Akt Regulates Glutamate Receptor Trafficking and Postsynaptic Membrane Elaboration at the *Drosophila* Neuromuscular Junction

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ABSTRACT: **The Akt family of serine-threonine kinases integrates a myriad of signals governing cell proliferation, apoptosis, glucose metabolism, and cytoskeletal organization. Akt affects neuronal morphology and function, influencing dendrite growth and the expression of ion channels. Akt is also an integral element of PI3Kinase-target of rapamycin (TOR)-Rheb signaling, a pathway that affects synapse assembly in both vertebrates and *Drosophila*. Our recent findings demonstrated that disruption of this pathway in *Drosophila* is responsible for a number of neurodevelopmental deficits that may also affect phenotypes associated with tuberous sclerosis complex, a disorder resulting from mutations compromising the TSC1/TSC2 complex, an inhibitor of TOR (Dimitroff et al., 2012). Therefore, we examined the role of Akt in the assembly and physiological function of the *Drosophila* neuromuscular junction (NMJ), a glutamatergic synapse that displays developmental and activity-dependent plasticity. The single *Drosophila Akt* family member, *Akt1* selectively altered the postsynaptic targeting of one glutamate receptor subunit, GluRIIA, and was required for the expansion of a specialized postsynaptic membrane compartment, the subsynaptic reticulum (SSR). Several lines of evidence indicated that *Akt1* influences SSR assembly by regulation of Gtaxin, a *Drosophila* t-SNARE protein (Gorczyca et al., 2007) in a manner independent of the mislocalization of GluRIIA. Our findings show that *Akt1* governs two critical elements of synapse development, neurotransmitter receptor localization, and postsynaptic membrane elaboration.** Develop Neurobiol 73: 723–743, 2013

Keywords: **Akt1; glutamate receptor; Gtaxin; subsynaptic reticulum; membrane trafficking**

Additional Supporting Information may be found in the online version of this article.

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INTRODUCTION

Synaptic plasticity requires molecular and morphological changes that allow previous activity to shape the physiological properties of synaptic communication. Secreted protein growth factors such as brain-derived neurotrophic factor play essential roles in synaptic plasticity, directing developmental and activity-dependent changes at these specialized cell junctions (Lauterborn et al., 2007). While an expanding set of growth factors are being identified as important determinants of synaptic plasticity, the molecular outputs of these signaling systems are less well understood (Rawson et al., 2003; Salinas, 2003). One signaling molecule of central importance for the integration of many growth factor inputs is the serine-threonine kinase Akt (Franke, 2008). In mammalian systems, three Akt isoforms govern a range of cellular and physiological processes from cell growth to membrane trafficking (Zhang et al., 2002; Manning and Cantley, 2007). *Akt1* plays critical roles in cell growth and cell survival (Chen et al., 2001). Akt phosphorylation of AS160 influences exocytosis of glucose transporter-containing vesicles, providing an increased capacity for glucose transport across the plasma membrane (Gonzalez and McGraw, 2006; Watson and Pessin, 2006; Grillo et al., 2009). Consistent with a role of Akt in glucose uptake and homeostasis, mice null for *Akt2*, expressed ubiquitously in all cell types, show defects in insulin-stimulated glucose uptake (Nakatani et al., 1999; Cho et al., 2001; Bae et al., 2003; Easton et al., 2005; McCurdy and Cartee, 2005). *Akt3*, the isoform expressed most abundantly in the central nervous system, is essential for normal brain growth affecting both the number and size of neurons (Tschopp et al., 2005). Akt signaling is also known to govern neuronal morphology and synapse development directly (Dudek et al., 1997; Grider et al., 2009; Lee et al., 2011). Phosphorylation of the type A GABA receptor by Akt increases its localization to the synapse (Serantes et al., 2006). Akt regulates dendrite formation in *Drosophila* peripheral sensory neurons, demonstrating the capacity of this kinase to govern membrane processes that influence synaptic function (Parrish et al., 2009). The central role of Akt in signal integration prompted us to explore its function in the development of the *Drosophila* neuromuscular junction.

The *Drosophila* neuromuscular junction is a powerful model for molecular analysis of synapse development and plasticity. Each muscle of the larval body wall is innervated by identifiable motoneurons, and these peripheral synapses are well described at the molecular, morphological, and physiological levels (Jan and Jan, 1976; Gramates and Budnik, 1999; Ruiz-Canada and Budnik, 2006; Schuster, 2006). The *Drosophila* NMJ is a synapse that expands greatly during larval growth, and the dynamic matching of pre- and postsynaptic elements is critical for its assembly. The growth of the NMJ is accompanied by the expansion of a specialized postsynaptic membrane, the subsynaptic reticulum (SSR), as well as the regulated expression of specific glutamate receptor subunits. GluRIIA is critical for the functional strengthening and morphological growth of the synapse that accompanies muscle expansion during development (Petersen et al., 1997; Sigrist et al., 2002).

We have explored the function of the single *Akt* gene in *Drosophila*, *Akt1*, in synapse assembly and function using the NMJ as a model. We demonstrate that *Akt1* is required for the developmentally regulated expansion of the SSR, in addition to regulating glutamate receptor composition. These findings demonstrate that *Akt1* serves a critical role in two fundamental elements of synapse development.

MATERIALS AND METHODS

Fly Stocks

All fly strains were raised in standard cornmeal food at 25°C during embryogenesis and 30°C during larval development under a 12-h/12-h day/night cycle, unless otherwise stated. *Oregon-R* strain served as the wild type stock. $Akt1^{1}/TM3$ and $Akt1^{04226}/TM3$ were obtained from the Bloomington *Drosophila* Stock Center (BDSC). $Akt1^{04226}$ is a *P*-element insertion and hypomorphic allele. The null allele $Akt1^{1}$ is embryonic lethal, but $Akt1^{1}/Akt1^{04226}$ transheterozygotes are semi-viable and some survive to the adult stage. *G14-GAL4*, *24B-GAL4*, *Mef2-GAL4*, and *elav-GAL4* transposon-containing stocks (BDSC) were used for muscle and neuronal-specific expression of $UAS\text{-}Akt1^{RNAi}$ (Vienna Drosophila RNAi Center (VDRC) #103703), $UAS\text{-}Gtx^{RNAi}$ (VDRC #105113), *UAS-Gtaxin* (from V. Budnick, University of Massachusetts (Gorczyca et al., 2007)), *UAS-GluRIIA-mRFP* (Kittel et al., 2006), and *UAS-mCD8-GFP* (BDSC #5137) constructs, respectively. Protein trap line *Bsg-GFP* (Flytrap #G00311) directs the expression of GFP-tagged Basigin under the control of its endogenous promoter. *UAS-DicerII* was used together with *elav-GAL4* to increase the effectiveness of RNA interference in neurons (Dietzl et al., 2007). The temperature-sensitive GAL80 repressor, $GAL80^{ts}$ under tubulin promoter ($Tubp\text{-}GAL80^{ts}$, from BDSC), was combined with *Mef2-GAL4* line for temporal control of $UAS\text{-}Akt1^{RNAi}$ expression in the muscle (Zeidler et al., 2004). $GAL80^{ts}$ suppressed GAL4 function at the permissive temperature (18°C). At the restrictive temperature (30°C), $GAL80^{ts}$ released GAL4, allowing its

binding to the UAS, and inducing the expression of $Akt1^{RNAi}$. To inhibit the expression of *Akt1* at the early developmental stage, embryos were kept at 30°C for 2 days and then raised at 18°C until they reached third instar larval stage. In contrast, animals, which *Akt1* was suppressed at the late stage, were raised at 18°C until second instar stage and then shifted to 30°C for 2 days before immunohistochemistry. The constitutively active forms of *Akt1* ($Akt1^{CA}$) and GFP tagged $Akt1^{CA}$ ($Akt1^{CA}$-*GFP*) were generated using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), resulting in the replacement of amino acids Threonine 342 (ACC) and Serine 505 (AGC) with Aspartic Acid (GAC). The $Akt1^{CA}$ and $Akt1^{CA}$-*GFP* constructs were cloned into *pUAST-attB* vector and then integrated into the third chromosome (99F8) by site-specific *P*-element mediated germline transformation (Rainbow Transgenic, CA).

Immunohistochemistry and Confocal Microscopy

The third instar larval muscles were dissected in ice-cold Ca^{2+} free HL-3 media and fixed with either 4% paraformaldehyde for 30 min or Bouin's fixative solution for 5 min (for glutamate receptor subunits antibody immunostaining) or 15 min (for Gtaxin antibody immunostaining). All subsequent washes were performed in PBST (0.5% triton X-100 in phosphate buffered saline (PBS)). A total of 5% normal goat serum in PBST was used for sample blocking and antibody incubations. Primary antibodies mouse anti-glutamate receptor IIA antibody (1:50, 8B4D2, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA), rabbit anti-glutamate receptor IIB and IIC antibodies (1:2000 from D. Featherstone, University of Illinois at Chicago), mouse anti-DsRed (1:500, Santa Cruz Biotechnology), rat anti-Syndapin (1:100, from M. Ramaswami, University of Arizona), rat anti-Gtaxin (1:200, from V. Budnick, University of Massachusetts), rabbit anti-Dorsal and Cactus antibodies (1:1000, from S. Wasserman, University of California, San Diego), mouse anti-Discs large (1:500, 4F3, DSHB), mouse anti-Cysteine string protein (1:1000, 6D6, DSHB), mouse anti-α-Spectrin (1:1000, 3A9, DSHB), and mouse anti-Bruchpilot (1:1000, nc82, DSHB) were incubated with sample for at least 12 h at 4°C. Alexa-fluorescence conjugated secondary antibodies were obtained from Life Technologies (Grand Island, NY).

Images were acquired using an Olympus Fluoview FV1000 laser scanning confocal microscope (Olympus America, Lake Success, NY). Quantification of protein levels were performed using Imaris 7.3 (Bitplane, Saint Paul, MN) and ImageJ1.42q (NIH) software for image processing and analysis. Serial images taken by confocal microscopy were reconstructed into 3D images using Imaris without any other processing. Immunoreactivity-positive voxels were then assayed by counting the total number of voxels (Abundance) and by measuring their average fluorescent intensity. Both of the values were further normalized by muscle size for each preparation.

Western Blotting Analysis

Total protein was prepared from dissected third instar larval muscle tissues in SDS-loading buffer and ran on 9% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), polyvinylidene difluoride (PVDF) membrane was incubated overnight with anti-phosphorylated Akt1 or anti-β-Actin antibodies (Cell Signaling Technology, MA) in blocking solution (5% w/v nonfat dry milk in 0.5% Tween-20 in Tris-buffer saline) at 4°C. Signals were amplified using horseradish peroxidase (HRP) conjugated secondary antibody and detected using Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, IL).

Transmission Electron Microscopy

The third instar larval muscles were dissected in ice-cold Ca^{2+} free HL-3 media and fixed in buffer (1.5% glutaraldehyde, 2.5% paraformaldehyde, 1.8 m*M* Ca^{2+} in 0.1*M* Na-cacodylate, pH 7.4) at 4°C overnight. Postfixation was done in 1% osmium tetroxide, and en bloc staining was performed with 2% uranyl acetate in dark condition. The samples were rinsed in 0.1*M* sodium cacodylate buffer (pH 7.4), dehydrated and infiltrated, embedded in Spurr's resin, and sectioned to 70 nm slices. The images were taken with a transmission electron microscope (JEOL1200, Tokyo, Japan) and analyzed by ImageJ1.42q (NIH).

Electrophysiology

Excitatory junction potentials (EJPs) and miniature excitatory junction potentials (mEJPs) were recorded at room temperature from muscle 6 of abdominal hemi-segment A3 in third instar larvae (Rawson et al., 2003). The third instar larvae were dissected in ice-cold Ca^{2+} free HL-3 media and recordings were performed with larvae in HL-3 media containing 1.2 m*M* Ca^{2+}. Muscle 6 of A3 was impaled with the recording electrode and before stimulation, recordings were taken for 1 min to measure spontaneous activities (mEJPs) (Stewart et al., 1994). Following the recording of mEJPs, evoked EJPs were elicited in the same muscle with 1 Hz pulses. A total of 1 nA of current was injected for 200 ms to record plasma membrane resistance and capacitance. Recordings were acquired with Axoclamp 2B amplifier and Clampex 9.2 software (Axon Instruments, CA). Only the recordings with resting membrane potentials lower than −60 mV were included in this analysis. EJP and mEJP amplitudes and kinetics were analyzed with MiniAnalysis (Synaptosoft, Fort Lee, NJ).

Statistical Analysis

Statistical analyses for quantitative data were performed in Minitab Release 16 (Minitab, State College, PA). All data points were presented as mean ± SEM and analyzed using Student's *t*-tests for normally distributed data or *post hoc* Tukey–Kramer for pairwise comparisons of data with non-normal distributions.

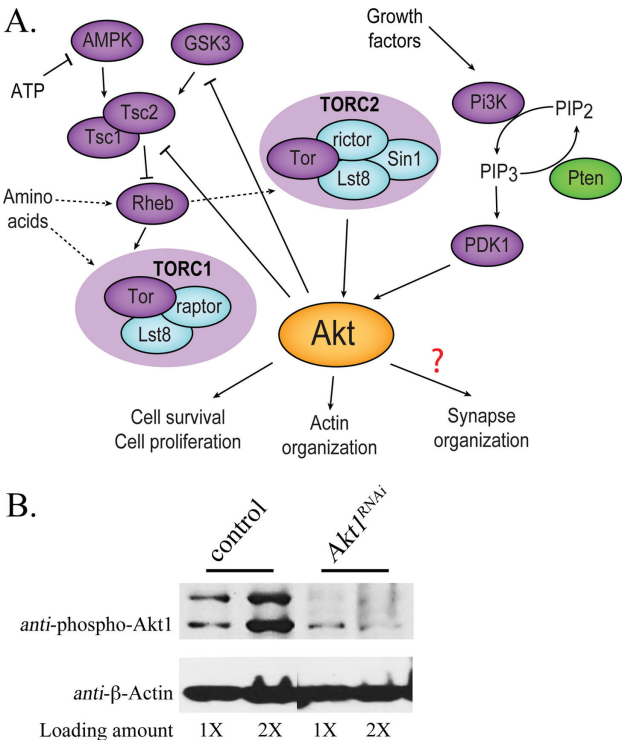

Figure 1 The Akt signaling system and level of Akt1 knockdown using RNA interference in *Drosophila*. A: In this summary, kinases Rheb, and Tsc1/2 are purple symbols, phosphatases are green, and other components of TOR1 and TOR2 complex are blue. Akt1 is activated by growth factors via Pi3K and PDK1 and by nutritional sensing through Tsc1/2 and TOR complexes. Relationships that are not fully understood or have several possible intermediary steps are shown as dashed arrows or a question mark (adapted from Dimitroff et al., 2012). B: Akt1 function was compromised by muscle-specific expression of an $Akt1^{RNAi}$ construct using the *GAL4-UAS* system. The level of phosphorylated Akt1 was measured by Western blot. Total muscle proteins were prepared from third instar larval muscles of control animals (*UAS-*$Akt1^{RNAi}$ transgene only; *UAS-*$Akt1^{RNAi}$/+) or $Akt1^{RNAi}$ animals with muscle specific knockdown of Akt1 using *24B-GAL4* driver (*24B-GAL4>UAS-*$Akt1^{RNAi}$). Akt1 was dramatically decreased in muscle tissue expressing $Akt1^{RNAi}$ as compared with controls. Measures of β-Actin were used as a protein loading controls. Total proteins extracted from either one (1×) or two larvae (2×) were loaded.

RESULTS

Akt1 plays a central role in a number of signaling processes, acting both downstream and upstream of growth factor and target of rapamycin-directed events. The Akt1 kinase governs a number of cellular activities including cell proliferation, cell survival, and cytoskeleton organization [Fig. 1(A)]. Given these diverse and critical functions, we explored the role of *Akt1* in synapse assembly. In addition to the well-described *Akt1* mutant alleles (Staveley et al., 1998; Mozden and Rubin, 1999; Guo and Zhong, 2006), we used an $Akt1^{RNAi}$ transgene (Dietzl et al., 2007) to inhibit *Akt1* function selectively in either motoneurons or muscle cells. To assess the level of inhibition achieved by the $Akt1^{RNAi}$ construct, we measured the level of phosphorylated Akt1 (active form of Akt1) by western blot. Using a muscle-directed *GAL4* to drive the expression of *UAS-*$Akt1^{RNAi}$, phosphorylated Akt1 protein was reduced to 24.2% of wild-type level in third instar larval muscle tissue [Fig. 1(B)].

We began assessing the role of *Akt1* in NMJ assembly by examining the distribution and level of glutamate receptor IIA (GluRIIA), one of the neurotransmitter receptor subunits at this glutaminergic synapse. Glutamate is the major excitatory neurotransmitter at the type I bouton of the *Drosophila* larval NMJ (Brunner and Okane, 1997; Collins and DiAntonio, 2007). The NMJ glutamate receptor (GluR) is a heterotetramer comprised of three invariant subunits: GluRIIC, D, and E. The fourth subunit, either GluRIIA or B, determines the type and the electrophysiological properties of the receptor

(DiAntonio et al., 1999; Featherstone et al., 2005; Qin et al., 2005a; DiAntonio, 2006). Subunit GluRIIA and B competitively bind to GluRIIC; hence, the preferential expression of these two subunits constitutes one element of developmental plasticity exhibited by this synapse (Marrus et al., 2004). We examined the levels and distributions of GluRIIA using a well-characterized monoclonal antibody, anti-GluRIIA (Featherstone et al., 2002; Qin et al., 2005a; Karr et al., 2009). The specificity of this antibody has been well documented by showing that immunoreactive signal is lost in GluRIIA null mutant (Marrus et al., 2004). Partial loss of *Akt1* function, achieved with the heteroallelic combination $Akt1^{1}$/$Akt1^{04226}$, altered GluRIIA distributions and levels, with a reduction at postsynaptic structures and the appearance of GluRIIA immunoreactivity within repeated bands throughout the muscle cells [Fig. 2 compare (A), (B) to (C), (D)]. This latter phenotype was more prominent in muscles 15 and 16 and was observed to a lesser extent in muscles 6 and 7, the postsynaptic cells typically used for electrophysiological analysis [arrowheads in Fig. 2(C,D)].

Directed expression of an $Akt1^{RNAi}$ in either muscle or neuron using the *GAL4-UAS* binary system (Brand and Perrimon, 1993) provided the means of assessing the cell-type specific requirements for *Akt1*. We used multiple muscle-specific *GAL4* driver lines, *G14, 24B*, and *Mef2* to confirm that the associated phenotypes were due to muscle-directed RNAi expression, but not expression in some alternative cell types. These *GAL4* drivers showed some differences in the level of transcriptional activity, but all induced similar *Akt1* knockdown phenotypes for GluRIIA localization and SSR expansion. Muscle-specific expression of $Akt1^{RNAi}$ produced a dramatic loss of GluRIIA at the synapse and its redistribution into intracellular bands in the muscle cell, confirming the phenotype observed in $Akt1^{1}$/$Akt1^{04226}$ mutants [Fig. 2 compare controls shown in (E–H) to muscle cell-directed $Akt1^{RNAi}$ animals in (I–L)]. Knockdown of *Akt1* in the motoneuron had no effect on GluRIIA distribution (data not shown). *GAL4*-directed transcriptional activation is temperature-dependent, allowing for different levels of $Akt1^{RNAi}$ expression and consequently loss of *Akt1* function, by simply rearing the animals at different temperatures. At 18°C, GluRIIA distributions were normal, but with decreasing levels of *Akt1* function produced at 25°C and 30°C, GluRIIA was progressively lost from the postsynaptic site and increasingly localized within intracellular bands [Fig. 2 compare control animals, panels (E–H), to muscle-specific $Akt1^{RNAi}$, panels (I–L); in enlarged images (H) and (L), arrows indicate synaptic boutons; arrowheads indicate GluRIIA in bands]. Although GluRIIA failed to localize to the postsynaptic specialization upon inhibition of *Akt1* function, we did note a net and significantly increased level of GluRIIA within intracellular structures [Supporting Information Fig. 1(A), animals reared at 30°C]. These findings established that localization of GluRIIA was affected by reductions of *Akt1* function mediated by $Akt1^{RNAi}$ transgene expression in the postsynaptic cell.

To further explore the mechanism of the dramatic redistribution of GluRIIA achieved by knockdown of *Akt1*, we examined the expression pattern of an mRFP-tagged GluRIIA derived from a UAS-transgene. This provided the opportunity to visualize the transgenic GluRIIA-mRFP by both fluorescence of the mRFP protein, and immunodetection of the polypeptide with an anti-RFP antibody, anti-DsRed. Consistent with our earlier results looking at endogenous GluRIIA, compromising *Akt1* function produced loss of GluRIIA-mRFP at the synapse, detected by either mRFP fluorescence or anti-RFP antibody [Fig. 3 compare (B), (C) to (F), (G)]. Interestingly, the redistribution of GluRIIA-mRFP to intracellular bands was only detected with the anti-RFP antibody, but not by monitoring the fluorescence of the mRFP-tagged receptor subunit [Fig. 3(F,G)]. In control animals, the RFP-fluorescence pattern precisely overlaps the anti-RFP signal [Fig. 3(B,C)]. This result suggests that reduction of *Akt1* function may disrupt the structural integrity of GluRIIA-mRFP, resulting in loss of its native fluorescence, whereas the RFP-epitope is found redistributed to intracellular membrane structures.

We have also examined the developmental window during which *Akt1* is essential for GluRIIA localization by using the temperature-sensitive $GAL80^{ts}$ system (Zeidler et al., 2004). When a $GAL80^{ts}$ transgene is present with *GAL4-UAS* components, the GAL80 suppresses the activity of the transcriptional activator GAL4, preventing expression of the UAS-transgene, in this case, *UAS-*$Akt1^{RNAi}$. Raising the temperature to restrictive level inactivates $GAL80^{ts}$ and permits expression of the $Akt1^{RNAi}$. We used this system to inactivate *Akt1* during different developmental stages. Reduction of *Akt1* function during a 2-day window early in development (embryo-first instar larva) produced some redistribution of GluRIIA into intracellular stripes, whereas a later 2-day inactivation window in third instar larval stage merely reduced the levels of GluRIIA at the synapse [Fig. 3(I–P)]. These data suggest that the redistribution of GluRIIA observed with reduction of *Akt1* throughout development is not merely the result of a failure of synaptic stabilization

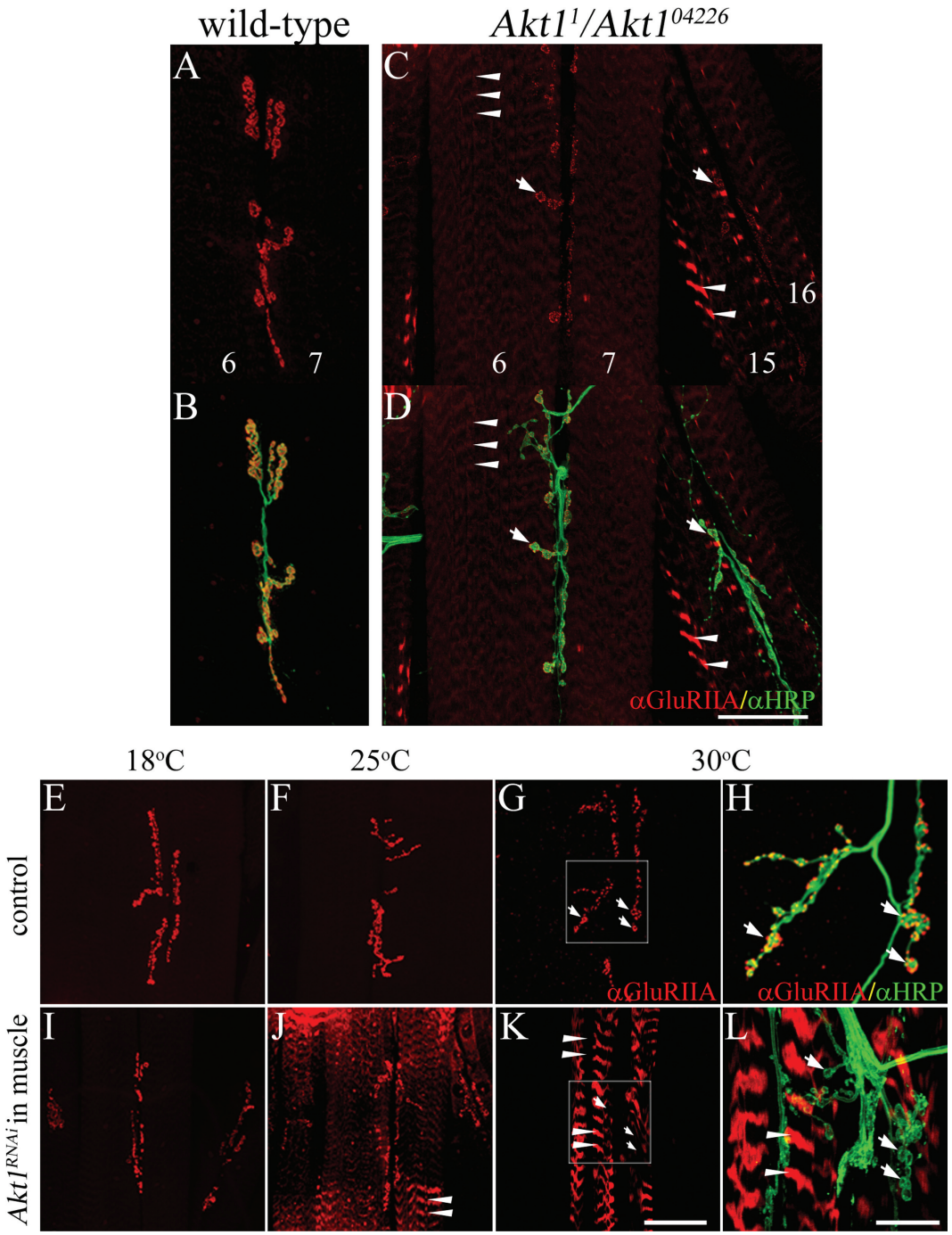

Figure 2 GluRIIA localization was modified in *Akt1* mutants and animals with muscle-specific inhibition of Akt1. GluRIIA localization was examined in muscles 6 and 7 using monoclonal anti-GluRIIA antibody (red). Anti-HRP antibody detected neuronal projections (green). A and B: In wild-type animals, GluRIIA was located in the postsynaptic specialization that surrounds the motoneuron boutons. C and D: $Akt1^{1}/Akt1^{04226}$ mutants showed reduction of GluRIIA at synaptic boutons (see arrows) and redirection to intracellular bands (faint staining in muscles 6 and 7, and more prominent in muscles 15 and 16; see arrowheads). E–L: Akt1 function was compromised by muscle-specific expression of an $Akt1^{RNAi}$ construct using the *GAL4-UAS* system. *UAS-*$Akt1^{RNAi}$/+ animals served as controls. GAL4 transcriptional activation shows temperature dependence, permitting a graded level of *Akt1* blockade from 18°C (low level of inhibition) to 30°C (high level of inhibition). E–H: In control larvae, GluRIIA immunoreactivity was concentrated in the postsynaptic region surrounding boutons at all temperatures. H: Enlarged view of white box area in (G), arrows show the motoneuron boutons surrounded by GluRIIA. I–L: In $Akt1^{RNAi}$ expressing larval muscle (*24B-GAL4>UAS-*$Akt1^{RNAi}$), GluRIIA mislocalization (arrowheads) was more severe with greater inhibition of Akt1 function at increasing temperature (larvae reared at 18°C (I), 25°C (J), or 30°C (K and L)). L Enlarged view of white box area in (K), arrows show synaptic boutons lacking GluRIIA immunoreactivity; arrowheads mark ectopic GluRIIA within intracellular bands. Scale bar in (A–G) and (I–K), 50 μm, in (H) and (L), 5 μm.

because the levels of GluRIIA would likely recover quickly from new synthesis (Rasse et al., 2005) but is affecting a process occurring in early development that alters GluRIIA production and delivery to the synaptic specialization.

We also examined the effect of *Akt1* on two potential downstream targets, Dorsal and Cactus (*Drosophila* homologs of NF-κB and Iκ-B, respectively). These two proteins have recently been shown to localize to postsynaptic specializations and regulate glutamate receptor levels at the NMJ (Heckscher et al., 2007). While Dorsal and Cactus have been well characterized as transcriptional activator proteins, their activity at the NMJ is posttranscriptional, affecting the localization or stabilization of glutamate receptors in the SSR (Heckscher et al., 2007). To determine whether *Akt1*'s effects on GluRIIA localization could be mediated at least in part by an influence on Dorsal or Cactus, the levels and distributions of these two proteins at the NMJ were evaluated. As previously described, Dorsal and Cactus were concentrated in postsynaptic specializations at type Ib boutons in control animals [Fig. 4(A,B,E,F)]. Upon RNAi knockdown of *Akt1*, both Dorsal and Cactus levels significantly decreased at the NMJ [Fig. 4(C,D,G,H)] [Supporting Information Fig. 1(B)]. In addition to the reduction of Dorsal levels at the NMJ, Dorsal was mislocalized in a number of animals (23.8% penetrance) and partially colocalized with GluRIIA into intracellular bands in the muscle cell [Fig. 4(L–N); arrowheads indicate the bands of GluRIIA and Dorsal, arrows indicate synaptic boutons]. Although the penetrance of this phenotype was modest, it was reproducible across three different sets of experiments. These findings showed that Akt1 affects the levels of two potential Akt1 downstream targets known to regulate GluRIIA levels, and suggest the possibility that Akt1 regulates GluRIIA at least in part via the control of Dorsal and Cactus.

The ability of *Akt1* to affect the trafficking of one glutamate receptor subunit to the postsynaptic specialization suggested the possibility that this mechanism could regulate GluR subunit composition. The distributions of glutamate receptor subunits IIB and IIC were therefore examined in animals with knockdown of *Akt1* in the muscle. In the animals with reduced *Akt1* function, GluRIIB, the functional alternative to IIA, remained at the synapse under conditions where GluRIIA was localized almost exclusively within intracellular bands [Supporting Information Fig. 2(H–K)]. The correct delivery of GluRIIB to the postsynaptic specialization when *Akt1* function was compromised with $Akt1^{RNAi}$ was confirmed by showing its spatial colocalization with Bruchpilot, a presynaptic protein required for active zone function [Supporting Information Fig. 2(E–G) for control and (L–N) for *Akt1* knockdown] (Wagh et al., 2006). The correct delivery of GluRIIB is consistent with the observation that these larvae were motile, and that a functional receptor must contain either GluRIIA or GluRIIB. Likewise, the essential subunit GluRIIC was appropriately localized to the postsynaptic specialization in the face of reduced *Akt1* function (data not shown). *Akt1* is therefore selectively regulating the delivery of GluRIIA to the synapse, and reductions in *Akt1* result in mislocalization of IIA to an intracellular compartment.

The selective requirement for *Akt1* function to correctly localize GluRIIA but not the other receptor subunits begs the question as to whether other proteins require *Akt1* for correct targeting to the postsynaptic specialization. Therefore, we have examined three other synaptic components: Discs-Large (DLG), the homolog of mammalian PSD-95; Syndapin, an F-BAR domain-containing protein; and Basigin, a transmembrane protein located principally in the SSR. Both DLG and Syndapin promote SSR expansion (Lahey et al., 1994; Budnik et al., 1996; Guan et al., 1996; Kumar et al., 2009) and associate with SSR membrane following their translation in the cytoplasm (Thomas et al., 2000). Basigin is a synaptic transmembrane protein located principally in the postsynaptic SSR and is required for synaptic function (Besse et al., 2006, 2007). Reduction of *Akt1* function in the muscle to a degree that completely disrupted GluRIIA localization did not alter the selective targeting of Basigin to the synapse [Supporting Information Fig. 3, compare (A) to (B)]. DLG and Syndapin, the two cytoplasmically synthesized and SSR-associated proteins, showed normal localization to the postsynaptic specialization of the NMJ [Supporting Information Fig. 3 compare controls without *GAL4* driver, (C–E) to muscle-specific *24B-GAL4>UAS-*$Akt1^{RNAi}$ animals in (F–H)]. Quantitation of the immunofluorescence signal for these proteins did show significantly reduced levels of Basigin and Syndapin, whereas DLG signal was lower but did not achieve statistical significance (Supporting Information Fig. 4). Taken together these findings demonstrated that the mislocalization of GluRIIA upon reduction of *Akt1* is specific, and does not affect the localization of other transmembrane (Basigin, GluRIIB) or cytoplasmically synthesized (Dlg, Syndapin) postsynaptic proteins.

The SSR is a complex postsynaptic membrane specialization that requires the activity of a number of proteins for its growth and maintenance, including DLG, Syndapin, and the *Drosophila* t-SNARE

Gtaxin (Lahey et al., 1994; Budnik et al., 1996; Gorczyca et al., 2007; Kumar et al., 2009). Given that reductions in *Akt1* function affected the levels of synaptic proteins Syndapin and Basigin, it was of interest to determine if *Akt1* affected the elaboration of the SSR. The ultrastructure of the SSR was evaluated in animals with reduced *Akt1* function using transmission electron microscopy (TEM) of NMJ synaptic

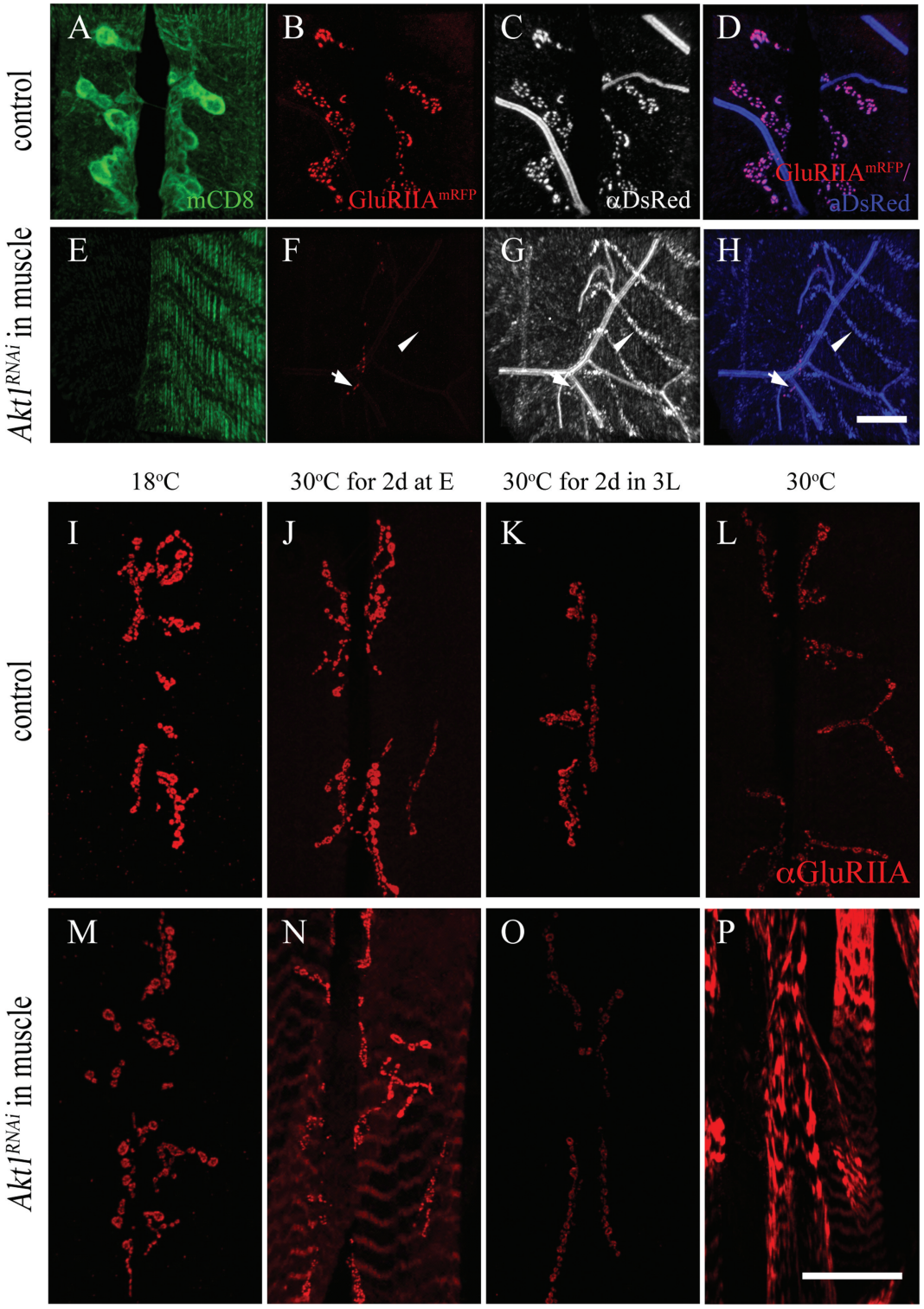

Figure 3

boutons. At the *Drosophila* NMJ, the motoneuron boutons are "embedded" in the surface of the muscle cell (Jia et al., 1993). The tubulo-membranous SSR is seen as a complex set of multilayered membranes within the muscle cell and surrounding the nerve terminal [Fig. 5(A)]. The dimensions and complexity of the SSR were reduced in larvae expressing $Akt1^{RNAi}$ in the muscle cell without affecting the length of the presynaptic active zones [Fig. 5(A–C)]. These experiments demonstrated that *Akt1* was required for the proper expansion of the SSR.

Gtaxin mutant shows reduced SSR elaboration as well as changes in the complex membrane architecture of the muscle cell (Gorczyca et al., 2007). This architecture was revealed by labeling all muscle membranes with a mouse transmembrane protein, mCD8-GFP, and performing 3D reconstruction of optically sectioned cells. The mCD8-GFP integral membrane protein tag uncovered a cortical membrane compartment above the muscle nuclei, and a subcortical membrane network below the nuclei intermingled with the contractile apparatus. The cortical membrane compartment was at the same level as the SSR and was greatly reduced in *Gtaxin* mutant (Gorczyca et al., 2007). There is evidence that DLG traffics through the cortical membrane compartment on its way to the SSR (Thomas et al., 2000; Gorczyca et al., 2007). Given that reductions of both *Gtaxin* and *Akt1* affected SSR, we examined whether *Akt1* also influenced the organization of intracellular membrane compartments, as documented for *Gtaxin* mutant. As previously reported, mCD8-GFP expression in the muscle cell revealed a complex set of membranous structures including a cortical domain (c), the nuclear envelope (n), and a subcortical network (sc) [Fig. 5(D,H)]. The SSR was also prominently labeled by mCD8-GFP in the muscle, as evidenced by colocalization with DLG [Fig. 5(E–G)]. Reduction of *Akt1* function affected muscle cell membrane organization in a manner similar to that observed in *Gtaxin* mutant (Gorczyca et al., 2007). Namely, the cortical domain was nearly abolished and the subcortical domain was compressed, consistent with the much reduced muscle thickness in these animals [Fig. 5(H)]. The mCD8-GFP labeling of the SSR was also dramatically decreased, supporting the TEM findings of reduced SSR elaboration in animals with muscle-directed Akt^{RNAi} expression [Fig. 5(I–K)].

Based on the similar ultrastructural changes in the SSR and muscle membrane organization resulting from reductions in *Gtaxin* and *Akt1* function, Gtaxin was a logical candidate as a downstream target of Akt1 activity. To explore this possibility, we examined Gtaxin levels and distribution in animals with muscle-specific expression of $Akt1^{RNAi}$ (*Mef2-GAL4>UAS-*$Akt1^{RNAi}$) or a constitutively active form

Figure 3 *Akt1* affects GluRIIA trafficking to NMJ and is crucial in the early developmental stage. Two experiments are shown here. The first (panels A–H) shows the results from a study where the distribution of an engineered GluRIIA-RFP when Akt1 function was compromised with RNA interference. The GluRIIA-RFP was detected with either endogenous fluorescence from the mRFP or an anti-mRFP antibody (anti-DsRed). The second experiment (panels I–P) was designed to determine the developmental window when *Akt1* activity was critical for GluRIIA localization. Time-limited inhibition of *Akt1* function was achieved using $Akt1^{RNAi}$ and a temperature-sensitive GAL80 (see "Materials and Methods"). A–D: GluRIIA-mRFP (red) was colocalized with anti-DsRed signals (gray) at the NMJ in control animals (*G14-GAL4, UAS-mCD8-GFP; UAS-GluRIIA-mRFP/+*). E–H: GluRIIA-mRFP fluorescence was reduced significantly at the postsynaptic density (arrows) upon inhibition of *Akt1* function. The redistribution of GluRIIA-mRFP protein into an intracellular compartment was detected only with anti-DsRed immunostaining in *Akt1* compromised animals (*G14-GAL4, UAS-mCD8-GFP; UAS-GluRIIA-mRFP>UAS-*$Akt1^{RNAi}$) (arrowheads). I–P: To investigate the critical periods when Akt1 is required for GluRIIA localization at the NMJ during development, the temperature-sensitive *GAL80* repressor under tubulin promoter, *Tubp-*$GAL80^{ts}$, was used along with the *GAL4-UAS* binary system to allow temporal spatial regulation of $Akt1^{RNAi}$ expression (*Tubp-*$GAL80^{ts}$, *Mef2-GAL4>UAS-*$Akt1^{RNAi}$). I–L: At all temperatures, control (*Tubp-*$GAL80^{ts}$, *Mef2-GAL4/+*) animals showed normal GluRIIA distribution at the NMJ. M: In *Tubp-*$GAL80^{ts}$, *Mef2-GAL4/UAS-*$Akt1^{RNAi}$ animals at the permissive temperature (18°C), when expression of $Akt1^{RNAi}$ is minimal on account of $GAL80^{ts}$ blockade of transcription, the animals displayed a normal GluRIIA distribution. N: Incubation at the restrictive temperature (30°C) for 2 days right after egg laying induced modest GluRIIA mislocalization in muscles while much of the GluRIIA remained at the NMJ. O: Temperature shift from 18°C to 30°C for 2 days at the third instar larval stage produced reduced levels of GluRIIA immunoreactivity at the NMJ but no abnormal localization. P: Animals reared at 30°C throughout the entire developmental stages displayed severe GluRIIA mislocalization. Scale bar in (A–H), 10 μm, in (I–P), 50 μm.

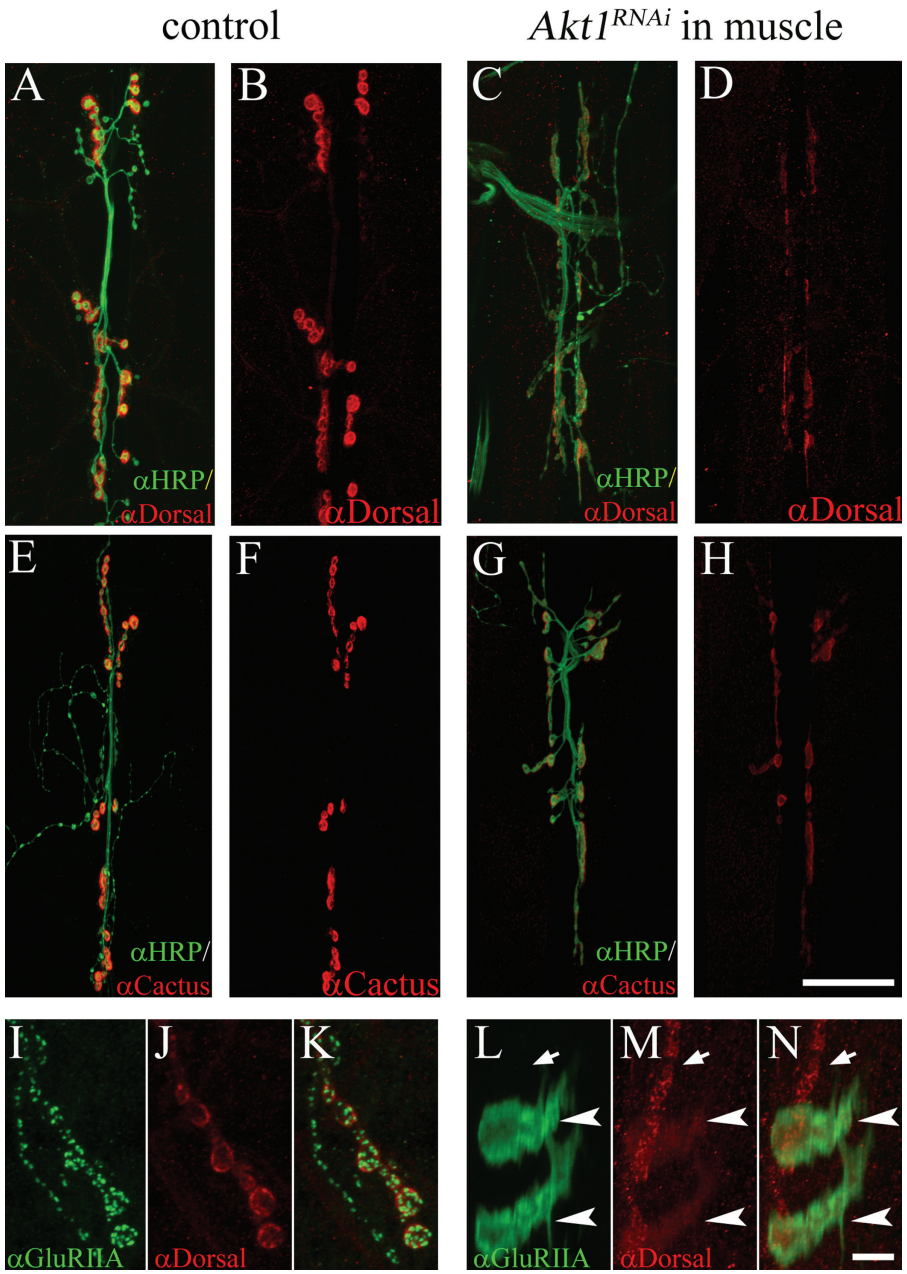

Figure 4 Influence of *Akt1* on Dorsal and Cactus levels and distribution at the NMJ. A and B: In control animals (*UAS-Akt1*^{RNAi}/+), Dorsal (detected by anti-Dorsal antibody; red) is localized to the postsynaptic specialization. Neuronal projections were labeled by anti-HRP staining (green). C and D: Akt1 function was compromised by expressing *UAS-Akt1*^{RNAi} under the muscle-specific 24B-GAL4. Dorsal levels at the NMJ were significantly reduced. E and F: Cactus (red) was concentrated at the NMJ in control animals. G and H: Inhibition of Akt1 function in the muscle (*24B-GAL4/UAS-Akt1*^{RNAi}) resulted in reduced levels of Cactus at the NMJ. I–K: In control animals, Dorsal immunoreactivity (red) colocalized with GluRIIA (green) immunoreactivity at the postsynaptic specialization. L–N: In muscles where *Akt1* expression was inhibited, both Dorsal and GluRIIA redistributed into intracellular bands, although the effect on Dorsal was less and incompletely penetrant. Mislocalized Dorsal partially overlapped with GluRIIA (arrowheads indicate bands of Dorsal and GluRIIA; arrows indicate synaptic boutons). Scale bar in (A–H), 50 μm, in (I–N), 5 μm.

of *Akt1* (*Mef2-GAL4>UAS-Akt1*^{CA}). In wild-type animals, Gtaxin immunoreactivity is concentrated at the SSR [Fig. 6(B,C)], and muscle-directed RNAi of *Akt1* greatly reduced Gtaxin levels at this postsynaptic specialization [Fig. 6(F,G)]. *Gtaxin* has been implicated in SSR formation not only on account of the reduction of SSR complexity in *Gtaxin* mutant but also from the production of ectopic, mCD8-GFP labeled membranous structures in animals overexpressing wild-type *Gtaxin* (Gorczyca et al., 2007). Muscle-directed expression of *Akt1*^{CA} produced membranous structures with the same visible features. In these animals, Gtaxin was present at increased levels and localized to patches throughout the muscle [Fig. 6(I–K)]. These ectopic membrane elaborations were confirmed at the TEM level and are structurally similar to those documented in animals overexpressing *Gtaxin* in the muscle [Fig. 6(M–O)].

The formation of mCD8-GFP-labelled membrane patches mediated by *Akt1*^{CA} was also found to be dependent on *Gtaxin*. The ectopic membranous patches induced by *Akt1*^{CA} expression in the muscle were visualized by mCD8-mRFP and showed some features of SSR, namely concentration of α-Spectrin and DLG (Pielage et al., 2006) [Fig. 7]. Reduction of *Gtaxin* by RNA interference blocked the *Akt1*^{CA}-mediated formation of these "ectopic" SSR structures [Fig. 7(B,D)]. The ectopic membrane patches induced by *Akt1*^{CA} overexpression were not reduced by expression of a control UAS-transgene, excluding the possibility that suppression of *Akt1*^{CA} function was due to titration of GAL4 proteins in *Gtaxin*^{RNAi} expressed animals (data not shown). Inhibition of Gtaxin by *Gtaxin*^{RNAi} expression in muscle induced loss of mCD8 at the SSR but DLG remained at the postsynaptic specialization [Fig. 7(F,H)]. In addition, GluRIIA localization was not disrupted by *Gtaxin*^{RNAi}, indicating that *Gtaxin* does not play a role in this aspect of *Akt1* function and is consistent with published findings (Gorczyca et al., 2007) [Fig. 7(J)]. These results demonstrated that *Gtaxin* is required for *Akt1*^{CA}-mediated formation of ectopic membranous structures. It is of interest that Gtaxin bears a consensus sequence (RXRXXS/T) for Akt1 phosphorylation, indicating a potential phosphorylation site at Serine 255, suggesting that Gtaxin could be a direct target of Akt1 activity (Datta et al., 1999; Zhang et al., 2002).

The experiments described above establish that *Akt1* is required for correct delivery of the GluRIIA subunit to the postsynaptic membrane and elaboration of the SSR. To assess the effects of *Akt1* on NMJ physiology, we conducted single cell recordings on the muscles of both *Akt1* mutants and animals with muscle-specific knockdown of *Akt1* using RNA interference. In animals with *Akt1*^{RNAi} directed to the muscle, amplitudes of miniature excitatory junctional potentials (mEJPs) were dramatically reduced, consistent with earlier reports that this measure of muscle response to spontaneous neurotransmitter release is greatly reduced [Fig. 8(A)] (Petersen et al., 1997). In larvae bearing a combination of mutant alleles (*Akt1*^{1}/*Akt1*^{04226}), mEJPs amplitudes were reduced but not to a statistically significant level [Fig. 8(A,B)]. However, *Akt1*^{1}/*Akt1*^{04226} transheterozygotes showed significantly reduced EJP amplitudes, the response of the muscle to a single suprathreshold stimulus of the motoneuron [Fig. 8(C,D)]. The shape of the EJP was also altered in *Akt1*^{1}/*Akt1*^{04226} animals, with measures of the EJP decay indicating a significantly decreased time to restore the membrane voltage to resting levels [Fig. 8(E)]. We noted these same changes in EJP properties in animals with *Akt1* function compromised in the muscle with targeted expression of *Akt1*^{RNAi}. The temperature sensitivity of the *GAL4-UAS* system allowed the graded reduction of *Akt1* function in the muscle. At elevated temperatures, *Akt1*^{RNAi} expression was higher and thus the reduction in *Akt1* function was more pronounced. At 24°C, expression of *Akt1*^{RNAi} in the muscle produced a significant reduction in the EJP amplitude, as observed in *Akt1*^{1}/*Akt1*^{04226} animals [Fig. 8(F,G)]. The EJP amplitudes were not significantly altered in animals reared at 18°C where *Akt1* function was compromised modestly [Fig. 8(F,G)]. However, at both temperatures, significant differences in the decay times of the EJPs in *Akt1*^{RNAi} expressing animals were observed [Fig. 8(H)].

A number of factors can influence the dynamics of the EJP, including membrane capacitance and resistance, as well as changes in voltage-gated channels in the membrane. To determine if the muscle membrane showed any changes in baseline resistance or capacitance, muscle cell responses to small current injections (1nA) were examined. *Akt1*^{RNAi} expressing animals did not show any significant changes to these small current applications (data not shown), suggesting that nonvoltage-dependent membrane properties could not account for the changes we observed in the EJPs of animals with decreased *Akt1* function.

It is important to note that electrophysiological changes observed in *Akt1* mutant and muscle-specific *Akt1* RNAi animals match the morphological and molecular alterations at the NMJ. In *Akt1*^{RNAi} expressing animals at 24°C, mEJP amplitudes were decreased to a nearly undetectable level [Fig. 8(A)], consistent with the previous characterization of

GluRIIA null mutant (DiAntonio et al., 1999). Furthermore, the decreased EJP decay times recorded in $Akt1^{1}/Akt1^{04226}$ and muscle-directed $Akt1^{RNAi}$ animals also mimicked a prominent phenotype of *Gtaxin* mutant animals (Gorczyca et al., 2007), supporting the hypothesis that changes in SSR and muscle membranous system were the reason for these physiological changes.

DISCUSSION

We explored *Akt* function in synapse development and function using a well-characterized model system, the *Drosophila* neuromuscular junction. There is a single *Akt* homolog in *Drosophila, Akt1*, facilitating the genetic and cellular studies of *Akt* function in synapse assembly. Our findings are summarized in

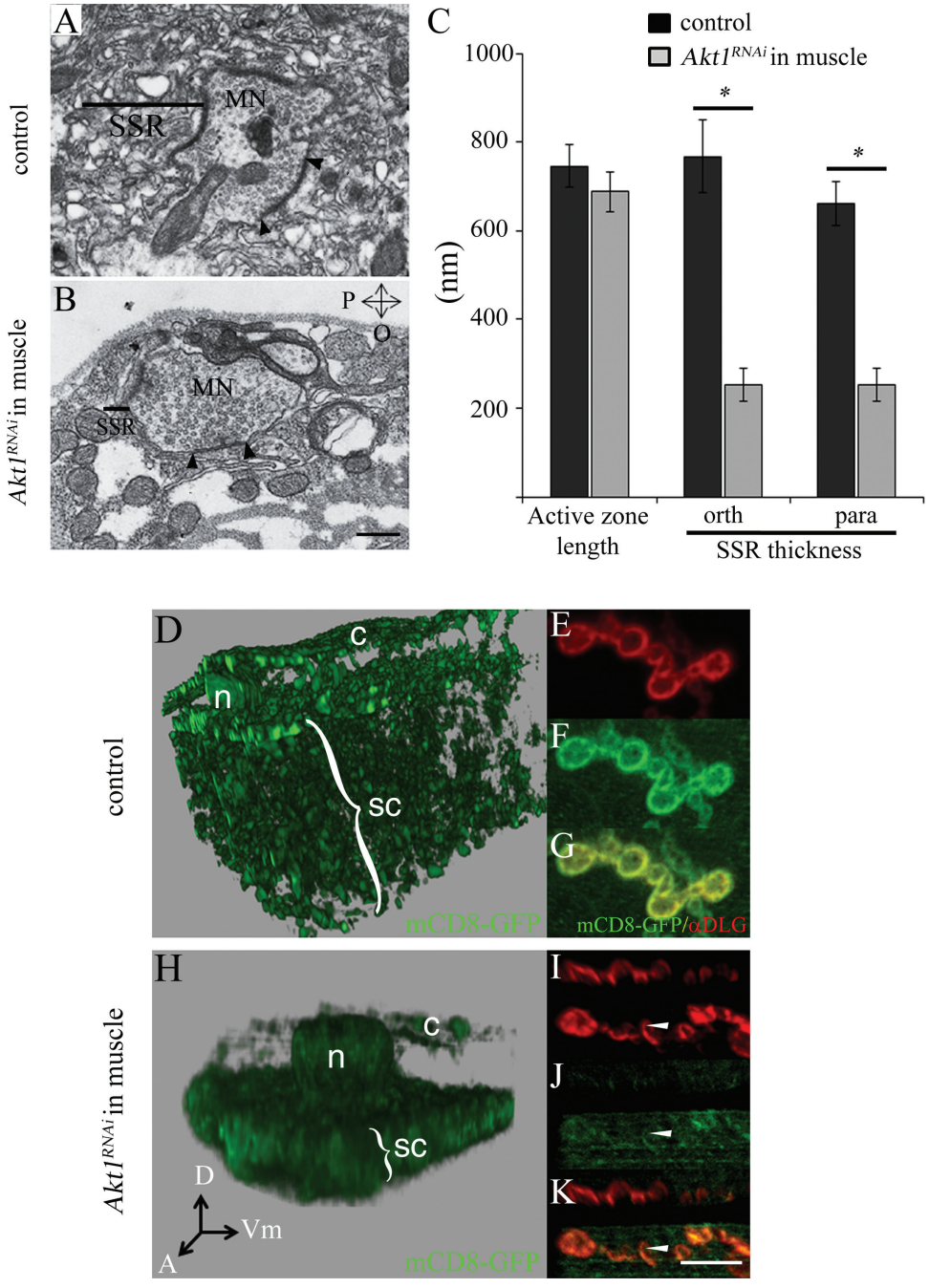

Figure 5

Figure 9. We found that *Akt1* was specifically required for the correct assembly of A-type glutamate receptors. Reductions of *Akt1* function either by mutation or RNA interference resulted in a loss of GluRIIA at the synapse paired with accumulation into intracellular structures. Reduction of *Akt1* influenced the levels and localization of proteins shown to affect GluRIIA, Dorsal, and Cactus. Therefore, Akt1 could affect GluRIIA at least in part via control of these proteins. *Akt1* was also required for the normal expansion of a specialized postsynaptic membrane compartment, the SSR. We provide evidence that *Akt1* mediates its effects on SSR via control of the t-SNARE Gtaxin. RNA interference of *Gtaxin* did not affect GluRIIA localization, showing that the control of SSR expansion and glutamate receptor composition mediated by *Akt1* occurs via different molecular mechanisms.

The analysis of *Akt1* reported here examined physiological, morphological, and cellular phenotypes, using both traditional *Akt1* mutant alleles and cell-type directed knockdown achieved with either of two different *UAS-Akt1*^{RNAi} lines. The results from these different genetic tools were consistent and showed that *Akt1* function is critical for both GluRIIA localization and SSR expansion. In particular, combinations of *Akt1* alleles resulted in the redistribution of GluRIIA into intracellular bands, a phenotype found to be even more pronounced in muscle-directed RNAi of *Akt1*. This remarkable phenotype was also observed in larvae expressing both *Akt1*^{RNAi} and a UAS-transgene-derived GluRIIA-RFP in the muscle, the latter detected by either endogenous fluorescence or anti-RFP antibody. It was of note that fluorescent signal from the GluRIIA-RFP was reduced at the synapse but receptor mislocalization to intracellular compartments was detected only with anti-RFP antibody. *Akt1*-dependent events were clearly required for the proper formation of the folded RFP domain of the recombinant GluRIIA protein while the polypeptide, detected with the anti-RFP antibody was present and redirected to an alternative cellular location, as we observed for the endogenous GluRIIA. These data implicate *Akt1* in processes of folding, stabilization, or assembly of GluRIIA.

A number of experiments were conducted to evaluate if *Akt1* was required for the localization of specific postsynaptic proteins, or rather served a more generalized role in directing a variety of proteins to this membrane specialization. The correct localization of GluRIIB, GluRIIC, Basigin, Discs large, and Syndapin in animals with *Akt1* knockdown in the

Figure 5 Muscle-specific inhibition of Akt1 affects the elaboration of the subsynaptic reticulum (SSR), and Akt1 is required for the integrity of the endomembrane system. A: Transmission electron microscopy shows SSR surrounding the motoneuron terminal in a control animal (*UAS-Akt1*^{RNAi}/+). Bar indicates approximate dimension of SSR. For both (A) and (B), the size of the SSR was determined by measuring its thickness in two-dimensions: parallel and orthogonal to the muscle surface. B: The dimensions and complexity of the SSR were dramatically reduced in *Akt1*-compromised larvae (*24B-GAL4>UAS-Akt1*^{RNAi}) without affecting the length of the presynaptic active zones (electron dense region, between two black arrowheads), where synaptic vesicles are released. C: Quantification of the length of presynaptic active zones, parallel and orthogonal SSR thicknesses. When compared with control animals, SSR thicknesses significant decreased in all dimensions with *Akt1*compromised (*24B-GAL4>UAS-Akt1*^{RNAi}). "*" denotes $p < 0.0005$, $n = 15$ each. P, parallel; O, orthogonal to the axis of muscle surface. D–K: To evaluate the organization of muscle membranes, mCD8-GFP, a transmembrane protein that tags cellular membranes, was used. mCD8-GFP expressed in the muscle (*G14-GAL4>UAS-mCD8-GFP*) localizes to membrane compartments, including plasma membrane, t-tubules, nuclear envelope, and the endoplasmic reticulum. The panels (D) (Control; *G14-GAL4,UAS-mCD8-GFP*/+) and (H) (muscle-specific Akt1 knockdown: *G14-GAL4, UAS-mCD8-GFP>UAS-Akt1*^{RNAi}) show 3D rendered images of serial confocal sections, representing the entire muscle cell thickness in a region where there are no synaptic boutons. The nuclear layer is located at the top of the image separating cortical (c) and subcortical (sc) membrane domains. Muscle-specific knockdown of *Akt1* produced a decrease in overall muscle cell thickness and reduced the complexity of membrane compartments (H). *Akt1*^{RNAi} results in a more compact subcortical membrane domain and notable reduction in the cortical membrane domain compared to control animals (D). c, the cortical membrane domain; sc, the subcortical membrane domain; n, nucleus; A, anterior; D, dorsal; and Vm, ventral midline. E–G: Visualization of the SSR by mCD8-GFP, and of the postsynaptic specialization with anti-DLG antibody (red). I–K: The extent of mCD8-GFP tagged SSR was dramatically reduced (arrowheads) by loss of *Akt1* function. DLG was correctly localized and modestly reduced in comparison with mCD8-GFP in these animals. Scale bar in (A and B), 0.5 μm, in (E–G) and (I–K), 5 μm.

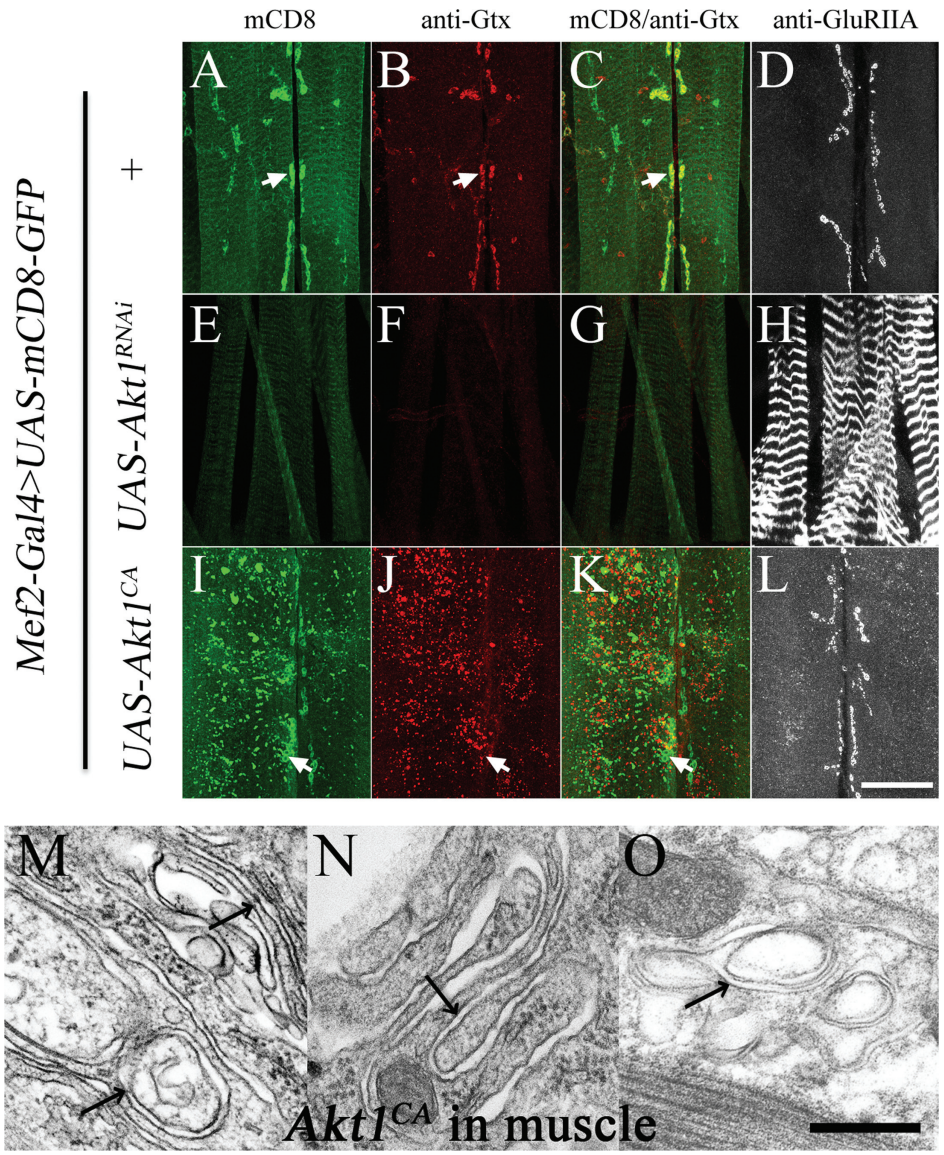

Figure 6 The localization and levels of Gtaxin at the postsynaptic specialization are *Akt1*-dependent, and overexpression of a constitutively active form of *Akt1* creates ectopic membranes distant from the synaptic region. Gtaxin localization and levels were examined in animals with reduced *Akt1* function or muscle directed expression of a constitutively active form of *Akt1*, $Akt1^{CA}$. All animals in this experiment also carried the muscle-specific driver *Mef2-GAL4* and *UAS-mCD8-GFP* transgenes. Gtaxin and GluRIIA were detected with anti-Gtx (red) and anti-GluRIIA (grayscale) antibodies. Panels (A–C), (E–G), and (I–K) are each from a single animal. Panels (D), (H), and (L) are anti-GluRIIA staining each from a single larva. A–D: Control animals had no *Akt1*-bearing transgene either $Akt1^{RNAi}$ or $Akt1^{CA}$ (labeled as +). Gtaxin is concentrated at the SSR, colocalizing with mCD8-GFP (arrows). GluRIIA is also highly concentrated at the NMJ specialization. E–H: Animals expressing *UAS-*$Akt1^{RNAi}$ showed dramatic reductions in the levels of mCD8-GFP at the SSR (E) and loss of Gtaxin at the synapse (F), as well as mislocalization of GluRIIA (H). I–L: Overexpression of $Akt1^{CA}$ caused ectopic mCD8 patches throughout the muscle (I), as well as increased levels of Gtaxin (J). The normal distribution of Gtaxin at the SSR was lost, with mislocalized Gtaxin patches evident throughout the muscle cell (J). Correct GluRIIA localization was maintained in these animals (L). M–O: Transmission electron microscope photomicrographs show ectopic membranous structures in muscles overexpressing the constitutively active form of *Akt1* (*Mef2-GAL4>UAS-Akt1CA*). Arrows point to infoldings of multilayered membranes in the cytosol or underneath the plasma membrane. Scale bar in (A–L), 50 μm, in (M–O), 0.1 μm.

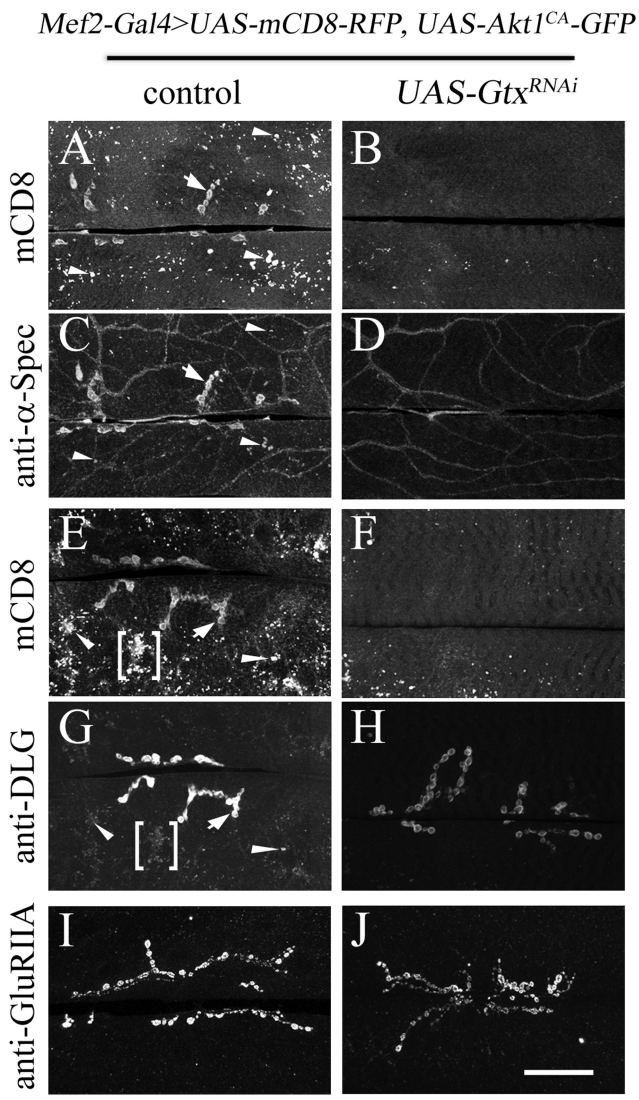

Figure 7 *Gtaxin* is required for ectopic membranous patches produced by expression of a constitutively activated form of Akt1. Synapse organization was assessed with mCD8-RFP and anti-α-Spectrin or anti-DLG antibody staining. All animals carried *Mef2-GAL4, UAS-mCD8-RFP, UAS-Akt1^{CA}-GFP*, and over either *OreR* as a control or *UAS-Gtx^{RNAi}*. Either anti-α-Spectrin or anti-DLG with mCD8-RFP images for each genotype were from the same animal, with a second preparation providing the anti-GluRIIA data. A and C: In animals with muscle-directed expression of *Akt1^{CA}-GFP*, ectopic patches of mCD8 were observed throughout the muscle (panel A, arrows), while leaving the SSR structure intact (on set of postsynaptic specializations shown with small arrow). Some of the ectopic mCD8 membrane patches also showed anti-α-Spectrin antibody staining (arrowheads in C). Inhibition of *Gtx* with RNAi abolished the mCD8 patches and greatly reduced the anti-α-Spectrin staining (panels B and D). Using anti-DLG to examine SSR structure in animals with muscle-directed expression of *Akt1^{CA}* also revealed that the membranous patches show some SSR-properties as evidenced by anti-DLG colocalization (arrowheads and a bracket in panels E and G). Inhibition of *Gtx* produced loss of mCD8-concentrated SSR membrane but not DLG localization to the postsynaptic specialization (panels F and H). As reported earlier and confirmed here, loss of *Gtx* did not compromise GluRIIA localization (panels I and J). Scale bar for A–J, 50 μm.

muscle demonstrated that *Akt1* has specific targeting functions for GluRIIA and is not a general factor for delivery of all postsynaptic proteins. Levels of these postsynaptic proteins were reduced in *Akt1^{RNAi}* bearing animals, not surprisingly given the substantial size reduction in the SSR.

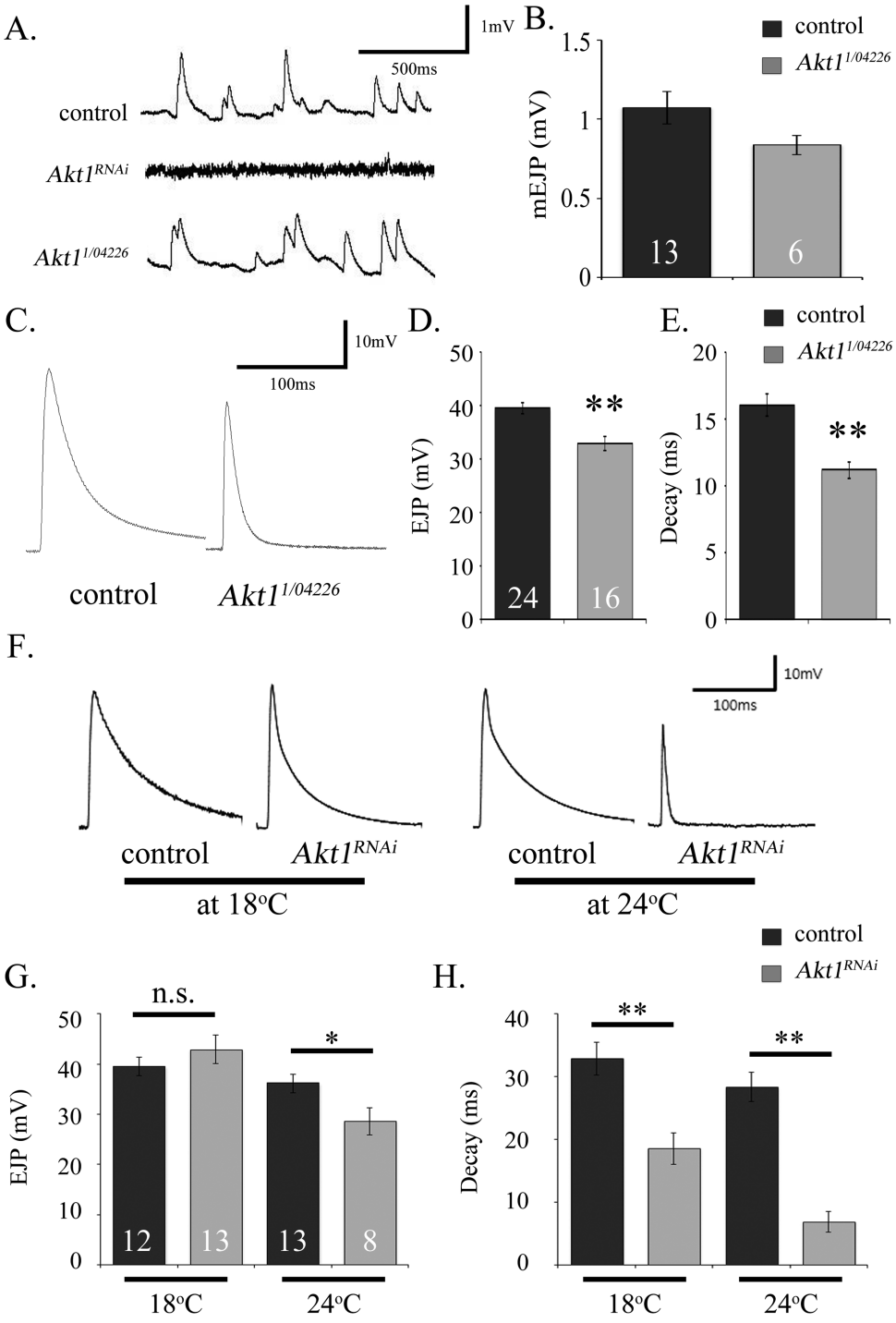

Figure 8 *Akt1* is required for normal electrophysiological response at neuromuscular synapses. A: Representative traces of miniature excitatory junction potentials (mEJPs) for *OreR* strain (as control), *Akt1* knockdown in the muscle, and $Akt1^{1}/Akt1^{04226}$ transheterozygous mutant animals. The $Akt1^{RNAi}$ expressing animals showed no readily detectable mEJP. B: $Akt1^{1}/Akt1^{04226}$ mutants, a mild hypomorphic combination of alleles, displayed somewhat reduced but not statistically significant different mEJP amplitude compared with controls ($p = 0.08$). C: EJP responses, detected in the muscle following motoneuron stimulation for control and $Akt1^{1}/Akt1^{04226}$ mutant. D and E: $Akt1^{1}/Akt1^{04226}$ mutant larvae exhibited significantly decreased EJP amplitudes and decay time compared to control ($^{**}p < 0.005$, $n = 24/16$). F: Traces of EJP in control (*Mef2-GAL4>UAS-mCD8-GFP*) and $Akt1^{RNAi}$ expressing larvae (*Mef2-GAL4>UAS-*$Akt1^{RNAi}$) reared at two different temperatures. G: EJP amplitude showed no difference at 18°C (low level of inhibition, n.s., no significant, $n = 12/13$), but was significantly decreased at 24°C (greater degree if *Akt1* inhibition,$^{*}p < 0.05$, $n = 13/8$). H: Similar to the electrophysiological changes observed in the $Akt1^{1}/Akt1^{04226}$ mutant animals, EJP decay time was abbreviated in $Akt1^{RNAi}$ expressing larvae, both at 18°C or 24°C ($^{**}p < 0.005$).

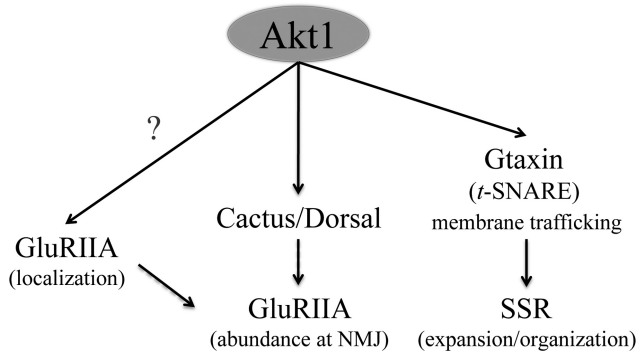

Figure 9 Model for *Akt1*'s regulatory role at the NMJ. *Akt1* selectively affects A-type glutamate receptor abundance at the NMJ. Compromising *Akt1* function reduced the levels of Dorsal and Cactus at the NMJ, two potential *Akt1* targets that regulate GluRIIA levels. Loss of *Akt1* also results in GluRIIA mislocalization to intracellular membrane compartments, suggesting that other *Akt1* targets are involved as well. *Akt1*-dependent subsynaptic membrane expansion is mediated through a separate pathway where the *Drosophila* t-SNARE protein Gtaxin acts downstream of *Akt1* function.

At the *Drosophila* NMJ, two types of glutamate receptors have been defined by their distinct compositions and physiological properties (DiAntonio et al., 1999; DiAntonio, 2006). The shifting between A- and B-type receptors provides a mechanism for modulating postsynaptic responses to variable presynaptic inputs during development (Sigrist et al., 2002). There is considerable evidence that modulation of GluRIIA and B representation at the NMJ is governed by different signaling systems. Coracle, a homolog of protein 4.1 in *Drosophila*, has been shown to specifically influence the targeting of GluRIIA but not IIB (Chen et al., 2005). A physical interaction between Coracle and GluRIIA was essential for actin-dependent trafficking of GluRIIA-containing vesicles to the plasma membrane. Conversely, DLG has been shown to be required for GluRIIB but not GluRIIA localization at the NMJ (Chen and Featherstone, 2005). Our finding supports the conclusion that A and B receptor subunits are differentially regulated and show that Akt1 serves a role in A but not B subunit control.

There is evidence that the assembly and localization of GluRIIA into the postsynaptic density at the NMJ is accomplished following delivery to the plasma membrane (Broadie and Bate, 1993; Rasse et al., 2005). This conclusion is based upon the observation that fluorescence photobleaching of the entire muscle delays accumulation of new GluRIIA to synaptic sites more so than local bleaching at the NMJ (Rasse et al., 2005). The effects of *Akt1* on GluRIIA localization could therefore be mediated by either regulated delivery of GluRIIA-containing vesicles to the plasma membrane, or by affecting the localization to the postsynaptic density following insertion into the plasma membrane. The accumulation of GluRIIA into an intracellular membrane compartments argues for a trafficking-based mechanism. This model is further supported by the results from the developmental timing experiments, where *Akt1* function was removed during different stages in synapse assembly. Loss of *Akt1* in a 2 day window early in development produced the phenotypes observed with continuous loss of *Akt1*, whereas a 2 day loss in third instar did not. If *Akt1* simply served to retain GluRIIA at the synapse, there should have been time for new synthesis to repopulate the NMJ. Therefore, we favor a model where *Akt1* affects developmental processes required for the selective delivery of GluRIIA from the endoplasmic reticulum into functional receptor units that arrive at the plasma membrane. It is notable that in mammalian systems, Akt is critical for the insulin-stimulated exocytosis of glucose transporter containing vesicles to the plasma membrane (Gonzalez and McGraw, 2006; Grillo et al., 2009). Perhaps *Akt1* governs similar exocytic processes at synapses. *Akt1* signaling has also shown to be essential for AMPA receptor trafficking in hippocampal neurons, further supporting a role for *Akt1* in trafficking of synaptic proteins (Qin et al., 2005b; Hou et al., 2008; Pratt et al., 2011).

A striking phenotype of animals with reduced *Akt1* function in muscles was a severe reduction in the SSR and disruption of intracellular membrane organization. These phenotypes were similar to those found in a *Gtaxin* mutant and suggested the possibility that *Akt1* and *Gtaxin* are involved in the same cellular process (Gorczyca et al., 2007). A number of

observations reported here indicate *Akt1* activity is mediated at least in part by control of Gtaxin. First, Gtaxin levels at the SSR are greatly reduced in animals with reduced *Akt1* function in the muscle cells. Second, muscle-directed overexpression of a constitutively active form of *Akt1* ($Akt1^{CA}$) produced ectopic membranous structures; a phenotype also observed with Gtaxin overexpression and elevated levels of Gtaxin. Third, inhibition of *Gtaxin* blocks the effects of the constitutively active *Akt1* in the muscle cell. Gtaxin does contain a consensus site for Akt1 phosphorylation and could therefore be a direct target of Akt1 kinase activity in regulating SNARE complex assembly.

The regulatory roles of *Akt1* in glutamate receptor composition and postsynaptic membrane expansion could be accomplished through separate or identical downstream effectors. The fact that *Gtaxin* mutants did not disrupt GluRIIA distribution suggests different downstream effectors regulated by *Akt1*. The regulation of GluRIIA localization by *Akt1* does not involve *Gtaxin* but could be mediated via Dorsal and Cactus. Dorsal and Cactus influence glutamate receptor delivery and are known effectors of Akt activity in mammalian cells (Heckscher et al., 2007; Dan et al., 2008). The levels of both Dorsal and Cactus were reduced in animals with knockdown of *Akt1* in the muscle. Notably, in some animals expressing $Akt1^{RNAi}$ in the muscle, Dorsal showed an altered intracellular distribution that overlapped with the mislocalized GluRIIA. However, because Dorsal and Cactus mutants are not reported to mislocalize GluRIIA into intracellular bands, *Akt1* is likely to have additional downstream targets that influence GluRIIA localization and delivery to the postsynaptic specialization.

Physiological measures of synaptic transmission showed that *Akt1* function is required for normal synapse function. *Akt1* transheterozygous mutants ($Akt1^{1}/Akt1^{04226}$) showed reduced EJP amplitudes and altered decay kinetics of the EJP. These same phenotypes were observed in animals with muscle-specific inhibition of *Akt1* function, with the severity correlating to the degree of *Akt1* inhibition. These changes in EJP kinetics were not accompanied by alterations of nonvoltage-dependent membrane capacitance or resistance, suggesting that voltage-gated channels contributing to EJP rise and decay times may be affected by *Akt1*. These findings contrast published work with *Akt1* mutant animals describing changes in long-term depression but not in EJP properties (Guo and Zhong, 2006). However, we note that our physiological studies were conducted at a higher Ca^{2+} concentration, which could account for these different measures of EJP properties in *Akt1* mutants. It is important to point out that the physiological changes we document were observed in both *Akt1* mutant larvae as well as animals with RNA interference of *Akt1* in the muscle cell. The physiological changes we observed in *Akt1* compromised animals are logical consequences of observed changes in NMJ composition. Loss of GluRIIA-containing receptors and an overall decrease in functional GluRs at the synapse could decrease the EJP amplitude. The altered EJP decay pattern in animals with reduced *Akt1* is consistent with the involvement of *Gtaxin*, as we have documented here. *Gtaxin* mutants showed similar changes in EJP decay, indicating that this feature of *Akt1* mediated physiological change is associated with the consequences of compromising the function of this t-SNARE.

There is a precedent for Akt-mediated regulation of neurotransmitter receptor localization to the cell surface. The NMDA receptor subunit NR2C is developmentally regulated in cerebellar granule cells and Akt-mediated phosphorylation is critical for cell surface expression of NR2C-containing receptors (Chen, 2009). Akt has also proven to be important in the elaboration of dendritic complexity in *Drosophila* sensory neurons, suggesting that this kinase is of general importance in the control of nervous system receptive fields (Parrish et al., 2009). Selective control of Akt or its downstream targets could provide a powerful method of influencing synaptic transmission and the receptive properties of neurons.

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