Published in final edited form as:

Dev Neurobiol. 2017 January ; 77(1): 39–60. doi:10.1002/dneu.22407.

Cyclic Nucleotide Signaling is Required during Synaptic Refinement at the *Drosophila* Neuromuscular Junction

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Abstract

The removal of miswired synapses is a fundamental prerequisite for normal circuit development, leading to clinical problems when aberrant. However, the underlying activity-dependent molecular mechanisms involved in synaptic pruning remain incompletely resolved. Here we examine the dynamic properties of intracellular calcium oscillations and test a role for cAMP signaling during synaptic refinement in intact *Drosophila* embryos using optogenetic tools. We provide *in vivo* evidence at the single gene level that the calcium-dependent adenylyl cyclase *rutabaga*, the phosphodiesterase *dunce*, the kinase PKA, and Protein Phosphatase 1 (PP1) all operate within a functional signaling pathway to modulate Sema2a-dependent chemorepulsion. We find that presynaptic cAMP levels are required to be dynamically maintained at an optimal level to suppress connectivity defects. We also propose that PP1 may serve as a molecular link between cAMP signaling and CaMKII in the pathway underlying refinement. Our results introduce an *in vivo* model where presynaptic cAMP levels, downstream of electrical activity and calcium influx, act via PKA and PP1 to modulate the neuron's response to chemorepulsion involved in the withdrawal of off-target synaptic contacts.

Keywords

pruning; chemorepulsion; activity-dependent; critical period; growth cone

INTRODUCTION

The guidance of axons toward their synaptic targets depends on multiple diffusible and surface-associated chemotropic signals (Kolodkin and Tessier-Lavigne, 2011). Early synaptic development is generally accompanied by the withdrawal of inappropriate contacts, a mechanism known as synaptic refinement or pruning. This form of error correction typically involves neural and/or synaptic activity (Kano and Hashimoto, 2009). Although neural activity was found to play a crucial role in neural network refinement over fifty years ago (Wiesel and Hubel, 1963), much remains to be learned about the underlying molecular pathways and their dynamics. Interest in the mechanisms governing synaptic pruning is

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The authors declare no competing financial interests.

Author contributions: FV and HK designed research and wrote the paper; FV performed research and analyzed data.

motivated by its potential role in clinical disorders, including schizophrenia and autism (Tang et al., 2014; Sekar et al., 2016).

A remarkable feature of activity-dependent synaptic refinement is the role played by low frequency electrical oscillations, often in the range of 0.01 Hz. The oscillations may coordinate dynamic changes in the activity of second messenger systems, or be part of a homeostatic mechanism to maintain signaling molecules within an optimal range. Low frequency oscillatory activity is observed during the refinement of synaptic connections in the visual system and spinal cord of vertebrates, as well as at the embryonic and larval *Drosophila* NMJ (Meister et al., 1991; Wong, 1999; Zhang and Poo, 2001; Hanson and Landmesser, 2004; Nicol et al., 2007; Carrillo et al., 2010; Ackman et al., 2012).

Previous work showed that synaptic refinement of the *Drosophila* NMJ also depends on an early critical period of electrical excitability (Jarecki and Keshishian, 1995). A low frequency (0.01 Hz) oscillation of membrane potential activates presynaptic voltage-gated calcium (Ca^{2+}) channels at the developing NMJ (Carrillo et al., 2010). Ca^{2+} levels modulate the motoneuron's response to the muscle-secreted transsynaptic chemorepellant Sema2a (Carrillo et al., 2010). Mutations that affect electrical excitability (such as the Na(v)1 channel *paralytic* and the Ca(v)2.1 channel *cacophony*), or of genes that encode Sema2a, its presynaptic receptor PlexinB, or CaMKII all share similar defects: ectopic neuromuscular contacts on up to 40% of bodywall muscles (Carrillo et al., 2010).

In addition to CaMKII, other Ca^{2+} -dependent effectors may also influence the chemorepellant response to Sema2a, including the Ca^{2+} -dependent adenylyl cyclase encoded by the *rutabaga* gene. A role for cyclic nucleotide signaling in growth cone chemotropic-dependent motility *in vitro* is well-established, but its role *in vivo* during synapse formation is not as well understood. Using molecular and optogenetic methods we monitored the intracellular Ca^{2+} signals at the developing neuromuscular boutons of intact embryos, and examined how the Ca^{2+} -dependent adenylyl cyclase, cAMP phosphodiesterase, cAMP-dependent protein kinase A (PKA), and protein phosphatase 1 (PP1) collectively regulate synaptic refinement. Our results indicate that the refinement of synaptic connections *in vivo* requires dynamically varying second messenger systems, including Ca^{2+} and cAMP, to engage multiple downstream effectors, including PKA and PP1, for the modulation of chemorepulsion and the refinement of synapses.

MATERIAL AND METHODS

Fly stocks

Animals were reared on standard cornmeal medium at 25 °C, except where noted. The GeneSwitch (GS) GAL4 experiments used a molasses food with or without the steroid activator RU486 (Osterwalder et al., 2001). The wild-type control used throughout was the isogenic Canton S (CS5) line.

We thank the following organizations and individuals for providing stocks: Bloomington Drosophila Stock Center: *rut^l* (BL9404), *rut^{Df}* (*Df(1)ED7261*, BL9218), *dnc^{Df}* (*Df(1)BSC531*, BL25059), *elav^{C155}*-GAL4 (BL458), UAS-*rut* (isolated from BL9405),

UAS-GFP (BL35786; control for TRiP RNAi lines), UAS-rut-RNAi (BL27035), UAS-dnc-RNAi (BL53344), UAS-PKAC1-RNAi (BL31599), UAS-PKAC2-RNAi (BL55859), UAS-PKAC3-RNAi (BL55860), UAS-PKAR2-RNAi (BL34983), pp1 α 96A² (BL23698), pp1-87^{87Bg-3} pp1 α 96A² (BL23699), UAS-pp1 α 96A² (BL23700), UAS-pp187B-RNAi (BL32414), UAS-pp1 α 96A-RNAi (BL40906), UAS-TNT-E (BL28838), UAS-GCaMP5attP40 (BL42037), UAS-myr-TdTomato (BL32223). Vienna Drosophila Resource Center: UAS-PKARI-RNAi (VDRC 103720), UAS-PKAR2-RNAi (VDRC 101763), UAS-pp1-13C-RNAi (VDRC 107770), UAS-pp1 α 96A-RNAi (VDRC 105525), UAS-pp2A-RNAi (VDRC 35172). G Boulianne, University of Toronto: UAS-dnc (Cheung et al., 1999). A Kolodkin, Johns Hopkins Med. School: *Sema2a*^{B65} (Wu et al., 2011), *pka R1^{EP(2)2162}* (Park et al., 2000), UAS-PlexinB (Hu et al., 2001). R Ordway, Penn State, State College: *cac*^S, *cac*^{L13}/FM7i, UAS-cac1-eGFP-786C. P Garrity, Brandeis University: UAS-dTrpA1. T Littleton, MIT: *cac*^{NT27}. M Schwaerzel, Freie Universitaet Berlin, Germany: UAS-bPAC (Stierl et al., 2011). S Sigrist, Freie Universitaet Berlin, Germany: GluRIIA-mRFP (Rasse et al., 2005). P Verstreken, Katholieke Universiteit Leuven, Belgium: n-syb-GAL4 (Khuong et al., 2010). CF Wu, University of Iowa: *y rut*² (Bellen et al., 1987), *y dnc*^{M11} *cv v/X^AX y f/Y*, *y dnc*^{M14} *cv/X^AX y f/Y*, (Byers et al., 1981), *dnc*^I (Dudai et al., 1976), *y dnc*² *ec f* (Bellen and Kiger, 1988). B White, NIH and D Kalderon, Columbia University: *yw*; UAS-PKA^{MC*} (UAS-PKA^{act}), *yw*; UAS-BDK35 (UAS-PKA^{inh}) (Li et al., 1995; Davis et al., 1998). S Bernstein, San Diego State University: *Mhc*^I (Wells et al., 1996). A DiAntonio, Washington University: MHC-Sh-CD8-GFP (Zito et al., 1997; Zito et al., 1999). Other lines used were the pan-neuronal elav-GS-GAL4 driver (Osterwalder et al., 2001), and the pan-muscular MHC-GAL4 driver (Schuster et al., 1996). All RNAi experiments included a UAS-Dicer transgene.

For the conditional genetic rescue experiments using GeneSwitch (GS), the elav-GS-GAL4 driver was activated in embryos by feeding parental flies for at least four days with food containing 10 μ g/ml RU486 (mifepristone) prior to collecting eggs. For the bPAC experiments, parental feeding was limited to 24, 48 or 96 hrs, as noted. Larvae with GS activation were also raised on media containing 10 μ g/ml RU486. Control animals were raised under identical conditions, but with media lacking RU486. For the induced expression of UAS-PKA^{act}, parental flies were fed RU486 for four days, and larvae were allowed to develop in RU486-containing food until late first instar stage, after which they were transferred to media lacking RU486.

Immunolabeling of larvae and analysis of ectopic contacts

Crawling third instar larvae were fillet dissected, fixed in 4% paraformaldehyde for 1h at room temperature, and washed in PBS. Primary antibodies were diluted in a solution of 1% BSA diluted in PBS with 0.3% Triton X-100 and incubated at 4° C overnight, whereas secondary antibodies were diluted in PBS for a 4h incubation at room temperature. Primary antibodies and dilutions from stock concentrations were: 1:250 Goat anti-HRP (MP Biomedicals), 1:5000 Rabbit anti-GluRIIC (DiAntonio, Washington U.), 1:250 mouse anti-Brp NC82 Mab (Developmental Studies Hybridoma bank), 1:1000 Rabbit anti-Synaptotagmin (H. Bellen, Baylor College of Medicine). Fluorescent visualization was with Alexa-conjugated secondary antibodies (Molecular Probes) at a 1:1000 dilution. Rhodamine

conjugated phalloidin 1:100 (Molecular Probes) was used to label F-actin in muscle. Labeling of NMJs was with anti-HRP immunocytochemistry, using diaminobenzidine (DAB) visualization (Berke et al., 2013). Frequency of positive immunolabeled ectopic contacts is presented as mean $\pm$ the standard error of the mean, s.e.m.

Ectopic contacts were scored on muscle fibers 7 and 6 in the abdominal segments A2–A7 in 3rd instar larval fillet preparations. An ectopic contact was counted if (1) the native innervation was present, (2) the ectopic innervation was not localized to the native innervation site, and (3) the muscle fibers had no evident defects (tissue damage or mislocalization). Data were collected from 3rd instar larvae where at least 8 hemisegments could be scored. An ectopic contact frequency was determined for each larva, calculated as the percentage of hemisegments scored, in which at least one ectopic contact was observed on muscle fiber 7&6 pairs. These values were then averaged over the number (n) of larvae examined, and are presented as the mean ectopic contact frequency $\pm$ the standard error of the mean, s.e.m. To account for natural variability and independent biological replicates, data for each group was collected from at least three crosses that contained multiple parents (typically 5 females and 3 males) and were set at different time points with the corresponding controls, unless otherwise stated in the text. We defined one data point as the average frequency of ectopic contacts observed in one animal where at least 8 different hemisegments could be scored. This means, for n=10 animals, at least 80 different hemisegments were scored. All data points were included, no outliers were excluded. Data from females and males were pooled unless otherwise indicated. Power analysis for ectopic counts was calculated using GPower software 3.0.10 (University of Duesseldorf, (Faul et al., 2007)). For an alpha level of 0.05 and a power level of 0.8, a sample size of n=9 was calculated for the comparison of two independent means with typical values of 15 $\pm$ 12% and 30 $\pm$ 12% based on the current and previous studies (Jarecki and Keshishian, 1995; White et al., 2001; Carrillo et al., 2010). In this study, sample sizes range from 8 to 51 animals, with a median of 18 and a total average of 20.3 animals considering all analyzed data groups. Statistical significance was calculated using t-test or ANOVA, using Prism 6 Software (GraphPad Software, Inc., La Jolla, CA). * = p<0.05, ** = p<0.01, *** = p<0.001.

Live imaging of *Mhc*¹ mutants

The UAS-GCaMP5-attP40 construct was recombined in a *Mhc*¹ mutant background localized on the second chromosome (Wells et al., 1996). Homozygous *Mhc*¹ mutant embryos are paralyzed (Mogami and Hotta, 1981) but show generally normal presynaptic terminals (Yoshihara et al., 2000). The panneuronal elav^{C155}-GAL4 was used to express UAS-GCaMP5 and UAS-myr-TdTomato. Embryos from 1hr egg lays were incubated at 25 °C for 20hr, then manually dechorionated and mounted on a coverslip in #700 halocarbon oil (Halocarbon Products Corp., River Edge, NJ). Live imaging of GCaMP5 in intact embryos was performed using 488nm excitation on a ZEISS LSM 880 airyscan using a 40 $\times$ W, 1.3 N/A, or a Biorad 1024 confocal microscope using a 16 $\times$ oil, 0.5 N/A or a 40 $\times$ oil, 1.3 N/A objective. Images were taken at 512 $\times$ 512 or 1024 $\times$ 1024 resolution using no averaging function at different imaging rates as noted in the text. 3D reconstruction of the RFP-tagged glutamate receptors was performed using the Airy-scan deconvolution algorithm of ZEN (black edition). For the suppression of synaptic transmission, the UAS-TNT-E (Sweeney et

al., 1995) construct inserted on the 2nd chromosome was recombined in a *Mhc^l* mutant background. Data was collected from motoneuron terminals on muscles 7 and 6 of the abdominal segment A3. Unless noted otherwise, images were taken every 5 seconds, based on the duration of peristaltic waves (Pereanu et al., 2007). Fluorescence values were calculated using ImageJ. The baseline fluorescence (F) was defined as the lowest mean gray value of the ROI after background subtraction for every single image stack.

Optogenetic stimulation of bPAC

The photoactivatable adenylyl cyclase of the bacterium *Beggiatoa* (bPAC) is a second generation light-gated adenylyl cyclase, with low activity in the dark. It increases its activity up to 300-fold after stimulation with 455 nm blue light (Stierl et al., 2011; Efetova et al., 2013; Stierl et al., 2014). In flies, the panneural expression of bPAC strongly elevates brain cAMP levels (Stierl et al., 2011). We used the panneural RU486-inducible *elav-GS-GAL4* driver to target bPAC expression to all neurons in a *rut^l* mutant background. Embryos of the same genotype and from the same parental crosses were collected for a control group reared with no RU486 (non-induced expression), and were compared to the experimental group where RU486 was fed to parents for 24h prior to egg collection (induced expression). Eggs were collected for two hours at 25°C on 3.5 cm yeasted apple juice agar egg-lay plates, and incubated in the dark at 18°C until stage 16. Embryos from both groups were placed in a light-tight illuminating chamber containing three 455nm high power LEDs (Philips Luxeon royal blue) at a distance of ~3 mm away, with a net output power of 4mW, as determined by a Newport 818-SL detector (Newport Instr., Irvine, CA). Photostimulation was for 24h at 22°C, starting at embryonic stage 16 until early larval stage L1, using various illumination protocols. The induction of bPAC activity thus occurred during the previously determined phenocritical period for activity-dependent synaptic refinement at the NMJ (Jarecki and Keshishian, 1995; White et al., 2001). Four oscillatory photostimulation protocols were tested: (1) 8 s illumination followed by 80 s of darkness; (2) 15 s illumination followed by 150 s of darkness; (3) 30 s illumination followed by 300 s darkness; and (4) 30 s illumination followed by 600 s darkness. The net illumination for the first three protocols is the same (1:10 ratio of light:dark), and matches the parameters previously tested for induced Ca^{2+} influx to rescue the miswiring phenotype of the *cac* mutation (Carrillo et al., 2010). In the experiments with continuous light stimulation, embryos were constantly illuminated at a specific light intensity for 24h. After bPAC photostimulation, both groups were kept in the dark until late (wandering) third instar stage, when the larvae were dissected and immunolabeled with anti-HRP. All of the animals analyzed were hemizygous males of the genotype *rut^l/>*; *UAS-bPAC/+*; *elav-GS-GAL4/+*.

Analysis of embryonic motor activity

Embryos were collected as described above, and mounted in halocarbon oil in groups of 2–4 on cover slips. Embryonic movements were recorded at 1 frame per second for 15 minutes at room temperature (21 °C). The number of complete forward and backward peristaltic waves was calculated over the interval or until hatching. Statistical difference was calculated by ANOVA using Prism 6 Software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Low frequency calcium oscillations are observed at growth cones and embryonic motoneuron terminals

During embryonic development *Drosophila* motoneuron growth cones extend processes over multiple muscle fibers, and initially make inappropriate muscle fiber contacts (Halpern et al., 1991; Sink and Whittington, 1991; Chiba et al., 1993). The off-target contacts must be pruned away during an early critical period (late embryo to early 1st instar), otherwise they mature into functional ectopic synapses that remain throughout larval life (Jarecki and Keshishian, 1995). Silencing electrical activity in the motoneuron during this critical period increases the frequency of ectopic motoneuron contacts throughout the bodywall (Jarecki and Keshishian, 1995; White et al., 2001; Carrillo et al., 2010). Given the importance of oscillatory calcium (Ca^{2+}) signaling in the embryo to remove ectopic contacts (Carrillo et al., 2010), we first examined whether periodic Ca^{2+} pulses are indeed present at embryonic growth cones and developing synapses.

We live-imaged intracellular Ca^{2+} signals in growth cones and developing NMJs of intact late stage embryos by expressing the genetically encoded Ca^{2+} indicator GCaMP5, using the panneuronal driver elav-GAL4 (Fig. 1 A–D). Although robust GCaMP5 fluorescence signals were detected in the embryo at developing NMJs (not shown), the movement caused by associated muscle contractions made imaging challenging. Movement was suppressed using a mutation of the myosin heavy chain (*Mhc^I*) gene (Mogami and Hotta, 1981; Mogami et al., 1986; Yoshihara et al., 2000). The Mhc protein is required for contractile function in muscle fibers (Wells et al., 1996), and the mutation permits prolonged time-lapse imaging throughout embryonic development (Fig. 1). *Mhc^I* embryos also show evidence of normal muscle development, innervation, and motor system maturation, albeit with fictive motor activity (Fig. 1 A–H; movie 1–3). The frequency of Ca^{2+} pulses detected using GCaMP was similar to the frequency of muscle contractions due to endogenous motoneuron activity, as previously reported. Figure 1G shows a thirty minute record of Ca^{2+} signals from the developing NMJ of a single motoneuron, RP3, on the ventral longitudinal muscle fibers 7 and 6. Intracellular Ca^{2+} signals are observed in the form of low frequency oscillations (Movie 3), where increases in Ca^{2+} levels are spaced every 2–3 minutes, a pattern similar to that observed in recordings of muscle contraction frequency (Pereanu et al., 2007; Crisp et al., 2008).

Spontaneous wave-like Ca^{2+} activity propagates across the ganglion cells throughout the retina, activating both the superior colliculus and cortex of mice. This spontaneous activity is involved in synaptic refinement within the visual system (Wong, 1999; Ackman et al., 2012). We tested whether the patterned Ca^{2+} oscillations observed at the embryonic *Drosophila* NMJs were also due to spontaneous activity in the motoneurons, or were driven by a central pattern generator (CPG). We therefore blocked synaptic transmission throughout the CNS by expressing tetanus toxin (TNT-E) panneuronally to silence all network activity. Ca^{2+} oscillations at the NMJ were essentially abolished (Fig. 1D; movie 4), showing that they are not arising spontaneously in the growth cones or terminals. By contrast, we observed persistent and regular Ca^{2+} oscillations in a subset of embryonic sensory neurons, despite

tetanus toxin expression and the suppression of movement (Movie 4). This suggests that for those sensory neurons there is an endogenous or pacemaker neural activity that results in oscillatory Ca^{2+} pulses.

The adenylyl cyclase *rutabaga* is required presynaptically for the withdrawal of off-target contacts

The *Drosophila* larval bodywall offers an anatomically stereotypic genetic model system for studying many aspects of neuronal connectivity (Fig. 2A,B) (Budnik, 1996). Errors in connectivity, including those involving activity-dependent refinement, are readily detected in the embryo and larva (Jarecki and Keshishian, 1995). A potential link between neural activity and downstream regulatory events involves Ca^{2+} entry through voltage-gated ion channels, where changes in intracellular Ca^{2+} would activate or inhibit various effectors (Flavell and Greenberg, 2008). In order to unravel the molecular mechanisms underlying activity-dependent synaptic refinement, we first examined the Ca^{2+} -dependent adenylyl cyclase *rutabaga* (*rut*), which has multifaceted roles in the nervous system. These include the synaptic changes involved in long term memory (Dudai et al., 1976), growth cone motility (Kim and Wu, 1996; Berke and Wu, 2002), and in the size and complexity of the larval NMJ (Zhong et al., 1992; Lee et al., 2014). However, there have been no reports that mutations affecting cAMP levels affect either connectivity or synaptic refinement at the NMJ. We therefore examined whether mutations in *rut* would lead to miswired neuromuscular connections, as previously observed for manipulations affecting neuronal activity and Ca^{2+} influx.

The *rut¹* mutation leads to a strong reduction in basal adenylyl cyclase activity, and eliminates its Ca^{2+} /calmodulin activation (Dudai and Zvi, 1984; Livingstone et al., 1984). We observed a significant increase in the frequency of ectopic contacts on bodywall muscles of *rut¹* mutants (Fig. 2D,G) as compared to wild type (WT, Fig. 2C,G; $p < 0.001$). Heterozygous *rut¹/+* mutants had no miswiring phenotype, and resembled the WT controls (Fig. 2G). The weaker *rut²* allele reduces basal adenylyl cyclase activity, but in contrast to *rut¹*, retains its activation by Ca^{2+} (Feany, 1990). *rut²* mutants showed no significant increase in the frequency of ectopic contacts (Fig. 2G). However, a significant phenotype was observed in the *rut¹/rut²* allelic combination (Fig. 2E,G). To test whether the observed phenotype was indeed due to mutations in the *rut* gene, *rut¹* mutants were crossed to flies bearing a chromosomal deficiency that spanned the entire *rut* locus (*rut^{Df}*; Fig. 2H). The frequency of ectopic contacts observed in *rut¹/rut^{Df}* animals was comparable to *rut¹* mutants, showing that the synaptic refinement phenotype maps close to *rut*, and is unlikely due to a second site mutation.

We next examined whether the phenotype observed in *rut* mutants was due to the loss of function (LOF) of *rut* in neurons or in muscles. We expressed wild type Rut cDNA in either all neurons or all muscles in a *rut¹* mutant background, and tested for the suppression of the miswiring phenotype. Expression of Rut using the pan-muscular MHC-GAL4 driver showed no rescue of the *rut¹* phenotype (Fig. 3D). By contrast, expression of Rut using the RU486-inducible pan-neural elav-GSGAL4 driver significantly suppressed the *rut¹* ectopic phenotype when GAL4 activity was induced. A similar rescue was also observed using a

second pan-neural driver, nsyb-GAL4 ($17\% \pm 4\%$, $n=17$; versus *rut¹* $30\% \pm 3\%$). We next tested whether knocking down Rut using RNAi in a tissue-specific manner would phenocopy the connectivity defects observed in *rut¹* mutants. As expected, panneural expression of Rut RNAi using elav-GAL4 (Fig 3C,E; $p<0.01$) or nsyb-GAL4 ($21\% \pm 3\%$, $n=24$) significantly increased the frequency of ectopic contacts, whereas expression of Rut RNAi in all muscles using MHC-GAL4 (Fig. 3E) had no detectable effect.

We next asked whether overexpression of Rut, which would presumably elevate cAMP production, would show effects opposite to those of Rut LOF, and possibly lead to a decreased ectopic frequency. Surprisingly, over-expression of Rut in neurons but not in muscles significantly increased the frequency of ectopic contacts (Fig. 3E). This suggests that experimentally forcing cAMP levels to remain high or low disrupts the normal withdrawal of off-target contacts. To further test this idea we examined a second enzyme known to regulate intracellular cAMP levels, the cAMP phosphodiesterase Dunce.

Mutations in the cAMP phosphodiesterase *dunce* lead to connectivity defects

The *dunce* (*dnc*) gene encodes a phosphodiesterase enzyme that degrades intracellular cAMP (Chen et al., 1986; Qiu et al., 1991), and *dnc* mutants have elevated intracellular cAMP levels (Byers et al., 1981; Davis and Kiger, 1981), and like *rut* mutations have previously been shown to influence the size and complexity of the larval NMJ (Zhong et al., 1992; Koon et al., 2011). We reasoned that *dunce* mutant animals would have defects similar to overexpressed Rut. Indeed, animals with *dunce* mutations that completely eliminate phosphodiesterase activity, such as *dnc^{M11}* (Fig. 4A,C) and *dnc^{M14}* ($27.4\% \pm 3.2\%$, $n=27$), showed a significantly elevated ectopic frequency as compared to WT. The frequency of ectopic contacts was also elevated in the *dnc* alleles that only partially reduce phosphodiesterase activity, such as *dnc²* (Fig. 4C) and *dnc¹* ($25.2\% \pm 4.8\%$, $n=11$), as well as for heterozygotes of all *dnc* alleles tested. A high frequency of ectopic contacts was also observed in heterozygous animals for *dnc^{M11}* and a deficiency spanning the *dnc* locus (*dnc^{M11}/dnc^{DF}*), confirming the genetic specificity of the *dnc* phenotype (Fig. 4C). The miswiring phenotype observed in *dnc^{M11}* and *dnc²* mutants was rescued by crossing them to *rut¹* mutants to generate double heterozygotes that showed WT levels of ectopics (Fig. 4C). This result suggests that *rut* and *dnc* may work in the same functional molecular pathway, presumably in regulating intracellular cAMP in motoneurons during synaptic development.

We next tested whether the observed phenotype in *dnc* mutants was tissue specific. The connectivity phenotype observed in *dnc^{M11}* mutants was rescued by introducing Dnc cDNA exclusively in neurons, whereas pan-muscular expression of Dnc showed no rescue (Fig. 4D). Similarly, expressing Dnc-RNAi panneurally using elav-GAL4 (Fig. 4B,E) or nsyb-GAL4 ($28\% \pm 3\%$, $n=8$) phenocopied the miswiring defects observed in *dnc* mutants, whereas expression of dnc-RNAi in all muscles had no detectable effect (Fig. 4E). Overexpression of Dnc either in all neurons or all muscles did not lead to significant connectivity defects (Fig. 4E).

Ectopic contacts express key synaptic proteins

Several previous studies have shown that ectopic synapses are functional and express various synapse-specific markers (Chang and Keshishian, 1996; Carrillo et al., 2010). We also examined ectopic contacts for several key pre- and post-synaptic proteins that are typically found in functional NMJs (Fig. 5A–D). Larval NMJs fall into different groups according to their morphology and neurotransmitter type. These include glutamatergic type Ib and Is (Johansen et al., 1989), glutamate/octopamine type II (Monastirioti et al., 1995), and glutamate/peptidergic type III NMJs (Gorczyca et al., 1993). The presynaptic proteins Synaptotagmin (Syt) and Bruchpilot (Brp) were detected in over 90% of both type I and II ectopic contacts in both *rut* ($90.2 \pm 1.8\%$, $n=4$) and *dnc* mutants ($95.7 \pm 2.3\%$, $n=3$; Fig. 5C–D). In contrast to their seemingly normal presynaptic features, and consistent with an earlier study (Carrillo et al., 2010), postsynaptic molecular markers were less reliably observed. For example, robust GluRIIC staining was detected in only 20% of the type Ib ectopic contacts that arise in *dnc* mutants (Fig. 5B; $20.8 \pm 12.5\%$, $n=3$). These observations suggest that many ectopic contacts have aberrant postsynaptic features, or that some ectopic contacts are less stable and may start disassembling postsynaptically, before native synapses do, in preparation for metamorphosis.

Intracellular cAMP levels function in the same molecular pathway downstream of neural activity and Ca^{2+} to regulate Sema2a-mediated chemorepulsion

The suppression of embryonic motoneuron activity, resulting in the cessation of peristaltic movement and paralysis, leads to extensive ectopic synaptic contacts (Jarecki and Keshishian, 1995; White et al., 2001; Carrillo et al., 2010). Therefore, we tested the possibility that the miswiring phenotypes of *rut* and *dnc* might also be due to aberrant peristaltic motor activity during embryonic development. For both the *rut* and *dnc* mutations we observed no aberrant motor activity, or any significant change in the frequency of peristaltic waves, as compared to wildtype embryos (*rut*¹: 0.48 waves/min, $n=9$; *dnc*²: 0.46 waves/min, $n=7$; WT: 0.67 waves/min, $n=11$; $p>0.05$). In both mutations the peristaltic waves propagated in the same multisegmental fashion as wild type, suggesting that motor activity was not significantly impaired in either mutation. This is consistent with the hypothesis that cAMP signaling acts downstream of electrical activity and that the connectivity phenotype seen in *rut* or *dnc* is not due to reduced motoneuron excitability.

Elevated ectopic frequency has been observed in animals that bear mutations in the gene that codes for the $\text{Ca(v)}2.1$ channel *cacophony* (*cac*) (Carrillo et al., 2010). We therefore performed genetic interaction tests to determine whether *rut* participates in the same functional pathway as *cac*. We found that a genetic interaction exists between *rut* and the mild hypomorphic *cac*^s allele, which shows an ectopic contact frequency similar to that of control larvae (Fig. 6A,C). One possibility is that in *cac* mutants, low Ca^{2+} levels lead to decreased activation of Rut, which causes cAMP levels to fall lower than normal. In order to increase cAMP levels in a *cac* mutant background, we crossed *dnc*^{M11} mutant males that show elevated cAMP levels to the hypomorphic *cac*^{NT27} allele that shows increased ectopic frequency in *cac*^{NT27/+} heterozygotes, to generate *cac*^{NT27/dnc}^{M11} double heterozygotes. An elevated ectopic frequency was observed, that was no different than either *dnc*^{M11/+} or *cac*^{NT27/+} heterozygotes. Thus, a genetic background that would elevate cAMP levels

constitutively is not sufficient to rescue the *cac* mutant phenotype. Furthermore, in *cac^{NT27}/rut^l* double heterozygotes the ectopic frequency was similar to *cac^{NT27}/+* heterozygotes and was not further increased (Fig. 6C). This observation argues that the effect may not be due to separate pathways yielding an additive effect. These results indicate that synaptic refinement requires functional *cac*, *rut* and *dnc* activity, and that chronic manipulations of these genes lead to connectivity defects.

The chemorepellant semaphorin *Sema2a* is also necessary for normal refinement of neuromuscular connections (Winberg et al., 1998; Carrillo et al., 2010). Therefore, we performed genetic interaction tests between *rut* and *sema2a* and observed an elevated ectopic frequency in the double heterozygotes *rut^l/+ sema2a/+* (Fig. 6B,C). To further test a relationship between *rut* and chemorepulsion, we asked whether the ectopic phenotype observed in *rut^l* homozygotes could be rescued by the panneural over-expression of the *Sema2a* receptor, *PlexinB* (Ayoob et al., 2006). Indeed, *rut^l* hemizygous animals over-expressing *PlexinB* significantly reduced the ectopic frequency (Fig. 6C).

The molecular pathway underlying synaptic refinement includes *Rut*, *PKA*, and *PP1* function

We next examined proteins downstream of cAMP signaling for their potential roles in synaptic refinement. A good candidate is the type II Protein Kinase A (PKA), a heterotetrameric holoenzyme activated by cAMP that is widely expressed in the *Drosophila* CNS (Kuo and Greengard, 1969; Müller, 1997). To test for a potential role of PKA in synaptic refinement, we expressed PKA-RNAi constructs panneurally to selectively knock down either of the two regulatory subunits (R1, R2) or the three catalytic subunits (C1, C2, C3). Intracellularly, the PKA complex consists of a dimer of two catalytic subunits and a second dimer of two regulatory subunits, which dissociates upon cAMP binding, activating the catalytic subunits. Therefore, increased PKA activity is observed by knocking down the regulatory subunits, whereas knock down of the catalytic subunits decreases PKA activity (Li et al., 1995). We observed a significant connectivity phenotype in knockdowns of either the R2, C2, or C3 subunits (Fig. 7A), which is consistent with our model suggesting that too high or too low cAMP levels both lead to connectivity defects. Knockdown of the R1 subunit yielded a slight but not significant increase in the frequency of ectopics, and the RNAi for the C1 subunit had no detectable effect (Fig. 7A). A similar miswiring defect was observed by both the panneural expression of a mutated mouse catalytic subunit that constitutively increases PKA activity (PKA^{act}) or a mutant regulatory subunit that inhibits its activity (PKA^{inh}; (Li et al., 1995; Davis et al., 1998)) (Fig. 7A). Using a second specific PKAR2-RNAi construct yielded similar defects (23% ± 3%, n=20) as well as the examination of *PKA-R2^{null}* homozygous and heterozygous mutants (Fig. 7A). No significant change in the frequency of peristaltic waves was observed in *PKA-R2^{null}* mutant embryos as compared to wild type (PKA LOF: 0.73 waves/min, n=6 vs. WT: 0.67 waves/min, n=11; p>0.05), suggesting that PKA activity likely acts downstream of activity for normal synaptic refinement.

The roles of CaMKII and chemorepulsion have been previously described to be required for normal refinement at the *Drosophila* NMJ (Carrillo et al., 2010). The protein phosphatase 1

(PP1) is a serine-threonine phosphatase that is known to interact with CaMKII and PKA to regulate chemorepulsion (Strack et al., 1997; Ceulemans and Bollen, 2004; Wen et al., 2004). Three fly genes code for the PP1 alpha subtype catalytic subunit, (Lin et al., 1999; Kirchner et al., 2007). Therefore, we tested for a potential role of PP1 in synaptic refinement by expressing RNAi constructs to selectively knock down the three catalytic PP1 genes, pp1-13C, pp1-87B, and pp1 α 96A (Kirchner et al., 2007). RNAi knockdown of each gene led to increased frequency of ectopic contacts, of which pp1 α 96A RNAi showed the highest frequency of ectopic contacts (Fig. 7B). A similar result was also observed by using a second specific pp1 α 96A RNAi construct ($21\% \pm 3\%$, n=14). Pan-neural pp2A RNAi knock down did not show a detectable effect, suggesting that the miswiring phenotype is likely specific to reduced PP1 function, and not to knocking down any phosphatase. A miswiring phenotype was also observed in animals heterozygous for both null alleles *pp1-87^{Bg-3}* and *pp1 α 96A²*, as well as in animals heterozygous for the null allele *pp1 α 96A²* (Fig. 7B). Furthermore, over-expression of pp1 α 96A using elav-GAL4 also increases ectopic frequency (Fig. 7B). These results suggest an optimal range for cAMP levels as well as PKA and PP1 activity for normal refinement, where deviations in either direction lead to connectivity defects.

We next tested whether PP1 acts in the same signaling cascade as cAMP and chemorepulsion for synaptic refinement using genetic interaction tests. Crossing *pp1 α 96A* mutants to *rut* or *sema2a* mutants increases ectopic frequency (Fig. 7B). Interestingly, the elevated ectopic frequency observed in *cac^{L13}* heterozygotes and in *rut^l* homozygous could also be rescued by over-expressing pp1 α 96A (Fig. 7B), suggesting that PP1 function is decreased in these genetic backgrounds. These results indicate that *cac*, *rut*, *pp1* and *sema2a* are likely to function within the same functional genetic pathway. The results are consistent with a model where presynaptic Ca²⁺ signaling regulates cAMP, which acts through PKA and PP1 activity, to modulate the motoneuron's response to muscle-derived chemorepulsion.

cAMP levels are required to be dynamically maintained in an optimal range in embryonic neurons to suppress ectopic contacts

Oscillatory electrical activity at the embryonic NMJ is revealed by low frequency periodic muscle contractions throughout the bodywall musculature (Pereanu et al., 2007; Crisp et al., 2008). This results in an oscillation in Ca²⁺ levels in all developing motoneuron synapses, as revealed using transgenic Ca²⁺ indicators imaged *in vivo* in the embryo (Fig. 1). This raises the possibility that cAMP levels may be regulated in response to dynamically changing levels of presynaptic Ca²⁺. One possibility is that presynaptic cAMP levels oscillate with a pattern similar to that for Ca²⁺ and the resulting dynamic changes in cAMP could be necessary for regulating the withdrawal of off-target contacts. Alternatively, the Ca²⁺ oscillations may be necessary for the homeostatic maintenance of cAMP levels within a specific range. To test whether cAMP levels must oscillate, we expressed a bacterial photoactivated adenylyl cyclase (bPAC) to optogenetically manipulate presynaptic cAMP levels *in vivo* during the critical embryonic period for synaptic refinement. bPAC is an adenylyl cyclase from the bacterium *Beggiatoa* that shows low cyclase activity in darkness, but increases its activity 300-fold in the presence of blue light (455 nm; Stierl et al., 2011; Efetova et al., 2013; Stierl et al., 2014). First, we tested whether baseline bPAC activity in

the dark was sufficient to rescue the ectopic phenotype observed in *rut^l* homozygotes. We used the GeneSwitch GAL4 system to compare embryos with identical genotypes. In constant darkness, elevated ectopic frequency is observed in control animals without induced expression as well as in animals that were fed RU486 for 24h (Fig. 8C). Upon expressing bPAC panneurally in *rut^l* mutants, we used four different light activation protocols (Fig. 8A) to examine whether cAMP levels are required to oscillate with specific patterns for normal synaptic refinement.

The suppression of the *rut^l* ectopic phenotype was observed with periodic bPAC activation protocols that resembled the normal pattern of embryonic motor activity (a cycle of 15s light activation followed by a 150s darkness, 15:150 protocol; fig. 8A,B). By contrast, embryos that were stimulated with a cycle of 8s of light activation followed by a 80s dark interval (8:80 protocol), or of 30s of light activation followed by a 300s dark interval (30:300 protocol) both failed to suppress ectopic contacts (Fig. 8B). Note that the total time that bPAC was activated using the 8:80 and 30:300 protocol is identical to that of a 15:150 protocol. We also did not observe a rescue of the *rut* phenotype with a 30:600 protocol. Therefore, inducing adenylyl cyclase activity in embryonic and early larval neurons, at a low frequency that resembles the WT pattern of electrical activity, is sufficient to suppress the miswiring seen with loss of function of *rut*.

Although these experiments show that oscillations of cAMP at a specific frequency and pattern are sufficient to rescue miswiring, we next asked whether oscillations are also necessary. To test this, we performed two sets of experiments. First, we used constant light to stimulate embryos from parents that were fed RU486 for 24h, using different light intensities that would lead to different levels of constant bPAC activation. A rescue of the miswiring phenotype was observed only in animals that were continuously stimulated using moderate light intensity (0.5 mW/cm²; fig. 8C). By contrast, embryos stimulated using continuous light with low or high light intensities (0.1, 1 and 4 mW/cm², respectively) showed increased ectopic frequency (Fig. 8C). We also tested whether the level of induced bPAC expression would lead to different degrees of baseline cyclase activity in darkness, and consequently, different degrees of rescue. Indeed, animals that were exposed to RU486 for prolonged time periods, such as 48h and 96h, and maintained in constant darkness, showed a significant decrease in the frequency of ectopic contacts. These results suggest that oscillatory cyclase activity is sufficient but not necessary for synaptic refinement.

Finally, we performed genetic tests to determine whether *rut* is positioned downstream of *cac* and thus Ca²⁺ influx. If so, then increasing Ca²⁺ influx in a *rut^l* mutant background would not be expected to rescue the *rut^l* phenotype. By contrast, increasing cAMP levels in a *cac* mutant would suppress the *cac* phenotype. First, we expressed Cac or the temperature sensitive Ca²⁺ permeable channel TrpA1 (Hamada et al., 2008; Pulver et al., 2009) using elav-GSGAL4 in hemizygous *rut^l* animals. Expression of Cac only led to a slight, but significant decrease of the *rut^l* phenotype (Fig. 8D), whereas expression of TrpA1 showed no suppression of the phenotype (Fig. 8D), even though we used the same activation protocol of TrpA1 (15s ON: 150s OFF) known to rescue connectivity defects in *cac* mutants (Fig. 8D; Carrillo et al., 2010). By contrast, a complete rescue of the miswiring phenotype occurred after expression of Rut using elav-GSGAL4 in *cac^{NT27}* hemizygous mutants (Fig.

8D). Similarly, the miswiring phenotype was rescued by expression of bPAC followed by a 15s ON: 150s OFF activating light protocol or in constant darkness after 96h RU486 exposure (Fig. 8D). A similar rescue was observed in *cac^{L13}* heterozygous animals (*cac^{L13}/w;elavGS*, 29.4% $\pm$ 4.0%, n=17; *cac^{L13}/w;elavGS>rut*, 13.3% $\pm$ 5.5%, n=8; *cac^{L13}/w;elavGS>bPAC dark*, 5.0% $\pm$ 1.9%, n=8).

DISCUSSION

The pathway for activity-dependent refinement involves cAMP signaling

In *Drosophila*, motoneuron activity is required for the refinement of embryonic and larval bodywall NMJs (Jarecki and Keshishian, 1995; White et al., 2001; Carrillo et al., 2010). Our results support a model in which patterned electrical activity in motoneurons controls intracellular Ca^{2+} -levels in developing synapses, to regulate the embryonic neuron's response to chemorepulsion (Carrillo et al., 2010). Withdrawal of off-target contacts depends on *Sema2a*, a chemorepellant molecule secreted by muscle that binds to *PlexinB*, the corresponding motoneuron receptor (Matthes et al., 1995; Winberg et al., 1998; Hu et al., 2001; Ayoub et al., 2006).

A role for cAMP at the developing NMJ was first demonstrated by Zhong et al. (1992), showing that size and complexity of the mature larval NMJ was increased in *dunce* mutants. By contrast, the results described in this study point to a distinct and earlier embryonic role for cyclic nucleotide signaling in the regulation of the developing synapse's response to muscle derived chemorepulsion. Central to this effect is the role of activity-dependent Ca^{2+} -entry into the developing NMJ.

Our results show that pan-neural overexpression of *Dnc* did not increase ectopic frequency, although this manipulation should resemble decreased cAMP levels as in *rut* mutants. Overexpression of *Dnc* has been previously reported to only partially reproduce *rut* phenotypes, as in a study examining the formation of satellite synaptic boutons at the larval NMJ (Lee and Wu, 2010). One possibility involves homeostatic mechanisms by other genes, such as *rut* or PKA, to compensate for the phosphodiesterase gain of function. Compensatory changes in expression or activity levels of identified molecular components in different genetic backgrounds should be investigated in future studies. Our results are consistent with previous studies showing that similar phenotypes are observed in *rut* and *dnc* mutants caused by their opposite defects in cAMP synthesis or hydrolysis, which range from poor learning performance (Tully and Quinn, 1985), defects in growth cone behavior (Kim and Wu, 1996), and synaptic function and growth (Renger et al., 1999; Lee and Wu, 2010).

Our data indicate that *PlexinB* function and/or its downstream signaling pathway is likely regulated by Ca^{2+} -entry through *Cacophony*, the $\text{Ca(v)}2.1$ voltage-gated channel, and by multiple Ca^{2+} /calmodulin-dependent downstream targets, such as the adenylyl cyclase *Rutabaga* and *CaMKII* (Carrillo et al., 2010). Extensive molecular interactions exist among key signaling molecules downstream of the Ca^{2+} -oscillations. The rise in cAMP levels due to the Ca^{2+} -dependent activation of adenylyl cyclase would be countered by the cAMP phosphodiesterase, *Dunce* (Davis and Kiger, 1981; Dudai and Zvi, 1984). Cyclic AMP would also activate Protein Kinase A (PKA). PKA, in conjunction with *Calcineurin*, would

activate Protein Phosphatase 1 (PP1) to dephosphorylate CaMKII, reducing its activity (Shields et al., 1985; Blitzer et al., 1998; Oliver and Shenolikar, 1998; Wen et al., 2004). Whether the activity or levels of these downstream signaling molecules also oscillate, phase-locked to the Ca^{2+} oscillation, remains to be determined.

A role for cAMP signaling in synaptic refinement observed here is consistent with *in vitro* studies of cyclic nucleotides and chemotropic signaling (Song et al., 1997; Song et al., 1998; Hopker et al., 1999; Chalasani et al., 2003; Nicol et al., 2011). For example, in the visual system ephrin-A-dependent retraction of cultured retinal axons requires AC1, the Ca^{2+} -stimulated adenylyl cyclase (Nicol et al., 2006). Similarly, the calmodulin-activated adenylyl cyclase ADCY8 regulates slit-dependent repulsion in zebrafish retinal axons (Xu et al., 2010). While the focus of this study has been on cAMP and its downstream signaling, a balance between cAMP and cGMP levels is often a factor governing whether a response is chemoattractive or chemorepulsive *in vitro* (Song et al., 1997; Song et al., 1998; Nishiyama et al., 2003). A role for cGMP in activity-dependent synaptic refinement at the *Drosophila* NMJ remains untested.

Previous *in vitro* studies and mathematical models show that growth cones often display repulsive behavior when Ca^{2+} and cAMP levels are low, due in part to the roles of PKA and PP1 (Ming et al., 1997; Wen et al., 2004; Forbes et al., 2012). We have observed *in vivo* an increased frequency of ectopic synapses following genetic methods that decrease neural activity, Ca^{2+} channel function and cAMP levels, as well as PKA and PP1 function. However, we also observed similar miswiring phenotypes with manipulations that elevate cAMP. These results are consistent with a model where intracellular Ca^{2+} -oscillations may serve to keep cAMP levels and the activity of the downstream effectors rising and falling within physiologically optimal ranges.

In other systems cAMP may also be a factor in modulating Ca^{2+} activity, through its influence on Ca^{2+} -induced Ca^{2+} release (CICR) from internal stores (Gomez and Zheng, 2006; Zheng and Poo, 2007). Accordingly, Nicol et al. (2011) reported that the amplitude of Ca^{2+} transients in growth cones is modulated by cAMP, through the cAMP-dependent activation of the ryanodine receptor (Ooashi et al., 2005). We observed that expression of Rut or bPAC, to elevate cAMP in *cac* mutants led to a full rescue of the miswiring phenotype. Expression of TrpA1 to introduce a constitutive Ca^{2+} -flux into the neurons, failed to rescue the *rut¹* phenotype. We observed a small but significant reduction of the phenotype when *Cac* was overexpressed. One possibility for this difference may be that additional Ca^{2+} -dependent molecules in the pathway are distinctly activated by the overexpression of *Cac* than by the restricted, episodic activation of TrpA1. Although secondary effects on neural activity cannot be completely ruled out, our genetic results thus indicate that the Ca^{2+} -dependent adenylyl cyclase *rut* may act downstream of the Ca^{2+} -channel *cac*.

The major downstream targets of cAMP include PKA (Brandon et al., 1997), “exchange proteins directly activated by cAMP” (EPAC; Gloerich and Bos, 2010), or cAMP-gated ion channels (CNGs; Kaupp and Seifert, 2002) each with distinct activation kinetics or subcellular compartmentalization (Beene and Scott, 2007; Ponsioen et al., 2009).

Pharmacological studies of cultured rat cells show that cAMP mediates growth cone attraction or repulsion, by independently activating either PKA or EPAC (Murray et al., 2009). PKA antagonizes chemotropic axonal repulsion in several systems, and its inhibition promotes the withdrawal of processes (Dontchev and Letourneau, 2002; Chalasani et al., 2003; Parra and Zou, 2010).

In *Drosophila* embryos PKA regulates interaxonal repulsion or defasciculation, mediated by the interaction of membrane-bound Sema1a and its receptor PlexinA (Ayoob et al., 2004). The interaction between cyclic nucleotide signaling and Plexins depends on the A kinase anchoring protein (AKAP) *nervy* (Terman and Kolodkin, 2004). Nervy binds and anchors PKA, and serves to reduce PlexinA chemorepulsion. In our study we find that PKA is required for synapse withdrawal, suggesting that it enhances chemorepulsion when it remains within a specific range of levels. Whether a link exists between AKAPs, PKA, and PlexinB to enhance Sema2a-dependent repulsion at the developing NMJ remains an open question.

A proposed link between cAMP signaling, CaMKII, and synaptic pruning

Our results point to a significant role for cyclic nucleotide-mediated signaling in the motoneuron's response to the retrograde chemorepellant Sema2a, leading to the pruning of off-target synaptic contacts. In a previous study (Carrillo et al, 2010), it was found that CaMKII is required for this form of refinement, where its inhibition resulted in the appearance of excess ectopic synapses. In that study and the present one the relevant molecular systems are regulated by Ca^{2+} , entering the cell through the Cac channel. We hypothesize that the two signaling systems may be linked through the actions of cAMP and the protein phosphatase PP1.

PP1 has previously been shown to serve as a molecular link between cAMP signaling and CaMKII to promote growth cone repulsion (Wen et al., 2004; Forbes et al., 2012). In mammalian systems, PP1 directly dephosphorylates CaMKII to reduce its activity (Shields et al., 1985). In *Drosophila*, the mechanism underlying PP1-regulation and its modulation of chemorepulsion has not been explored in detail. Our results provide some insight as to how PP1 may be regulated at the developing synapse. Elevating PP1-expression in embryonic neurons *rescues* the miswiring phenotype due to the loss of function of either the Ca^{2+} channel Cac, or the Ca-activated adenylyl cyclase Rut (Fig. 7). This suggests that PP1 acts downstream of Ca^{2+} and cAMP signaling, and that its function is depressed when levels of either Ca^{2+} or cAMP are low. Driving PP1 activity well above wildtype levels also has deleterious effects, leading to an increased frequency of ectopic connections (Fig. 7). Assuming that PP1 suppresses CaMKII activity, then this result is consistent with the previous observation that the inhibition of CaMKII results in a miswiring phenotype (Carrillo et al., 2010). PP1 activity is also known to be regulated by the Ca^{2+} -dependent phosphatase Calcineurin (Klee et al., 1998; Oliver and Shenolikar, 1998). Consistent with this, we have recently observed that Calcineurin loss of function mutations similarly disrupt synaptic refinement (Vonhoff and Keshishian, unpublished observations).

Finally, there is additional evidence from the literature linking Ca^{2+} and cAMP signaling with PP1 function. In mammalian neurons, PP1 activity is regulated by protein Inhibitor 1

(I1+), a phosphoprotein that is phosphorylated by PKA, and dephosphorylated by Calcineurin (Blitzer et al., 1998; Oliver and Shenolikar, 1998). While I1+ has no identified fly homologue (Ceulemans et al., 2002; Bennett et al., 2006), known *Drosophila* proteins that inhibit PP1 include Inhibitor-2 (I-2Dm) and Nuclear Inhibitor of PP1c (NIPP1Dm) (Bennett et al., 2003). Whether any of these molecules are regulated in a Ca^{2+} or cAMP dependent manner, like I1+, or whether they play a role in activity-dependent refinement, remains to be tested.

The role of oscillatory activity in synaptic refinement

Neural activity plays a fundamental role during early critical periods for network assembly, as it regulates a variety of cellular events, such as dendritic development (Cline, 2001; Vonhoff et al., 2013), synaptic development (Kelsch et al., 2009), synaptic scaling (Desai et al., 2002), and the proper refinement of synaptic connections (Wiesel and Hubel, 1963; Hubel and Wiesel, 1970; Kakizawa et al., 2000). In many cases, the necessary activity is oscillatory.

An intriguing hypothesis is that synaptic refinement at the NMJ involves the dynamic regulation of chemotropic signaling, orchestrated by Ca^{2+} rising and falling within a specific range during neuromuscular exploration and withdrawal in the embryo. By episodically modulating the response of the motoneuron to muscle-derived Sema2a in an oscillatory fashion, the developing contacts could preferentially explore membrane surfaces when Ca^{2+} -levels are low, and then withdraw the less firmly-associated contacts when sensitivity to the Sema2a chemorepellant rises, during the bouts of Ca^{2+} -entry.

In vitro studies indicate that intracellular cAMP levels also need to oscillate to modulate growth cone responses to ephrin-A5 (Nicol et al., 2007) and to in turn induce filopodial Ca^{2+} -transients (Nicol et al., 2011). Using the photoactivated adenylyl cyclase bPAC we were able to suppress the miswiring phenotype observed in *rut¹* mutants when the cyclase was optogenetically oscillated at a frequency and pattern comparable to that for motor activity in the embryo. No rescue of the *rut¹* phenotype was observed when bPAC was activated using slow or fast activation protocols, or continuous light using either low or high intensities. However, we did find that a reduction in ectopic contacts occurred when bPAC-illumination was adjusted to a specific intermediate level. This suggests that the neuron must maintain an optimal range of cAMP levels for normal synaptic refinement, where levels that fall either too low or too high lead to miswiring defects.

Another possible role for Ca^{2+} -oscillation is to keep levels of cAMP within a physiologically appropriate range. Thus, the episodic rise and fall of Ca^{2+} that regulates cyclase activity, would have a homeostatic function, to maintain cAMP levels within a specific range of values. These observations provide an impetus to determine whether the other downstream Ca^{2+} -dependent signaling systems also oscillate, in a phase locked fashion to the Ca^{2+} -oscillation, and whether oscillations are both necessary and sufficient for chemotropic signal processing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Brett Berke, William Leiserson, Damon Clark, and John Carlson for their advice and comments on the manuscript, and Douglas Olsen for his preliminary analysis of the *rutabaga* and *dunce* mutations. The research was supported by grants to HK from the NIH (5R01NS031651 and 1R21NS053807). Imaging data was partially collected at the MBL in Woods Hole, MA supported by the Grass Foundation Fellowship to FV.

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Figure 1. Low frequency calcium waves in motoneuron terminals require synaptic transmission during late embryogenesis

A. A stage 17e *Mhc^l* embryo (21 hours after egg lay) shows the presence of morphological structures such as ventral dark denticles (asterisks), air-filled trachea (arrow), and uric acid crystals (arrowhead).

B. Bodywall musculature of a dissected *Mhc^l* embryo as revealed by phalloidin staining. Muscle fibers 7, 6, 13 and 12 are shown as orientation.

C–D. Localization of postsynaptic GFP-tagged potassium Shaker channels using the MHC-Sh-CD8-GFP construct (**C**) (Zito et al., 1997; Zito et al., 1999) and postsynaptic RFP-tagged glutamate receptor subunits IIA (**D**) (Rasse et al., 2005) in an intact *Mhc^l* embryo.

E. A stage 17e embryo (21 hours after egg lay (AEL)) expressing panneuronally the membrane-tagged red fluorescent protein Td-Tomato and the calcium indicator GCaMP5 in a homozygous *Mhc^l* mutant background. The central nervous system (CNS) is condensed, and all peripheral nerves and sensory cells are present in all segments. The white square depicts the area shown in B, where the SNb nerve innervates the internal ventral longitudinal muscles 7, 6, 12 and 13 in the abdominal segment A3.

F. Motoneuron terminals from the SNb nerve in segment A3 of a stage 17e embryo as revealed by the basal GCaMP5 fluorescence. Scale bar: 50μm for (A), 10μm for (B).

G. Example of oscillations of intracellular Ca²⁺ in boutons of a single motoneuron, RP3, innervating muscles 7 and 6 in segment A3 as measured by changes in GCaMP5 basal fluorescence (ΔF/F) imaged every 5 seconds. The pattern reveals short periods of activity followed by longer silent periods.

H. Frequency of activity events at the NMJ. For wildtype (white bar), the average frequency of peristaltic waves is shown. For the other two groups intracellular Ca²⁺ signals as

measured by increases in GCaMP5 fluorescence (peaks) per minute recorded from a single motoneuron as described in C in embryos with a control genetic background (elav-GAL4, UAS-myr-TdTomato; *Mhc^l*, UAS-GCaMP5. Gray bar) and in embryos expressing tetanus toxin in all neurons to block synaptic transmission (elav-GAL4, UAS-myr-TdTomato; *Mhc^l*, UAS-GCaMP5/ *Mhc^l*, UAS-TNT-E. Black bar). Scale bar: 50µm for (A), (C), and (E); 30µm for (B); 7µm for (D) and (F).

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Figure 2. The Ca^{2+} -dependent adenylyl cyclase is required for synaptic refinement

A, B. (A) Musculature of the ventral portion of one abdominal hemisegment of a WT third instar larva with a corresponding schematic (B). In (A), the musculature is labeled with rhodamine-conjugated phalloidin (red), while nerves and neuromuscular junctions are labeled with anti-HRP antibodies (green). The white dashed square indicates the area that is shown in images of ectopic contacts in figures 2–6. In (B), the ectopic contacts (red) are shown in the typical locations in which they are observed. TN: Transverse Nerve (green). SNb: Segmental Nerve b (blue). SNd: Segmental Nerve d (gray). Scale bar: 50 μm .

C, D. The transverse nerve (TN) of a wild type (WT) (C) and a *rut¹* mutant (D) third instar larva labeled as above. In (D) a type Ib ectopic contact is visible emerging from the transverse nerve onto muscle fiber 7 (arrow), which is distinct from the native innervation (arrowhead) of MF7 and 6. Scale bar: 20 μm .

E, F. Ectopic contacts in the heteroallelic combination *rut¹/rut²* (E) and the heterozygous *rut^{Df}/+* (F). Arrowhead indicates the native innervation of MF 7 and 6. Scale bar: 20 μm .

G. Frequency of ectopic contacts between WT and various *rut* alleles, calculated as the percentage of ectopic-containing hemisegments per animal, averaged over the number of animals (+ s.e.m.; n = 51, 33, 22, 19, 26, 13, and 12 animals, respectively).

Figure 3. The Ca^{2+} -dependent adenylyl cyclase is required presynaptically for refinement
A–C. The segmental border region of MF7 and 6 of a third instar larvae from WT (A), *rut^l* (B), and a larva expressing *rut*-RNAi in all neurons (C). Ectopic contacts in (B) and (C) are indicated by an arrow. The native innervation is indicated by an arrowhead. Scale bar: 40 μm for (A), 25 μm for (B) and (C).

D. Frequency of ectopic-containing hemisegments per animal in the indicated genotypes ($n=33, 13, 27, 45$, and 20 animals, respectively). Rescue of the *rut^l* phenotype was scored in *rut^l/>*hemizygous mutants bearing a copy of the UAS-*rut* construct in four different conditions: with no GAL4 driver present (*rut^l, UAS-rut* control); with the panneural RU486 inducible *elav*-GeneSwitch-GAL4 (*rut^l, elavGS*) in the uninduced (control) or induced condition; and with the panmuscle MHC-GAL4 driver.

E. Frequency of ectopic-containing hemisegments per animal + s.e.m. in larvae expressing GFP (as control), *rut*-RNAi, or UAS-*rut* either in all neurons ($n= 12, 36, 29$), or in all muscles ($n= 19, 14, 14$).

Figure 4. The cAMP phosphodiesterase is required presynaptically for synaptic refinement

A,B. Ectopic contacts are observed in *dnc^{M11}/+* hemizygous mutants as well as in animals expressing UAS-*dnc*-RNAi in all neurons. Ectopic contacts are indicated by arrows. Arrowheads indicate the native innervations. Scale bar: 20μm.

C. Frequency of ectopic-containing hemisegments in the indicated genotypes (n= 51, 16, 14, 24, 14, 12, 15, 14). To test for statistical significance, all *dnc* groups were compared to WT.

D. Tissue-specific rescue of the *dnc^{M11}* phenotype was assessed by scoring the indicated genetic conditions in a *dnc^{M11}* heterozygous background (n= 14, 22, 20, 21, 15) in a similar way as described in Fig 3D.

E. Frequency of ectopic-containing hemisegments per animal in larvae expressing GFP (as control), *dnc*-RNAi, or UAS-*dnc* panneurally (n= 12, 28, 24), or in all muscles (n= 19, 15, 16).

Figure 5. Ectopic synaptic contacts express both pre and postsynaptic molecular markers

A–D. The segmental border of MF 7 and 6 of third instar larvae stained with antibodies to HRP (blue) to reveal motoneuron endings from: wildtype WT (A), *dnc^{M11}* (B,C) and *rut¹* mutants (D).

B. The postsynaptic dGluRIIC receptor subunit (magenta) is localized in a type Ib ectopic contact (arrow) emerging from the transverse nerve (TN) onto MF 7 in a *dnc^{M11}/>* hemizygous larva. The inset shows a higher-magnification image of the region shown at the top right.

C,D. Ectopic contacts observed in both cAMP mutants contained presynaptic markers such as the active-zone associated protein Bruchpilot (Brp, red) and the vesicular vSNARE protein Synaptotagmin (Syt, green). Ectopic contacts are indicated by arrows. Arrowheads indicate the native innervations. Scale bar: 20µm for A–D; 10µm for inset in B.

Figure 6. Genetic interaction tests indicate that *rut* may function in a pathway that involves Ca^{2+} signaling and Sema2a-dependent chemorepulsion

A–B. Ectopic contacts (arrows) on muscle fiber 6 in double heterozygotes *cac^S/+, rut¹/+* (A), and *rut¹/+, sema2a/+* (B).

C. Frequency of ectopic-containing hemisegments per animal in the indicated genotypes (n= 27, 22, 28, 16, 13, 16, 16, 34, 29, 18). Data from different groups were accordingly compared to *rut¹/+* heterozygotes or homozygotes animals.

Figure 7. Synaptic refinement likely requires protein kinase A and protein phosphatase 1 in a pathway that involves Sema2a-dependent chemorepulsion

A. Frequency of ectopic-containing hemisegments in the indicated genotypes. RNAi constructs for the indicated subunits of PKA were panneurally expressed using the elav-GAL4 driver. To account for the different genetic backgrounds of the RNAi flies, control groups include elav-GAL4 flies crossed to either W1118 or UAS-GFP flies, and experimental lines were compared to their corresponding control for statistical significance. PKAact or PKAinh were panneurally expressed (n= 19, 12, 22, 21, 17, 17, 16, 14, 24). Data from *pka RII^{EP(2)2162}* homozygous (n=18) and heterozygous mutants (n=10) were compared to WT.

B. Frequency of ectopic-containing hemisegments in larvae expressing RNAi for the indicated protein phosphatase genes using the elav-GAL4 driver. The control group includes elav-GAL4 flies crossed to W1118 (n= 19, 22, 16, 11, 22). Data from animals expressing pp1α96A panneuronally (n=18) were compared to the elav-GAL4/W1118 control group. Data from heterozygous animals bearing the single mutation pp1α96A² or the double mutation pp1-87B^{87Bg-3} and pp1α96A² were compared to WT (n=18, 22, 23, 30). Rescue of the miswiring phenotype in *cac^{L13}* heterozygotes and *rut¹* hemizygous by the panneural expression of pp1α96A was assessed by comparing the uninduced to the induced conditions (n=23, 23, for *cac^{L13}*, and n=11, 15, for *rut¹*, respectively).

Figure 8. An optimal range of dynamic cAMP levels downstream of Ca signaling are required for synaptic refinement during embryonic development

A. The photoactivatable adenylyl cyclase bPAC was used for conditional light-dependent production of cAMP, expressed panneurally in *rut¹* mutants. Light intensity in mW/cm² at the stimulation chamber is shown for two activation protocols: 15 s light followed by 150 s dark (upper trace), or 30 s light followed by 300 s dark (lower trace). Embryos were stimulated with cycles of light activation followed by periods of dark from stage 16 to early L1. Larvae were then allowed to develop in darkness until third instar.

B. Frequency of ectopic-containing hemisegments in *rut¹/>*hemizygous larvae expressing bPAC using the panneural elav-GSGAL4 driver in the uninduced (control, light gray) and induced (after parental RU486 feeding, dark gray) condition (n= 45, 47, 41, 35, 29, 20, 12, 16). Embryos for both conditions had the same genotype: they were collected from the same parents with the only difference of RU486 induction of bPAC expression. Embryos were stimulated with a 1:10 duty cycle (either with 8 s light followed by 80 s dark, or with 15 s light followed by 150 s darkness, or with 30 s light followed by 300s dark) or with a 1:20 duty cycle (30 s light followed by 600 s dark).

C. Frequency of ectopic-containing hemisegments in *rut¹/>*hemizygous larvae expressing bPAC. Left bars show data from embryos that were stimulated using continuous light with different intensities (0, 0.1, 0.5, 1, and 4.5 mW/cm²; n=30, 23, 16, 17, 31) following 24h of parental RU486 feeding. Right bars show data from embryos that were kept in constant darkness following the uninduced (control, light gray; n=31) and induced (after parental RU486 feeding, dark gray) condition during different RU486 feeding periods (24, 48, or 96 hours; n=30, 16, 23).

D. Frequency of ectopic-containing hemisegments in the indicated genotypes. Rescue of the *rut¹* phenotype was scored in *rut¹/>*hemizygous larvae expressing cDNA of the voltage-gated Ca(v)2.1 Ca²⁺ channel Cacophony or the temperature sensitive Ca²⁺ permeable channel TrpA1 using the elav-GSGAL4 driver (n=33, 24, 14). Activation of TrpA1 occurred by shifting the temperature during a 1:10 duty cycle of 15 s at 28 °C followed by 150 s at 18 °C. Similarly, rescue of the *cac^{NT27}* phenotype was scored in *cac^{NT27}/>*hemizygous larvae expressing a copy of UAS-rut or UAS-bPAC using the elav-GSGAL4 driver (n=12, 8, 8). bPAC was activated by a pattern of 15s light followed by 150s darkness as described in B.