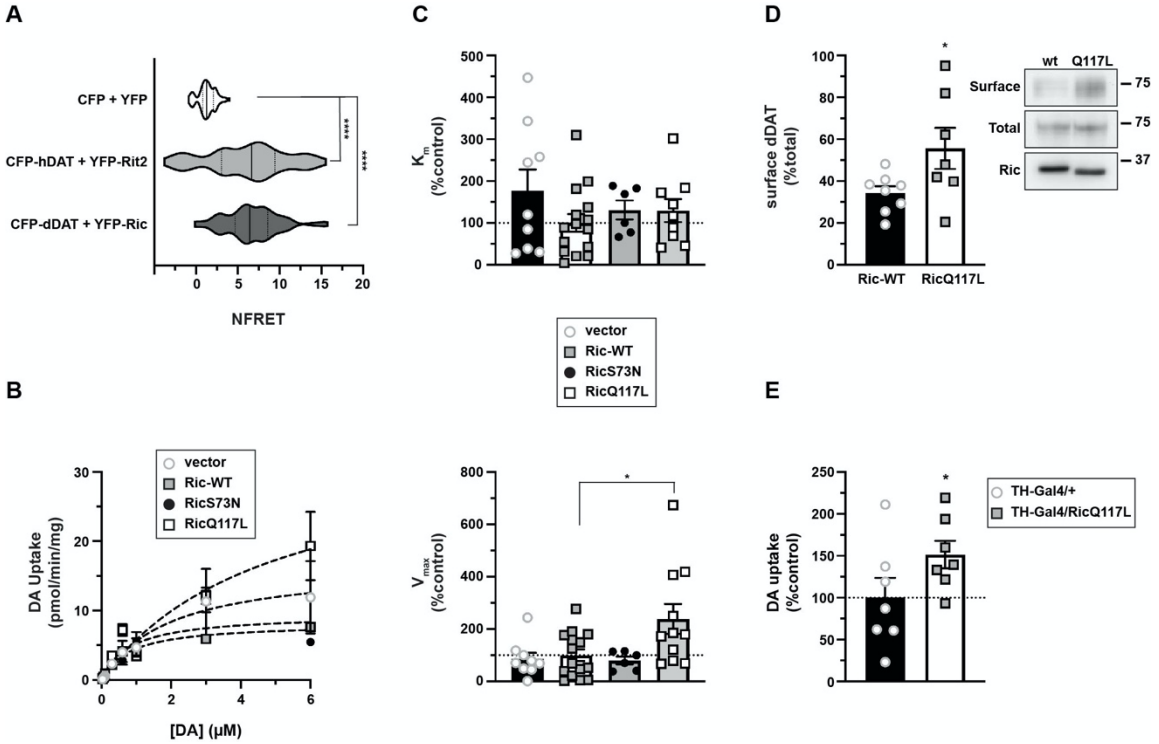

Figure 1: Ric interacts with dDAT and RicQ117L increases dDAT function and surface expression. A. *FRET microscopy*. HEK293 cells were transfected with the indicated constructs and live cell FRET microscopy was performed as described in *Methods*. A significant FRET signal was detected by Kruskal-Wallis test ($p<0.0001$, n=45-76 cells) compared to CFP/YFP controls in all FRET pairs (Dunn's multiple comparisons test): CFP-hDAT/YFP-Rit2 (****$p<0.0001$) and CFP-dDAT/YFP-Ric (****$p<0.0001$). CFP-hDAT/YFP-Rit2 and CFP-dDAT/YFP-Ric were not significantly different ($p=0.53$). **B.** *DA uptake kinetics*. HEK-293T cells were co-transfected with HA-dDAT, and either pcDNA3.1(+) (vector), HA-Ric-WT, HA-RicQ117L, or HA-RicS73N and [^{3}H]DA uptake kinetics were measured as described in *Methods*. **B.** Representative DA transport curves. **C.** *Average DA uptake kinetic values*. K_m (t*op*) and V_{max} (*bottom*) are expressed as a percentage of Ric-WT controls, normalized within each independent experiment. Neither WT nor Ric mutants significantly affected dDAT K_m ($p=0.36$, one-way ANOVA, $F_{(3, 35)} = 2.428$). HA-RicQ117L significantly increased dDAT Vmax as compared to WT-Ric controls (one-way ANOVA: $F_{(3, 37)} = 2.474$, $p=0.01$; *$p=0.01$, Dunnett's multiple comparisons test, n=6-15). **D**. *Surface biotinylation*. HEK293T cells were co-transfected with HA-dDAT, and either HA-WT-Ric or HA-RicQ117L, and dDAT surface expression was measured by surface biotinylation as described in *Methods*. *Inset:* Representative immunoblots. Average surface dDAT values expressed as %total dDAT (*$p=0.038$, one-tailed Student's *t* test with Welch's correction, n=7-8). **E.** *Ex vivo whole brain [^{3}H]DA uptake*. TH-Gal4 flies were crossed to +/+ (*TH-Gal4/*+), or UAS-RicQ117L (*TH-Gal4/UAS-RicQ117L*) flies. DA uptake was measured from dissected brains as described in *Methods*. DA uptake is expressed as %TH-Gal4/+ values (*$p=0.04$, one-tailed Student's t test, n=7).

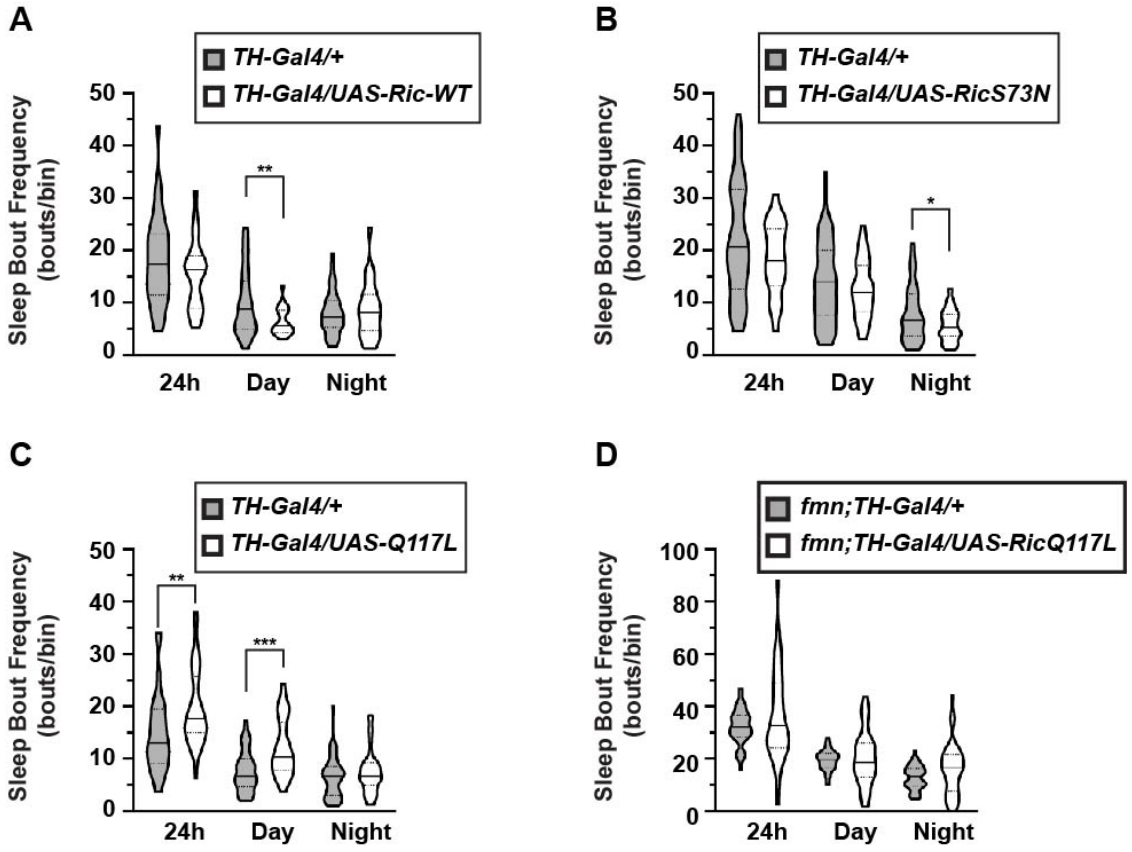

Figure 2. DAergic Ric activity modulates sleep bout frequency in a dDAT-dependent manner. *TH-Gal4* and *Ric-WT, UAS-RicS73N,* or *UAS-RicQ117L* flies were crossed and the number of sleep episodes in male progeny were counted for bins of 24h, daytime (12h lights-on), and nighttime (12h lights-off), and data were analyzed as described above. Ric-WT (**A**) had no effect on sleep bout number over 24h or at night, but significantly decreased sleep frequency during the daytime (**p=0.003, two-tailed Student's *t* test with Welch's correction, n=24-38). RicS73N (**B**) had no effect on sleep bout number over 24h or during the day, but significantly decreased sleep frequency during at night (*p=0.02, two-tailed Student's *t* test with Welch's correction, n=41-47). RicQ117L (**C**) significantly increased sleep bouts during the 24h bin (**p=0.005, two-tailed Student's *t* test, n=36-44) and during the day (***p=0.0001, two-tailed Student's *t* test with Welch's correction), but did affect sleep bout numbers at night. **D**. *Epistasis studies: TH-Gal4* and *UAS-RicQ117L* flies were crossed onto the dDAT-null background (*fmn*) and locomotor activity and sleep were measured in male progeny (*fmn; TH-Gal4/UAS-RicQ117L*). RicQ117L expression on the dDAT-null background did not affect the number of sleep episodes compared to *fmn; TH-Gal4/+* controls (24h: p=0.19; day: p=0.61; night: p=0.087, two-tailed Student's *t* test with Welch's correction, n=31-47).

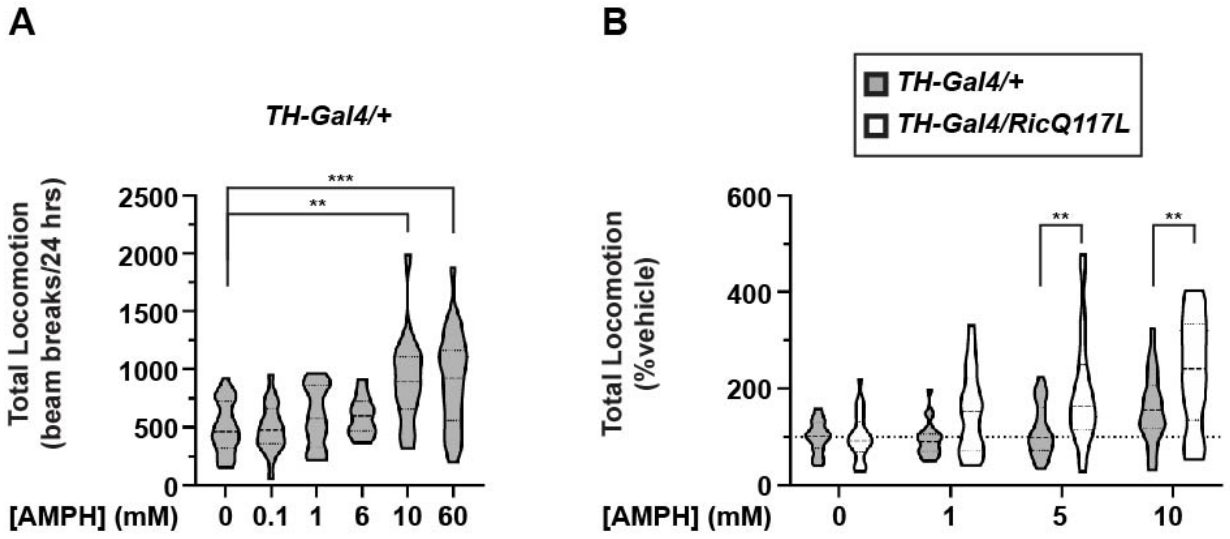

Figure 3. DAergic RicQ117L increases AMPH sensitivity in *Drosophila*. *Locomotor response to AMPH feeding.* (**A**) *TH-Gal4* flies were crossed to +/+ controls, and locomotor activity was measured in progeny for 24h ± the indicated AMPH doses, as described in *Methods*. AMPH significantly increased locomotion at 10mM and 60mM doses (Kruskal-Wallis test, $p<0.0001$; **$p=0.001$, ***$p=0.0004$, Dunn's multiple comparisons test, n=11-28). (**B**) *TH-Gal4/UAS-RicQ117L response to AMPH*, *TH-Gal4* flies were crossed to +/+ or *UAS-RicQ117L* and AMPH-dependent locomotion was measured in progeny. Data are presented as %response of vehicle-treated flies, measured in parallel. AMPH induced significantly higher locomotion in *TH-Gal4/UAS-RicQ117L* flies than *TH-Gal4/+* flies at 5mM and 10mM AMPH (two-way ANOVA: Interaction: $F_{(3, 168)} = 2.86$, *$p=0.04$; Dose: $F_{(3, 168)} = 13.48$, ****$p<0.0001$; Genotype: $F_{(1, 168)} = 22.22$, ****$p<0.0001$; 5mM AMPH: **$p=0.001$, 10mM AMPH: **$p=0.003$, Sidak's multiple comparisons test, n=16-27).

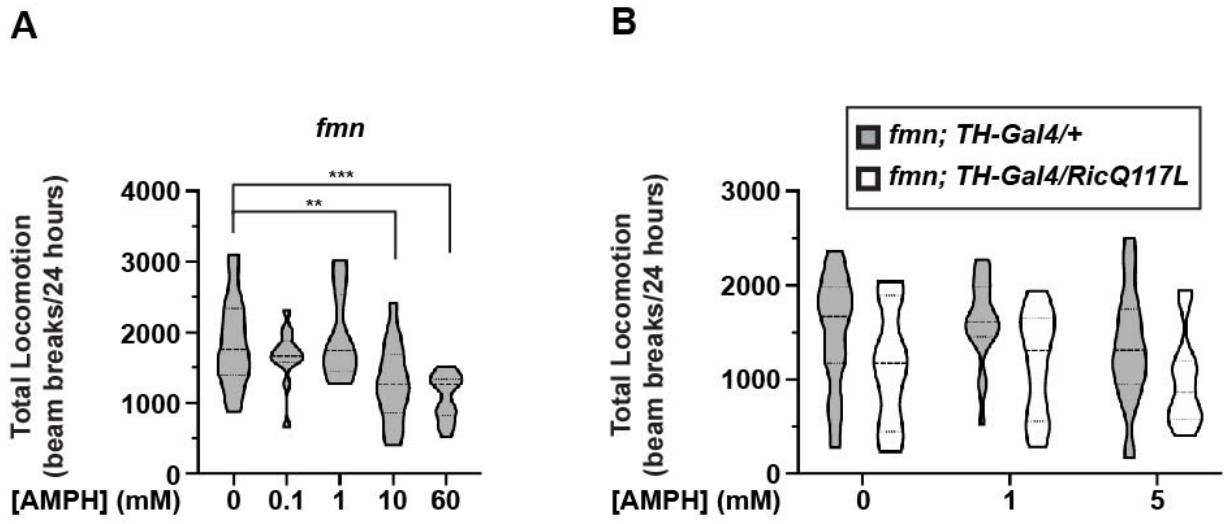

Figure 4. RicQ117L-enhanced AMPH sensitivity requires dDAT expression. *Locomotor response to AMPH feeding.* **(A)** *fmn fly response to AMPH*. AMPH did not significantly increase *fmn* locomotion, and significantly decreased locomotion at high doses as compared to vehicle-fed controls (one-way ANOVA $F_{(4, 73)} = 1.66$, $p<0.0001$; **p=0.007, ***p=0.0003, Dunnett's multiple comparisons test, n=13-21). **(B)** *TH-Gal4* and *UAS-RicQ117L* flies were crossed onto *fmn* background and AMPH-dependent locomotion was measured in male progeny (*fmn; TH-Gal4/UAS-RicQ117L*). AMPH did not cause an interaction, or an effect of dose. There was a significant effect of genotype (two-way ANOVA: Interaction: $F_{(2, 95)} = 0.057$, p=0.95; Dose: $F_{(2, 95)} = 1.43$, p=0.25; Genotype: $F_{(1, 95)} = 10.43$, **p=0.002, n=10-24). Sidak's multiple comparisons test did not detect any significant differences between the genotypes at either vehicle (p=0.24, 1mM (p=0.12), or 5mM (p=0.21) feeding conditions.

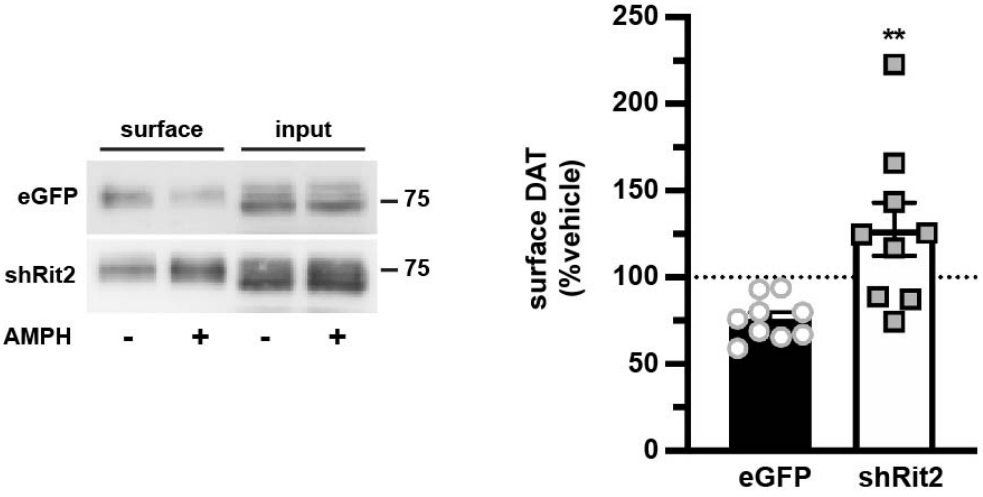

Figure 5. Rit2 is required for AMPH-stimulated DAT internalization in mouse striatum. *Ex vivo striatal slice biotinylation.* *Pitx3*$^{IRES2\text{-}tTA}$ VTA were injected with AAV9 viral particles encoding either eGFP or shRit2, and acute striatal slices were prepared 4-6 weeks post-injection, as described in *Methods.* Hemi-slices were treated ±10μM AMPH, 30 min, 37°C, and surface DAT was isolated by biotinylation. *Left:* Representative DAT immunoblot. *Right:* Average surface DAT values, expressed as %vehicle-treated hemislice. (**p=0.005, one-tailed Student's t test with Welch's correction, n=9).

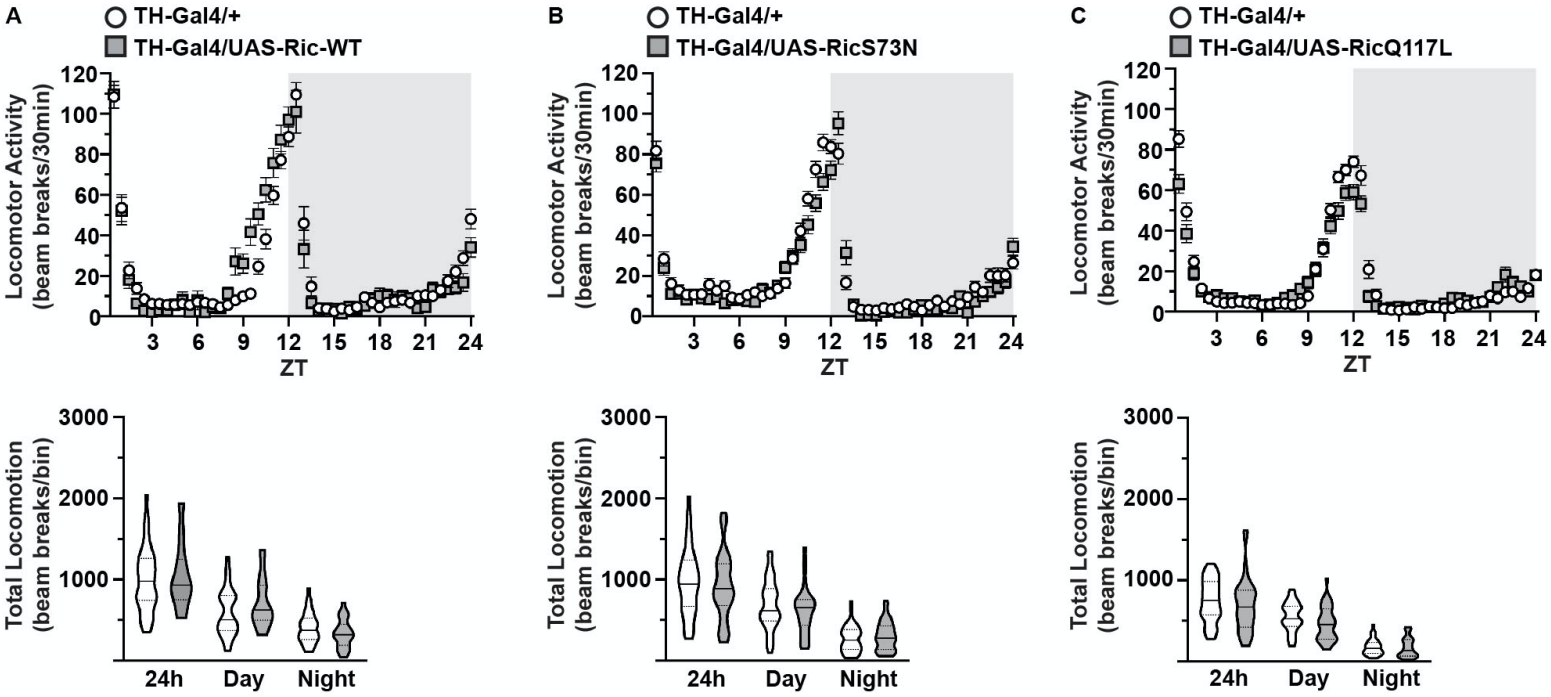

Figure S1. Ric activity does not alter total *Drosophila* locomotor activity. *TH-Gal4* and *UAS-Ric-WT*, *UAS-RicS73N*, or *UAS-RicQ117L* flies were crossed and locomotor activity was measured in progeny as the total number of beam breaks during 24h, day (12h lights-on), and night (12h lights-off) bins. Data were analyzed by two-tailed student's *t* test or Welch's correction (as determined using Bartlett's test) between *TH-Gal4/+* control and experimental animals. Neither Ric-WT (**A**), RicS73N (**B**), nor RicQ117L (**C**) had an effect on total activity counts during the 24h, day, or night bins (3-6 independent experiments, n=24-47 flies/genotype).

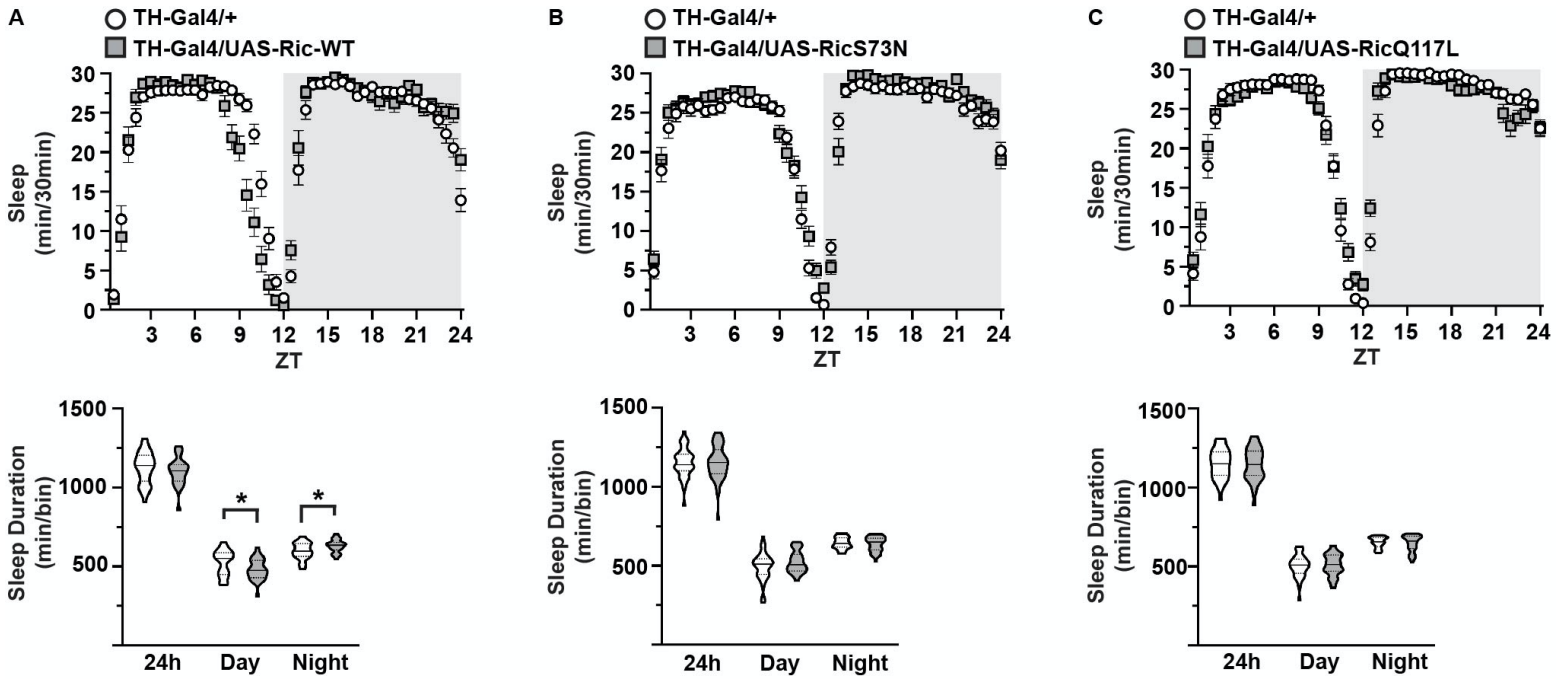

Figure S2. Ric activity does not alter total *Drosophila* sleep.
TH-Gal4 and *UAS-Ric-WT*, *UAS-RicS73N*, or *UAS-RicQ117L* flies were crossed and total sleep was measured in progeny as the minutes spent sleeping during 24h, day (12h lights-on), and night (12h lights-off) bins. Data were analyzed by two-tailed student's *t* test or Welch's correction (as determined using Bartlett's test) between *TH-Gal4/+* control and experimental animals. Ric-WT (**A**) had no effect on total sleep over the 24h period, but significantly decreased total sleep during the day (*p=0.028, n=24-38), and increased sleep during the night (*p=0.02, n=24-38). RicS73N (**B**) and RicQ117L (**C**) had no effect on total sleep in either the 24h, day, or night bins (3-6 independent experiments, n=24-47 flies/genotype).