© 2014. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2014) 217, 4091-4098 doi:10.1242/jeb.110098

RESEARCH ARTICLE

Targeted inactivation of the rickets receptor in muscle compromises *Drosophila* viability

Benjamin N. Harwood[1,2], Isabelle Draper[1,*] and Alan S. Kopin[1,2,*]

ABSTRACT

Bursicon is a hormone that modulates wing expansion, cuticle hardening and melanization in *Drosophila melanogaster*. Bursicon activity is mediated through its cognate G protein-coupled receptor (GPCR), rickets. We have developed a membrane-tethered bursicon construct that enables spatial modulation of rickets-mediated physiology in transgenic flies. Ubiquitous expression of tethered bursicon throughout development results in arrest at the pupal stage. The few organisms that eclose fail to undergo wing expansion. These phenotypes suggest that expression of tethered bursicon inhibits rickets-mediated function. Consistent with this hypothesis, we show *in vitro* that sustained stimulation of rickets by tethered bursicon leads to receptor desensitization. Furthermore, tissue-specific expression of the tethered bursicon inhibitor unraveled a critical role for rickets in a subset of adult muscles. Taken together, our findings highlight the utility of membrane-tethered inhibitors as important genetic/pharmacological tools to dissect the tissue-specific roles of GPCRs *in vivo*.

KEY WORDS: Bursicon, *Drosophila melanogaster*, G protein-coupled receptor, Membrane-tethered ligand, Rickets

INTRODUCTION

Bursicon is a heterodimeric cystine-knot protein required for wing expansion and cuticle hardening in a variety of insects. Early studies performed in blowflies and cockroaches showed that a hormone of unknown molecular identity released from the central nervous system was important for tanning and wing expansion (Fraenkel and Hsiao, 1962; Fraenkel et al., 1966). Approximately four decades later, the molecular identity of bursicon and its cognate receptor, rickets, was obtained in *Drosophila melanogaster* (Dewey et al., 2004; Luo et al., 2005). Rickets [rk or dLGR2 (*Drosophila* leucine-rich repeat receptor 2)] is a member of the leucine-rich repeat-containing subfamily of G protein-coupled receptors (GPCRs) (Van Loy et al., 2008), which is expressed both in the CNS and in the periphery (Diao and White, 2012). Previously, we have shown that a membrane-anchored single subunit fusion construct of the bursicon heterodimer (CFP-tBur-β-α) can activate rk *in vitro* in a concentration-dependent manner (Harwood et al., 2013). Membrane-tethered ligands (MTLs) are cDNA constructs that express genetically encoded peptide hormones anchored to a transmembrane domain via a protein linker. The generation of this complex bursicon heterodimeric tethered construct was done as part of an ongoing effort to sequentially develop a broad range of MTLs that selectively activate either insect or mammalian GPCRs (Choi et al., 2009; Fortin et al., 2011; Fortin et al., 2009).

A major advantage of using MTL technology *in vivo* is that it enables activation of receptors in a targeted tissue without the confounding effects of soluble ligand diffusion. The transgenic Gal4/UAS (upstream activation sequence) system in conjunction with membrane-tethered bursicon offers an excellent model system in which to better understand the tissue dependence of rk-mediated signaling. In *Drosophila*, wing expansion occurs within 1 h of eclosion. A tightly choreographed motor program is required for wing expansion and cuticle hardening. This series of events coincides with a biphasic release of bursicon from a subset of crustacean cardioactive peptide (CCAP)-positive neurosecretory cells. Bursicon is first released from the subesphogeal ganglion, followed by secretion from the abdominal ganglion into the hemolymph. The *Drosophila* circulatory system then disperses bursicon throughout the organism (Peabody et al., 2008; Peabody et al., 2009). While most studies have focused on the location of bursicon release, much less research has addressed the importance of tissue-selective rk activation (Honegger et al., 2008).

Previously, it was thought that rk was only required following eclosion, as a trigger for wing expansion and cuticle hardening. This postulate was based on data from two fly stocks, Rk^1 and Rk^4, which were thought to be receptor nulls (Baker and Truman, 2002). However, a more recent study has shown that these flies are hypomorphs. In fact, global knockdown of rk *in vivo* using RNAi results in developmental arrest, rather than just impaired wing expansion, melanization and cuticle hardening (Loveall and Deitcher, 2010). A recent study also showed that deletion of the bursicon β-subunit results in significant lethality throughout pupariation, specifically during ecdysis (Lahr et al., 2012; Loveall and Deitcher, 2010).

There has been no comprehensive study that has specifically examined what tissues require rk for proper development. A previous study, which utilized a green fluorescent protein (GFP) reporter, demonstrated rk expression in the epidermis (Diao and White, 2012). More detailed analysis of rk transcript levels as revealed by Fly Atlas and RNA-Seq studies have shown that this receptor is expressed at low levels throughout development (Chintapalli et al., 2007; Graveley et al., 2011; Robinson et al., 2013). However, which tissues or cells require rk expression for proper development remains an unexplored area of inquiry.

In our study, the bursicon MTL CFP-tBur-β-α offered a novel approach to investigate the tissue-specific requirements of rk. The use of tissue-specific Gal4 drivers to target expression of the fused heterodimer provided a means to selectively modulate rk without the confounding effects of soluble ligand diffusion. Parallel studies using rk RNAi transgenic flies enabled a complementary approach to confirm conclusions drawn through the use of the membrane-tethered ligand.

[1]Tufts Medical Center, Molecular Cardiology Research Institute, Molecular Pharmacology Research Center, 800 Washington St, Box 7703, Boston, MA 02111, USA. [2]Program in Genetics, Sackler School of Graduate Biomedical Sciences, Tufts University, 145 Harrison Avenue, Boston, MA 02111, USA.

*Authors for correspondence (idraper@tuftsmedicalcenter.org; akopin@tuftsmedicalcenter.org)

Received 25 June 2014; Accepted 23 September 2014

The Journal of Experimental Biology

The Journal of Experimental Biology (2014) doi:10.1242/jeb.110098

List of abbreviations

AMP	adult muscle precursor
Ap-Gal4	apterous-Gal4
Bur	bursicon
CCAP	crustacean cardioactive peptide
CFP-tBur-β-α	cherry fluorescent protein membrane-tethered bursicon
CRE	cAMP response element
Cy	Curly
GPCR	G protein-coupled receptor
HOW-Gal4	held out wing-Gal4
IFM	indirect flight muscle
LGR	leucine-rich repeat-containing G protein-coupled receptor
MTL	membrane-tethered ligand
rk	rickets
sBur	soluble bursicon
Tb	Tubby^{1}
tBur	membrane-tethered bursicon
UAS	upstream activation sequence
β2AR	β adrenergic receptor 2

Prior investigations by our group have shown that a membrane-tethered agonist can trigger long-term receptor activation in flies (Choi et al., 2009). In the current study, we illustrate that there is an alternative potential consequence that may result from membrane-tethered ligand expression *in vivo.* Specifically, the bursicon MTL triggers rk desensitization, resulting in functional blockade of the receptor. This mechanism is supported by *in vitro* data demonstrating that long-term bursicon stimulation, using either soluble ligand or the corresponding MTL, essentially eliminated further bursicon-mediated signaling.

In this study, we show that ubiquitous receptor inactivation using the CFP-tBur-β-α *in vivo* results in developmental lethality. Furthermore, by expressing membrane-tethered bursicon with a collection of increasingly focused tissue-specific Gal4 drivers, we were able to demonstrate that rk expression in adult muscle plays an essential role during eclosion and subsequent wing expansion. Parallel studies with rk RNAi constructs support each of the above conclusions.

RESULTS

Ubiquitous membrane-tethered bursicon causes developmental lethality

Previously, using a membrane-tethered bursicon construct (CFP-tBur-β-α), we have shown that a single fusion of both bursicon subunits can activate rk *in vitro*. As a follow-up to this project, we generated transgenic flies expressing CFP-tBur-β-α under the control of a UAS. These flies were used in combination with selected Gal4 *Drosophila* driver lines to express the bursicon MTL in a tissue-specific manner. The CFP-tBur-β-α line was first crossed to fly stock expressing ubiquitous Gal4 under the control of the actin-5C promoter. Unexpectedly, such expression of CFP-tBur-β-α resulted in a significant decrease in survival through eclosion. Developmental lethality was also observed upon ubiquitous knockdown of the rk receptor, but not from knock down of the bursicon ligand (bursicon α-subunit) as seen in Fig. 1A. In addition to developmental lethality, flies that ubiquitously expressed CFP-tBur-β-α yet survived through eclosion (escapers) failed to expand their wings 100% of the time. In contrast, with Burs-α knockdown, ~50% of flies failed to properly expand their wings. We observed no escapers with ubiquitous rk RNAi knockdown (Fig. 1B). As confirmation, CFP-tBur-β-α flies were crossed with a novel rk-specific driver, rk^{PAN}-Gal4 (Diao and White, 2012). With the rk-specific driver, significant amounts of developmental lethality occurred and escapers were unable to expand their wings (supplementary material Fig. S1).

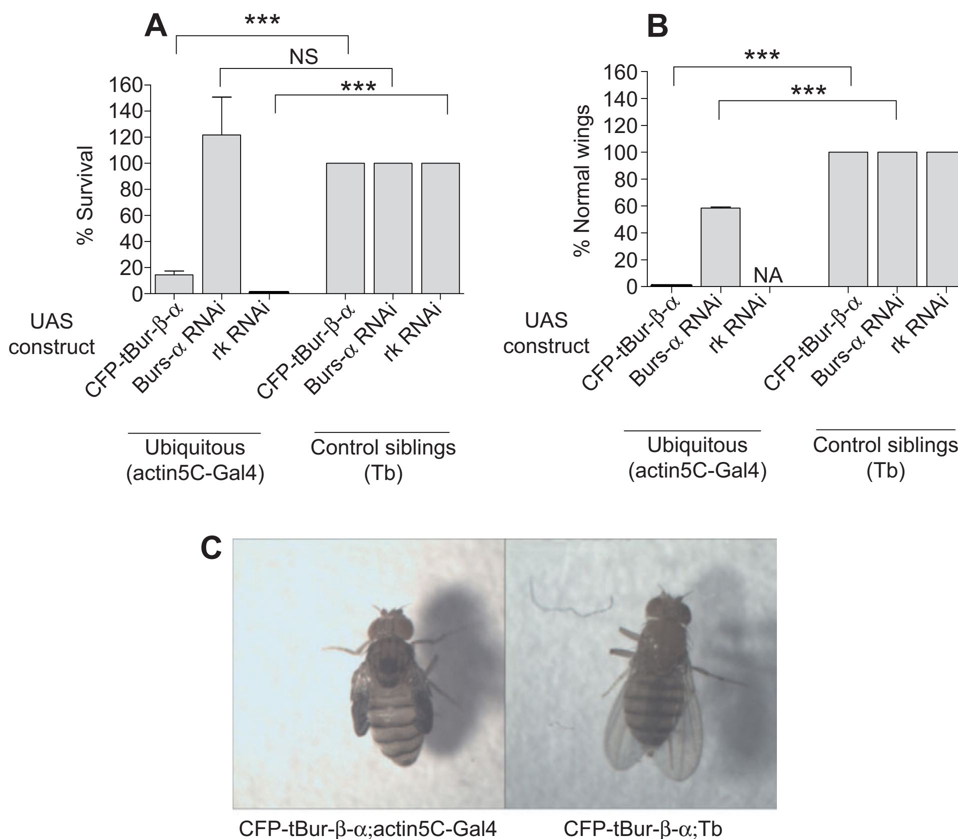

Fig. 1. Ubiquitous overexpression of membrane-tethered bursicon recapitulates the phenotypes induced by downregulation of its cognate receptor, rk. (A) Survival through eclosion of the indicated progeny. (B) Percentage of flies with normal expanded wings for the indicated progeny. (C) Representative images of wings of eclosed flies and corresponding wings from indicated genotypes. NA, wing expansion data not available because of 100% developmental lethality; UAS, upstream activation sequence; Tb, Tubby^{1}; CFP-tBur-β-α, cherry fluorescent protein membrane-tethered bursicon. Data represent means ± s.e.m. of experiments done in triplicate. N≥50–134 flies were counted for each independent cross. ***P<0.0001, NS P>0.05.

The Journal of Experimental Biology

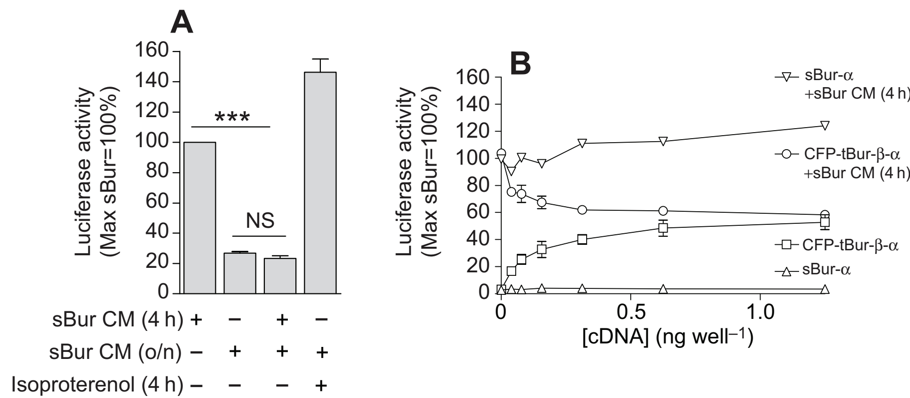

Fig. 2. Chronic *in vitro* stimulation results in rk desensitization. (A) Overnight stimulation with bursicon-conditioned media desensitizes rk receptors *in vitro*. HEK293 cells were transfected with rk, Pgl4.22 [a cAMP response element (CRE) luciferase reporter construct] and a β-galactosidase control gene. Twenty-four hours after transfection, cells were treated overnight (o/n) with bursicon-conditioned media. The next day, the cells were again stimulated with bursicon-conditioned media or isoproterenol for an additional 4 h. Activity was compared with that of a 4 h stimulation of rk-expressing cells without an overnight bursicon-conditioned media pre-treatment. (B) CFP-tBur-β-α desensitizes rk receptor activation in a cDNA concentration-dependent manner. HEK293 cells were transfected as described for A, but with the addition of increasing amounts of the indicated cDNA encoding soluble or tethered bursicon. Forty-four hours following transfection, cells were further stimulated for 4 h with a maximal concentration of bursicon-conditioned media or serum-free DMEM. Luciferase activity was then quantified. sBur CM, soluble bursicon-conditioned media; sBur-α, soluble bursicon α-subunit cDNA; CFP-tBur-β-α, cherry fluorescent protein membrane-tethered bursicon. Data represent the means ± s.e.m. from three independent experiments, each performed in triplicate. ***P<0.0001, NS P>0.05.

Chronic rk stimulation results in receptor desensitization

We were somewhat surprised that the observed phenotype with ubiquitous expression of CFP-tBur-β-α (an agonist) recapitulated results of the rk knockdown. One possible explanation was that chronic activation of rk during development led to receptor desensitization, thus functionally inactivating rk. To determine whether rk was desensitized with agonist stimulation, a previously described *in vitro* model (Harwood et al., 2013) was adapted to examine the effects of chronic stimulation of rk. Notably, rk was efficiently desensitized following chronic stimulation with bursicon-conditioned media. After an overnight incubation, rk was no longer able to respond to a second 4 h pulse of bursicon-conditioned media (Fig. 2A). Desensitization with bursicon appears to be rk specific. Following an overnight stimulation with bursicon-conditioned media, isoproterenol was added to the same cells to stimulate β2 adrenergic receptors (β2ARs), which are endogenously expressed on HEK293 cells. β2AR activation was observed, suggesting that rk desensitization is homologous rather than heterologous (Fig. 2A) (Kelly et al., 2008).

In addition to rk desensitization by soluble bursicon, expression of CFP-tBur-β-α triggered a similar pharmacological consequence. This effect was dependent on the amount of cDNA transfected. Desensitization increased in parallel with tether expression. Importantly, overexpression of the bursicon α cDNA subunit alone did not result in receptor desensitization, suggesting that the receptor must be activated by the full heterodimer (α plus β) for desensitization to occur (Fig. 2B).

rk is required in muscle for wing expansion and survival through eclosion

Based on the above data, it became evident that expression of CFP-tBur-β-α could be used as a tool to selectively inactivate rk in tissues of interest. A general screen of Gal4 drivers was conducted to determine what tissues are critical for rk-dependent survival (Table 1). We found that the muscle-specific driver 'held out wing' (HOW-Gal4) (Draper et al., 2009), which targets both larval and adult muscle, led to a significant decrease in survival when used to direct expression of either CFP-tBur-β-α or rk RNAi. CFP-tBur-β-α;HOW-Gal4 escapers had 100% wing expansion defects. In contrast, knockdown of Burs-α with How-Gal4 did not cause any lethality or wing expansion defects (Fig. 3A,B). This suggests that the bursicon tethered ligand is acting on muscle cells that express rk but do not release bursicon.

To extend our investigation of rk in muscle, we focused on adjacent motor neurons as the potential source of bursicon. Using the D42-Gal4 driver, Burs-α RNAi, rk RNAi and CFP-tBur-β-α were expressed in these cells. Wing expansion defects were seen when Burs-α RNAi constructs were expressed in motor neurons, indicating that at least some bursicon is released from these cells. Previous studies in larvae have also shown that bursicon is

Table 1. CFP-tBur- β-α screen with indicated Gal4 driver lines

Gal4 driver	Reduced survival	Wing expansion defect	Expression pattern
Actin5C-Gal4	+	+	Ubiquitous
CCAP-Gal4	–	+	CCAP neurons
D42-Gal4	–	+	Motor neurons
Twist-Gal4	–	–	Stem cells, adult muscle precursors
HOW-Gal4	+	+	Pan mesoderm, tendon cells
Ap-Gal4	+	+	Direct flight muscle, tendon cells, abdominal muscle, neurons
Mef2-Gal4	+	+	Differentiating adult muscle, neurons
Actin88F-Gal4	+	+	Indirect flight muscle, leg muscle, abdominal muscle
Tinman-Gal4	–	–	Cardiac muscle
Striped-Gal4	–	–	Tendon cells

The Journal of Experimental Biology

The Journal of Experimental Biology (2014) doi:10.1242/jeb.110098

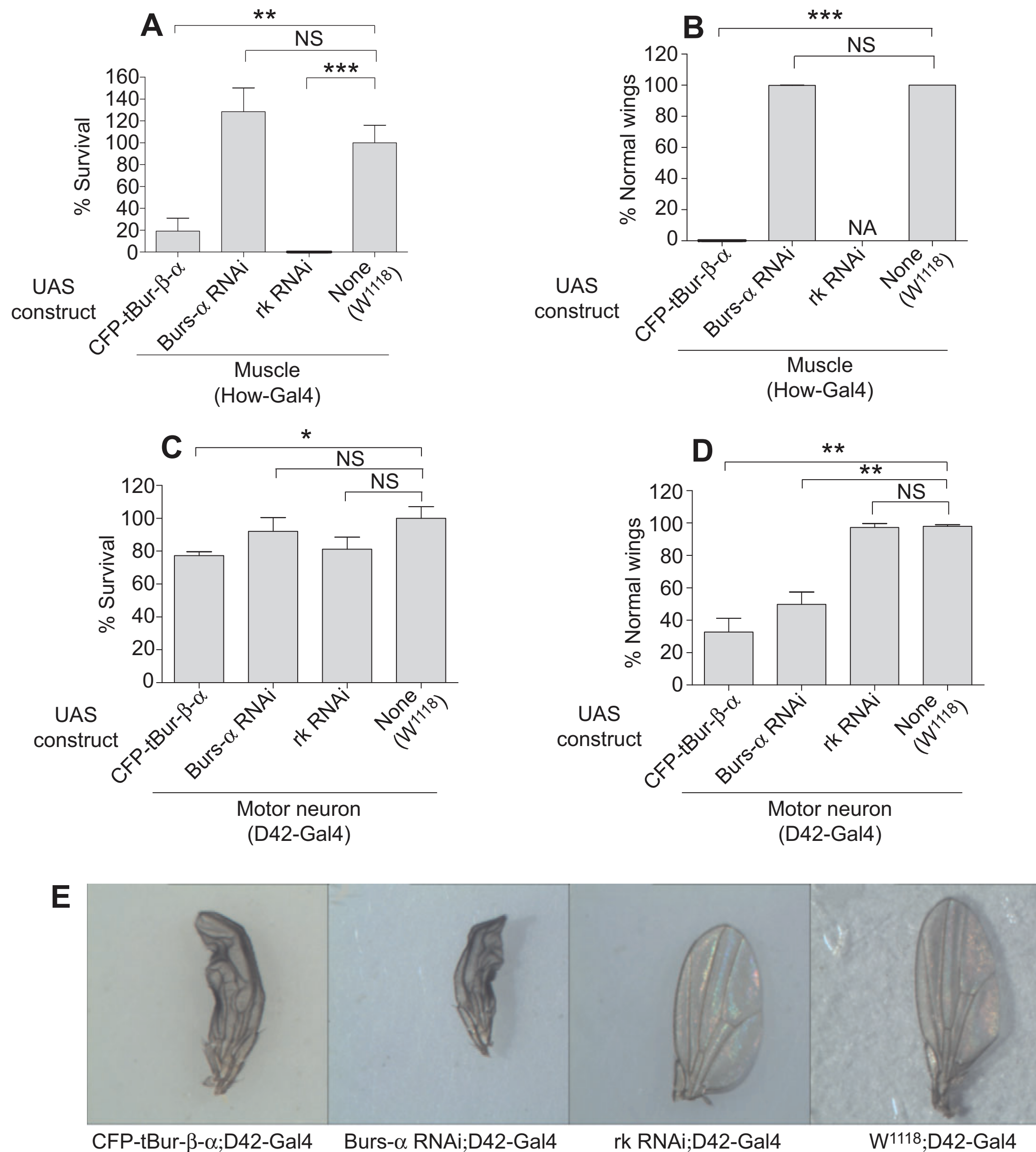

Fig. 3. Expression of membrane-tethered bursicon in muscle and motor neurons results in wing expansion defects. (A,B) Expression of CFP-tBur-β-α and rk RNAi, but not Burs-α RNAi, in muscle results in a reduction in survival through eclosion and wing expansion defects. (C) Expression of CFP-tBur-β-α but not Burs-α RNAi and rk RNAi in motor neurons results in a significant reduction in survival through eclosion. (D) Expression of CFP-tBur-β-α and Burs-α RNAi, but not rk RNAi in motor neurons results in wing expansion defects. (E) Representative images of wings following eclosion from the indicated genotypes. NA, wing expansion data not available because of 100% developmental lethality; UAS, upstream activation sequence; CFP-tBur-β-α, cherry fluorescent protein membrane-tethered bursicon; How, held out wing. Data represent means ± s.e.m. of experiments done in triplicate. N=85–172 progeny were counted for each independent control cross.***P<0.0001, **P<0.001, *P<0.01, NS P>0.05; data are normalized to three independent crosses of indicated Gal4 lines with W^{1118} flies. NA, not applicable.

expressed in selected motor neurons (i.e. those containing type III boutons) (Loveall and Deitcher, 2010). No wing expansion defects or lethality was observed when rk was knocked down in motor neurons (Fig. 3C–E). In contrast, CFP-tBur-β-α expression in motor neurons led to wing expansion defects (as well as a small decrease in survival). Taken together, these phenotypes suggest that CFP-tBur-β-α expression in motor neurons acts on adjacent muscle to inhibit rk.

rk activity is required during formation of adult muscle

As a next step, we investigated when during muscle differentiation rk downregulation leads to compromised survival and wing expansion. To address this question, selected myogenic Gal4 lines were tested (Table 1). Apterous (Ap) and Mef2 fly lines showed significant developmental lethality at the pupal stage with either CFP-tBur-β-α expression or RNAi-mediated rk knockdown (Fig. 4). Notably, the corresponding Ap-Gal4 flies that survived through development had wing expansion defects (Fig. 4A). A second driver, Mef2-Gal4, also showed decreased survival and wing expansion defects with expression of either CFP-tBur-β-α or rk RNAi. Although significant, the phenotypes with Mef2-Gal4 were less severe than those with the Ap-Gal4 and How-Gal4 drivers (Fig. 4B–D). The results with Ap-Gal4 and Mef2-Gal4, which target differentiating muscle cells, contrast with the absence of lethality or a wing phenotype when either the tethered bursicon or the rk RNAi construct was expressed earlier, in muscle progenitors (using the

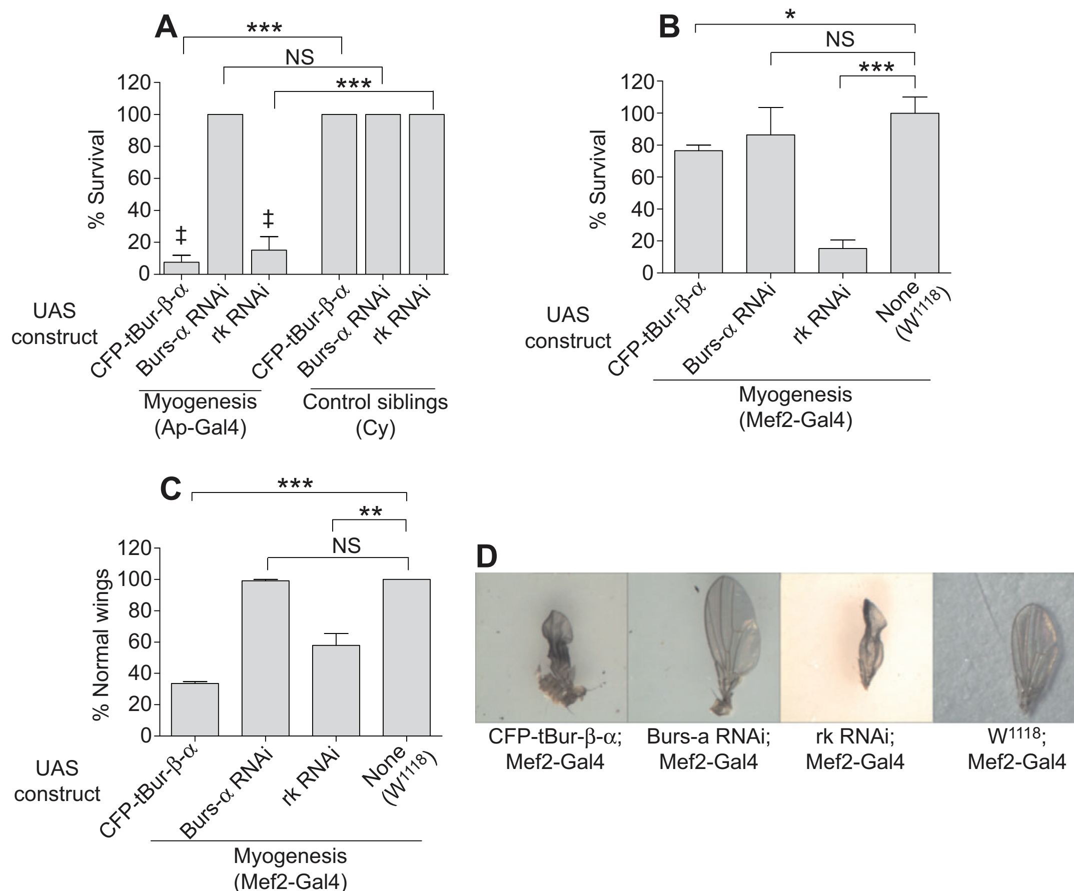

Fig. 4. CFP-tBur-β-α expression or rk knockdown during myogenesis causes developmental lethality and wing expansion defects. (A) CFP-tBur-β-α and rk RNAi expression with apterous-Gal4 results in reduced survival through eclosion. Escapers are unable to expand their wings. (B,C) Expression of CFP-tBur-β-α and rk RNAi, but not Burs-α RNAi, in Mef2-positive myotubes results in a reduction in survival through eclosion and wing expansion defects of escapers. (D) Representative images of wings following eclosion from the indicated genotypes. UAS, upstream activation sequence; CFP-tBur-β-α, cherry fluorescent protein membrane-tethered bursicon; Ap, apterous; Mef2, myocyte enhancer factor-2; Cy, Curly. ‡100% wing expansion defects. Data represent means ± s.e.m. of experiments done in triplicate. *N*=40–112 progeny were counted for each independent control cross. ****P*<0.0001, ***P*<0.001, **P*<0.01, NS *P*>0.05. Data are normalized to three independent crosses of indicated Gal4 lines with W^{1118} flies (B,C).

Twist-Gal4 driver) (Ciglar and Furlong, 2009; Sandmann et al., 2007). Taken together, these results highlight the importance of rk later in the process of muscle differentiation.

Finally, we wanted to determine whether rk plays a demonstrable role in adult muscle. To investigate this question, we targeted adult muscle, using the actin88F-Gal4 driver line. Using this driver line, with expression of either CFP-tBur-β-α or rk RNAi, significant lethality was observed prior to eclosion (Fig. 5A). We also observed wing expansion defects in all escapers (Fig. 5B). Again, no decrease in survival and no wing expansion defects were observed in parallel experiments expressing a Burs-α-subunit RNAi construct (Fig. 5A,B). Taken together, these results suggest that rk activation is required in developing muscle and formed adult muscles. rk-mediated activity on muscle thus appears to be important for both proper eclosion and wing expansion.

DISCUSSION

In this study we utilized a membrane-tethered bursicon construct (CFP-tBur-β-α) to probe the role of rk in development. Previous studies from our lab have shown that, when assessed *in vitro*, CFP-tBur-β-α is an rk agonist (Harwood et al., 2013). Initial *in vivo* investigations revealed that expression of CFP-tBur-β-α in otherwise rk/bursicon wild-type *Drosophila* (W^{1118}) led to lethality and wing expansion defects, both unanticipated phenotypes. Although it is difficult to predict the effect of hormonal imbalance *in vivo*, we had hypothesized that the agonist activity of CFP-tBur-β-α would provide a gain of function and would be useful to rescue bursicon mutant fly lines. Contrary to this expectation, the phenotypes resulting from ubiquitous expression of CFP-tBur-β-α were loss of function (i.e. lethality and wing expansion defects) and resembled those observed with knockdown of rk using RNAi (Fig. 1). Flies that expressed CFP-tBur-β-α or rk RNAi ubiquitously generally reached pupal development but failed to eclose. Dead flies were fully developed but did not emerge from their pupal cases. Based on the parallel phenotypes with the RNAi flies, we hypothesized that expression of CFP-tBur-β-α resulted in a decrease of rk-mediated signaling.

Prior pharmacological studies have shown that sustained activation of a GPCR can trigger receptor desensitization, in turn leading to decreased receptor-mediated signaling (Magalhaes et al., 2012). Based on this precedent, we postulated that CFP-tBur-β-α expression induces chronic stimulation of rk *in vivo*, resulting in receptor desensitization. The desensitized rk receptor may not adequately respond to endogenous bursicon at key developmental stages. To examine this possibility, we carried out *in vitro* studies comparing the effects of CFP-tBur-β-α and soluble bursicon-conditioned media on rk activation/desensitization. The results show

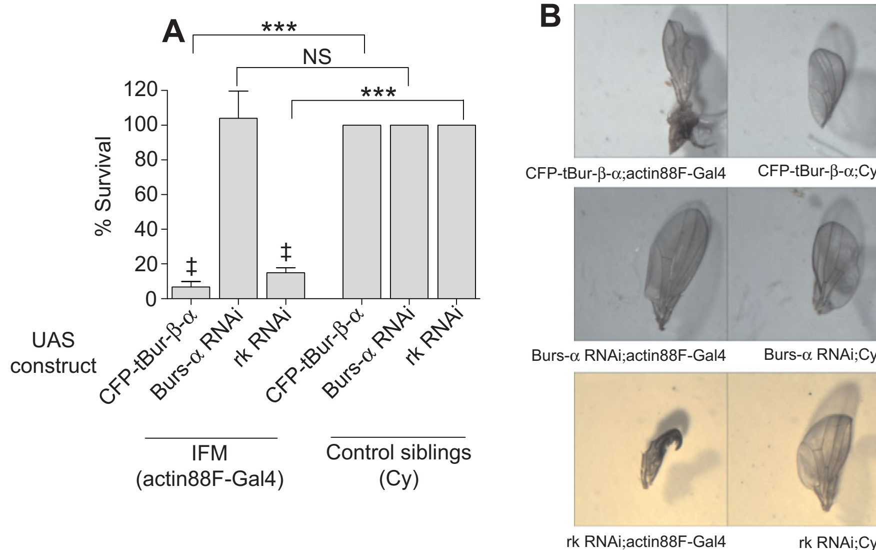

Fig. 5. rk knockdown and CFP-tBur-β-α expression in adult indirect flight muscle causes lethality and wing expansion defects. (A) Survival through eclosion is reduced for progeny expressing CFP-tBur-β-α and rk RNAi, but not Burs-α RNAi, in adult indirect flight muscle. (B) Representative images of wings from eclosed flies for the indicated genotypes. UAS, upstream activation sequence; CFP-tBur-β-α, cherry fluorescent protein membrane-tethered bursicon; Cy, Curly; IFM, indirect flight muscle. ‡100% wing expansion defects. Data represent means ± s.e.m. of experiments done in triplicate. *N*≥58–191 flies were counted for each independent cross. ****P*<0.0001, NS *P*>0.05.

that long-term stimulation of rk (overnight) with either soluble or membrane-tethered bursicon renders the receptor unable to further respond to bursicon (Fig. 2). Notably, the desensitization of rk was receptor specific. HEK293 cells expressing recombinant rk that had been desensitized to bursicon could still signal in response to β2AR agonist treatment via endogenously expressed β2AR, which also signals through $G\alpha_s$.

Desensitization has previously been documented among other leucine-rich repeat-containing GPCRs (LGRs). For example, the human receptor LGR5 is known to be constitutively internalized and is one of the most evolutionarily related orthologs to rk (Snyder et al., 2013b). Notably, the C-terminal region of both rk and LGR5 includes multiple serine residues, which, in the case of LGR5, has been shown to play an important role in desensitization (Snyder et al., 2013a). Taken together, we propose that tethered bursicon-mediated desensitization of rk may underlie the loss of function phenotypes observed *in vivo*. Transgenic flies expressing tethered bursicon under a UAS inducible promoter provide an important complementary genetic tool for studying the rickets bursicon system.

We showed that ubiquitous expression of CFP-tBur-β-α leads to developmental arrest at the pupal stage. Escapers that survive to adulthood show melanization and wing expansion phenotypes, features characteristic of rk knockdown. CFP-tBur-β-α induces phenotypes that are readily monitored, and provides a useful tool to further dissect the role of rk in the developing fly (complementing rk RNAi constructs).

During a previous screen performed in the Kopin lab, it was noted that RNAi-mediated knockdown of the heterotrimeric G protein subunit $G\alpha_s$, with muscle-specific drivers resulted in developmental arrest (I.D. and A.S.K., unpublished results). It was hypothesized that rk may be the receptor upstream of $G\alpha_s$ in muscle that led to this phenotype. To assess this possibility, CFP-tBur-β-α expression was restricted to muscle under the control of the pan-mesodermal driver HOW-Gal4 (Zaffran et al., 1997). CFP-tBur-β-α expression resulted in defects that phenocopied those seen with both ubiquitous expression of the tethered ligand and $G\alpha_s$ knockdown in muscle (i.e. lethality, wing phenotypes). Similar phenotypes were also observed when rk was downregulated using rk RNAi, confirming that the receptor was important in muscle. We subsequently used a series of Gal4 driver lines to target our tethered ligand to selected tissues and thus define the critical cell type(s) that requires rk and underlies the lethality/wing expansion phenotypes (Table 1). Although many studies have focused on the spatial and temporal release of bursicon (Lahr et al., 2012; Peabody et al., 2008; Peabody et al., 2009), the localization of the bursicon receptors that are essential for survival has remained elusive. Our study strongly supports that a peripheral rk, localized in muscle, plays an important role in *Drosophila* development.

Expression of either CFP-tBur-β-α or rk RNAi in muscle led to comparable lethality/wing phenotypes, indicating that the receptor is present postsynaptically. In contrast, expression of rk RNAi in motor neurons (using the D42-Gal4 driver) had no effect, suggesting the absence of a presynaptic receptor. Expression of CFP-tBur-β-α using the same driver, however, led to a wing defect phenotype. By design, CFP-tBur-β-α anchors in the membrane and projects into the extracellular space (Harwood et al., 2013). Its function is defined primarily by three structural elements: transmembrane domain, linker and peptide. We postulate that tethered bursicon anchored in the motor neuron may act on muscle rk in trans, leading to partial desensitization of the receptor, thus inducing the wing expansion defect.

In addition to defining a role for rk in muscle tissue, we probed the role of rk during myogenesis. It is well established that, during metamorphosis, most of the adult muscles are formed *de novo* from progenitor cells, i.e. adult muscle precursors (AMPs) located on the larval wing imaginal disc and leg imaginal disc (Raghavan et al., 1996). Undifferentiated AMPs express high levels of the helix-loop-helix transcription factor twist (Bate et al., 1991; Currie and Bate, 1991). As twist is downregulated, myoblasts commit to the muscle lineage and differentiate. This occurs in conjunction with an interplay of many transcription factors. A core regulatory network includes Tinman (cardiac muscle specification), myocyte enhancer factor-2 (Mef2, which plays a key role in myoblast fusion/formation of somatic muscles) and apterous (which specifies selected subtypes of somatic muscles) (Ciglar and Furlong, 2009; Cripps et al., 1998; Olson et al., 1995; Ranganayakulu et al., 1995; Sandmann et al., 2007; Weitkunat and Schnorrer, 2014; Yin et al., 1997). Based on the above, selected drivers were used to target rickets at different stages of the myogenic process using either CFP-tBur-β-α or rk RNAi. The results of this analysis are summarized in Table 1. Using the twist-Gal4 driver, we showed that downregulation of rk in AMPs had no effect on survival or wing expansion. This suggests that very early myogenesis is not altered by the absence of rk. In contrast, we showed that altering rk in differentiating myotubes (Mef2-positive cells) compromised survival and wing expansion.

It has previously been shown that bursicon and CCAP are co-packaged and released directly on to heart muscle (Woodruff et al., 2008). CCAP has an important role in modulating heart function (Dulcis et al., 2005; Woodruff et al., 2008). Given the coordinated synthesis and release of these two hormones, we explored whether rk-mediated eclosion may also be linked to receptors expressed on *Drosophila* heart muscle. Our analysis ruled out this possibility; decreased rk function in cardiac muscle did not compromise either eclosion or wing expansion (Table 1).

We next investigated whether expression of rk in selected subtypes of pharate adult muscles could account for the observed CFP-tBur-β-α-/rk RNAi-induced phenotypes. For these studies, we used the actin88F-Gal4 driver line, which targets major muscles in the adult fly, including the indirect flight muscles. Expression of CFP-tBur-β-α or knockdown of rk in adult muscle resulted in developmental lethality prior to eclosion while escaper flies failed to expand their wings (Fig. 5). Comparison of the tissues that are targeted by Ap-Gal4 (as outlined above) and actin88F-Gal4 enabled us to further define the muscle subtypes that potentially underlie the rk-mediated phenotypes. Actin 88F is a muscle actin predominantly expressed in the thoracic indirect flight muscles (IFMs) (Weitkunat and Schnorrer, 2014). Notably, apterous is absent in the IFMs, making this an unlikely target. Actin88F is also expressed in the mesothoracic leg (tibial depressor muscles), as well as in the abdominal muscles (both ventral and dorsal) (Ghazi et al., 2000; Nongthomba et al., 2001). These two muscle types thus emerge as potentially important for rk signaling-regulation of eclosion.

It is well established that both leg and abdominal muscles are important during metamorphosis. At 12 h after puparium formation, contraction of the abdominal muscles forces an air bubble forward, which in turn, triggers head eversion (Fernandes et al., 1991). In addition, at the end of the pupal stage, the newly formed adult uses its legs to free itself from the case. Consistent with the hypothesis that rk is present in leg muscle, bursicon immunoreactive neurons have been shown to directly innervate the leg of the cricket *Gryllus bimaculatus* (Honegger et al., 2008; Kostron et al., 1996). The potential role of rk in leg muscle could also explain the kinked leg phenotype observed with rk classical mutants (Baker et al., 2005; Loveall and Deitcher, 2010).

In conclusion, we have developed a membrane-tethered ligand (CFP-tBur-β-α) that negatively regulates rk function when expressed *in vivo*. Using this novel tool in conjunction with existing RNAi fly lines, we have identified adult muscle as a tissue that requires rk expression for survival and wing expansion in *Drosophila*. In particular, based on our analysis, it appears that leg and abdominal muscles, or a subset of these, could be important. Our studies set the stage for future investigations aimed at further understanding the role of muscle rk in fly development.

MATERIALS AND METHODS

Cell culture

Human embryonic kidney cells (HEK293) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, USA), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Life Technologies). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Luciferase assays

Luciferase assays were carried out as previously described with minor modifications (Harwood et al., 2013). In brief, HEK293 cells in 96-well plates were transfected in serum-free DMEM with antibiotics using polyethylenimine (1 μg ml^{-1}) for 48 h. Cells were transfected with rk receptor (1 ng $well^{-1}$), a cAMP response element (CRE) luciferase reporter construct (5 ng $well^{-1}$) and β-galactosidase plasmid (5 ng $well^{-1}$) as a transfection control. In addition, cells were transfected with various membrane-tethered or soluble bursicon cDNAs as indicated.

Desensitization assays

In brief, cells were transfected as described for luciferase assays. For soluble ligand-based desensitization, 24 h following transfection, bursicon-conditioned media or serum-free DMEM was added to cells, and plates were incubated overnight. The following day, bursicon-conditioned media or isoproterenol was added to the cells for an additional 4 h. Luciferase levels were then quantified. For membrane-tethered ligand-based desensitization, cells were transfected as previously described with slight modifications; increasing amounts of membrane-tethered ligand cDNA were used. Approximately 44 h after transfection, cells were then stimulated with bursicon-conditioned media for 4 h. Luciferase levels were then quantified. Bursicon-conditioned media were prepared as previously described (Harwood et al., 2013).

Plasmids

rk pcDNA1.1, β-galactosidase pcDNA1.1, membrane-tethered ligand cDNAs and bursicon plasmids were generated as previously described (Harwood et al., 2013). The cDNA encoding rk was generously provided by Dr Cornelis Grimmelikhuijzen. Bursicon α and β cDNAs in pcDNA3.1 were a gift from Dr J. Vanden Broeck. For the luciferase reporter gene plasmid, a 6× CRE was cloned into the promoter region pGL4.22, a destabilized luciferase gene (Madison, WI, USA).

Transgenic fly generation

The coding sequence of CFP-tBur-β-α pcDNA1.1 (Harwood et al., 2013) was subcloned into pUAST vector using *Eco*RI and *Xba*I restrictions sites. CFP-tBur-β-α pUAST plasmid was sequence verified and then sent to Duke University Model System Genomics *Drosophila* embryo injection service. Positive transformants on a W^{1118} genetic background were then selected by the presence of red eye color. Siblings were crossed and homozygous stocks were maintained.

Drosophila stocks and culture

The following were obtained from the Bloomington Stock Center: w^{1118} (FBal0018186), actin5C-Gal4 (y1 w*; P[Act5C-GAL4]17bFO1/TM6B, Tb1; FBst0003954), how-Gal4 (w*; P[GawB]how24B; FBst0001767), D42-Gal4 (w*; P[GawB]D42; FBst0008816), Mef2-Gal4 (y[1] w[*]; P[w[+mC]=GAL4-Mef2.R]3; 27390), Ap-Gal4/Cy (y[1] w[1118]; P[w[+mW.hs]=GawB]ap[md544]/CyO; FBst0003041) and actin88F-Gal4/Cy (w*; P[Act88F-GAL4.1.3]81B, P[Mhc-tauGFP]2/SM6b; FBst0038460). Burs-α RNAi (w^{1118}; P[GD3951]v13520; FBst0451049) was obtained from Vienna *Drosophila* RNAi Center (Vienna, Austria). rk RNAi (8930R-1;FBsf0000434575) was obtained from NIG-FLY (National Institute of Genetics, Mishima, Shizuoka, Japan). rk^{PAN}-Gal4 (Diao and White, 2012) was a gift from Dr Benjamin White at the National Institutes of Health. All fly stocks were maintained at 25°C on a standard 12 h:12 h light:dark cycle. *Drosophila* growth media contained cornmeal, agar, brewer's yeast, dextrose, sucrose and wheatgerm. All crosses between UAS stocks and Gal4 stocks were performed at 29°C using standard *Drosophila* growth media. For crosses, virgin females of the indicated UAS constructs or W^{1118} flies were collected and mated with Gal4 stock males. Flies were counted after eclosion and assessed for wing expansion defects. Each fly cross was performed on a minimum of three separate occasions.

Data analysis and statistics

All luciferase and fly cross data were graphed and analyzed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

Acknowledgements

We thank Ci Chen (Tufts Medical Center) for her help with tissue culture throughout the course of this research. We also thank Rob Jackson (Tufts University) and Martin Beinborn (Tufts Medical Center) for helpful discussions.

Competing interests
The authors declare no competing financial interests.

Author contributions
B.N.H. was responsible for conception, experimental design, execution of experiments and preparation of this manuscript. I.D. was responsible for conception, experimental design, execution of experiments, data analysis and preparation of this manuscript. A.S.K. was responsible for conception, experimental design, data analysis and preparation of this manuscript.

Funding
B.N.H. was supported by the synapse neurobiology training program (T32-NS061764). Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.110098/-/DC1

References

Baker, J. D. and Truman, J. W. (2002). Mutations in the *Drosophila* glycoprotein hormone receptor, rickets, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program. *J. Exp. Biol.* **205**, 2555-2565.

Baker, P. W., Tanaka, K. K., Klitgord, N. and Cripps, R. M. (2005). Adult myogenesis in *Drosophila melanogaster* can proceed independently of myocyte enhancer factor-2. *Genetics* **170**, 1747-1759.

Bate, M., Rushton, E. and Currie, D. A. (1991). Cells with persistent twist expression are the embryonic precursors of adult muscles in *Drosophila*. *Development* **113**, 79-89.

Chintapalli, V. R., Wang, J. and Dow, J. A. (2007). Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat. Genet.* **39**, 715-720.

Choi, C., Fortin, J. P., McCarthy, E., Oksman, L., Kopin, A. S. and Nitabach, M. N. (2009). Cellular dissection of circadian peptide signals with genetically encoded membrane-tethered ligands. *Curr. Biol.* **19**, 1167-1175.

Ciglar, L. and Furlong, E. E. (2009). Conservation and divergence in developmental networks: a view from *Drosophila* myogenesis. *Curr. Opin. Cell Biol.* **21**, 754-760.

Cripps, R. M., Black, B. L., Zhao, B., Lien, C. L., Schulz, R. A. and Olson, E. N. (1998). The myogenic regulatory gene Mef2 is a direct target for transcriptional activation by Twist during *Drosophila myogenesis*. *Genes Dev.* **12**, 422-434.

Currie, D. A. and Bate, M. (1991). The development of adult abdominal muscles in *Drosophila*: myoblasts express twist and are associated with nerves. *Development* **113**, 91-102.

Dewey, E. M., McNabb, S. L., Ewer, J., Kuo, G. R., Takanishi, C. L., Truman, J. W. and Honegger, H. W. (2004). Identification of the gene encoding bursicon, an insect neuropeptide responsible for cuticle sclerotization and wing spreading. *Curr. Biol.* **14**, 1208-1213.

Diao, F. and White, B. H. (2012). A novel approach for directing transgene expression in *Drosophila*: T2A-Gal4 in-frame fusion. *Genetics* **190**, 1139-1144.

Draper, I., Tabaka, M. E., Jackson, F. R., Salomon, R. N. and Kopin, A. S. (2009). The evolutionarily conserved RNA binding protein SMOOTH is essential for maintaining normal muscle function. *Fly (Austin)* **3**, 235-246.

Dulcis, D., Levine, R. B. and Ewer, J. (2005). Role of the neuropeptide CCAP in *Drosophila* cardiac function. *J. Neurobiol.* **64**, 259-274.

Fernandes, J., Bate, M. and Vijayraghavan, K. (1991). Development of the indirect flight muscles of *Drosophila*. *Development* **113**, 67-77.

Fortin, J. P., Zhu, Y., Choi, C., Beinborn, M., Nitabach, M. N. and Kopin, A. S. (2009). Membrane-tethered ligands are effective probes for exploring class B1 G protein-coupled receptor function. *Proc. Natl. Acad. Sci. USA* **106**, 8049-8054.

Fortin, J. P., Chinnapen, D., Beinborn, M., Lencer, W. and Kopin, A. S. (2011). Discovery of dual-action membrane-anchored modulators of incretin receptors. *PLoS ONE* **6**, e24693.

Fraenkel, G. and Hsiao, C. (1962). Hormonal and nervous control of tanning in the fly. *Science* **138**, 27-29.

Fraenkel, G., Hsiao, C. and Seligman, M. (1966). Properties of bursicon: an insect protein hormone that controls cuticular tanning. *Science* **151**, 91-93.

Ghazi, A., Anant, S. and VijayRaghavan, K. (2000). Apterous mediates development of direct flight muscles autonomously and indirect flight muscles through epidermal cues. *Development* **127**, 5309-5318.

Graveley, B. R., Brooks, A. N., Carlson, J. W., Duff, M. O., Landolin, J. M., Yang, L., Artieri, C. G., van Baren, M. J., Boley, N., Booth, B. W. et al. (2011). The developmental transcriptome of *Drosophila melanogaster*. *Nature* **471**, 473-479.

Harwood, B. N., Fortin, J. P., Gao, K., Chen, C., Beinborn, M. and Kopin, A. S. (2013). Membrane tethered bursicon constructs as heterodimeric modulators of the *Drosophila* G protein-coupled receptor rickets. *Mol. Pharmacol.* **83**, 814-821.

Honegger, H. W., Dewey, E. M. and Ewer, J. (2008). Bursicon, the tanning hormone of insects: recent advances following the discovery of its molecular identity. *J. Comp. Physiol. A* **194**, 989-1005.

Kelly, E., Bailey, C. P. and Henderson, G. (2008). Agonist-selective mechanisms of GPCR desensitization. *Br. J. Pharmacol.* **153 Suppl. 1**, S379-S388.

Kostron, B., Kaltenhauser, U., Seibel, B., Bräunig, P. and Honegger, H. (1996). Localization of bursicon in CCAP-immunoreactive cells in the thoracic ganglia of the cricket *Gryllus bimaculatus*. *J. Exp. Biol.* **199**, 367-377.

Lahr, E. C., Dean, D. and Ewer, J. (2012). Genetic analysis of ecdysis behavior in *Drosophila* reveals partially overlapping functions of two unrelated neuropeptides. *J. Neurosci.* **32**, 6819-6829.

Loveall, B. J. and Deitcher, D. L. (2010). The essential role of bursicon during *Drosophila* development. *BMC Dev. Biol.* **10**, 92.

Luo, C. W., Dewey, E. M., Sudo, S., Ewer, J., Hsu, S. Y., Honegger, H. W. and Hsueh, A. J. (2005). Bursicon, the insect cuticle-hardening hormone, is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2. *Proc. Natl. Acad. Sci. USA* **102**, 2820-2825.

Magalhaes, A. C., Dunn, H. and Ferguson, S. S. (2012). Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. *Br. J. Pharmacol.* **165**, 1717-1736.

Nongthomba, U., Pasalodos-Sanchez, S., Clark, S., Clayton, J. D. and Sparrow, J. C. (2001). Expression and function of the *Drosophila* ACT88F actin isoform is not restricted to the indirect flight muscles. *J. Muscle Res. Cell Motil.* **22**, 111-119.

Olson, E. N., Perry, M. and Schulz, R. A. (1995). Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Dev. Biol.* **172**, 2-14.

Peabody, N. C., Diao, F., Luan, H., Wang, H., Dewey, E. M., Honegger, H. W. and White, B. H. (2008). Bursicon functions within the *Drosophila* CNS to modulate wing expansion behavior, hormone secretion, and cell death. *J. Neurosci.* **28**, 14379-14391.

Peabody, N. C., Pohl, J. B., Diao, F., Vreede, A. P., Sandstrom, D. J., Wang, H., Zelensky, P. K. and White, B. H. (2009). Characterization of the decision network for wing expansion in *Drosophila* using targeted expression of the TRPM8 channel. *J. Neurosci.* **29**, 3343-3353.

Raghavan, K. V., Gendre, N. and Stocker, R. F. (1996). Transplanted wing and leg imaginal discs in *Drosophila melanogaster* demonstrate interactions between epidermis and myoblasts in muscle formation. *Dev. Genes Evol.* **206**, 46-53.

Ranganayakulu, G., Zhao, B., Dokidis, A., Molkentin, J. D., Olson, E. N. and Schulz, R. A. (1995). A series of mutations in the D-MEF2 transcription factor reveal multiple functions in larval and adult myogenesis in *Drosophila*. *Dev. Biol.* **171**, 169-181.

Robinson, S. W., Herzyk, P., Dow, J. A. and Leader, D. P. (2013). FlyAtlas: database of gene expression in the tissues of *Drosophila melanogaster*. *Nucleic Acids Res.* **41**, D744-D750.

Sandmann, T., Girardot, C., Brehme, M., Tongprasit, W., Stolc, V. and Furlong, E. E. (2007). A core transcriptional network for early mesoderm development in *Drosophila melanogaster*. *Genes Dev.* **21**, 436-449.

Snyder, J. C., Rochelle, L. K., Barak, L. S. and Caron, M. G. (2013a). The stem cell-expressed receptor Lgr5 possesses canonical and functionally active molecular determinants critical to β-arrestin-2 recruitment. *PLoS ONE* **8**, e84476.

Snyder, J. C., Rochelle, L. K., Lyerly, H. K., Caron, M. G. and Barak, L. S. (2013b). Constitutive internalization of the leucine-rich G protein-coupled receptor-5 (LGR5) to the trans-Golgi network. *J. Biol. Chem.* **288**, 10286-10297.

Van Loy, T., Vandersmissen, H. P., Van Hiel, M. B., Poels, J., Verlinden, H., Badisco, L., Vassart, G. and Vanden Broeck, J. (2008). Comparative genomics of leucine-rich repeats containing G protein-coupled receptors and their ligands. *Gen. Comp. Endocrinol.* **155**, 14-21.

Weitkunat, M. and Schnorrer, F. (2014). A guide to study *Drosophila* muscle biology. *Methods* **68**, 2-14.

Woodruff, E. A., III, Broadie, K. and Honegger, H. W. (2008). Two peptide transmitters co-packaged in a single neurosecretory vesicle. *Peptides* **29**, 2276-2280.

Yin, Z., Xu, X. L. and Frasch, M. (1997). Regulation of the twist target gene tinman by modular cis-regulatory elements during early mesoderm development. *Development* **124**, 4971-4982.

Zaffran, S., Astier, M., Gratecos, D. and Sémériva, M. (1997). The held out wings (how) *Drosophila* gene encodes a putative RNA-binding protein involved in the control of muscular and cardiac activity. *Development* **124**, 2087-2098.

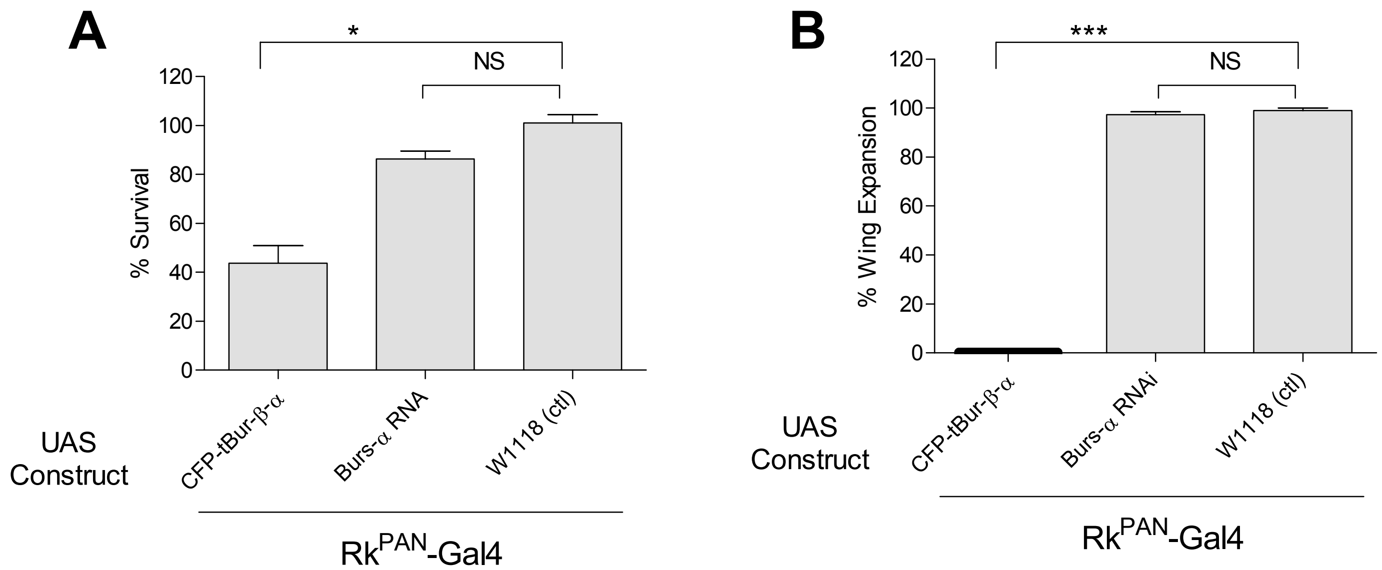

Fig. S1. Expression of membrane tethered bursicon with RkPAN-Gal4 results in reduced survival and in wing expansion defects. (A,B) Expression of CFP-tBur-β-α, but not Burs-α RNAi in rk expressing cells results in a reduction in survival through eclosion and wing expansion defects. Abbreviations: UAS= upstream activation sequence, CFP-tBur-β-α = cherry fluorescent protein membrane tethered bursicon, RkPAN= Gal4 expression under rk promoter sequence. Statistics: data represent mean + SEM of experiments done in triplicate. 134-143 progeny were counted for each independent cross.***P<0.0001, *P<0.05, ^{NS}P>0.05. Data is normalized to 3 independent crosses of the indicated Gal4 lines with W1118 flies.