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APPROVAL SHEET

The Role of the Myb-related Transcription Factor Adf-1 in Dendritic Plasticity

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ABSTRACT COVER PAGE

The Role of the Myb-related Transcription Factor Adf-1 in Dendritic Plasticity

By

Christina Kimberly Timmerman
B.S., Auburn University, 2007

Advisor: Subhabrata Sanyal, Ph.D.

A dissertation submitted to the Faculty of the Graduate
School of Emory University in partial fulfillment
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Department of Cell Biology

2013

Abstract

Across multiple animal models, there is a much-appreciated role of protein-synthesis in long-term memory formation. This protein-synthesis dependent form of neuronal plasticity requires the coordinated actions of transcription of new gene products and also local translation at relevant synaptic sites. Although, it is unsurprising that mutations in any of these processes affect long-term behavioral adaptations such as memory formation. We investigate a *Drosophila* long-term memory mutant (*nalyot*) and its role in neuronal plasticity. The *nalyot* mutant is a hypomorphic allele of the Myb-related transcription factor Adf-1. Previous studies have shown that these mutants exhibit mild presynaptic defects. Since a variety of transcription factors are known to influence both pre- and post- synaptic plasticity, we investigated if Adf-1 was involved in post-synaptic (dendritic) plasticity. Dendrites represent sites of signal integration and as such its morphology is intimately associated with how information is processed in any given neuronal circuit. In order to observe Adf-1's role in dendritic morphology *in vivo*, we turned to a system previously described by our laboratory that reproducibly labels a subset of motor neurons (RP2) in the *Drosophila* larval ventral nerve cord. Following 3D reconstructions of these RP2 neurons, we find that perturbations in Adf-1 expression result in dramatic loss of dendritic complexity and also lead to severe defects in motor behavior. Furthermore, we find that Adf-1 exerts these effects downstream of neuronal activity and CaMKII signaling. Finally, in order to identify neuronal targets of Adf-1 transcription, we performed ChIP-sequencing from larval brain tissue. We discover that Adf-1 transcriptionally regulates the neural cell-adhesion molecule FasII (NCAM homolog) and the local translation component Staufen in the process of dendritic plasticity.

COVER PAGE

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I. Chapter 1: Introduction to Memory Formation And Processes of Synaptic Plasticity

Chapter 1

Introduction to Memory Formation And Processes of Synaptic Plasticity

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The Identification of *Drosophila* memory mutants

The isolation and characterization of memory mutants in the model system *Drosophila melanogaster* began with the father of fruit fly behavior, Seymour Benzer. Earlier work had established a behavioral technique based on training fruit flies to associate a given odor with electrical shock. After subsequent training, normal flies learn to avoid the odor associated with the unpleasant shock (Quinn et al., 1974). Through ethyl methanesulfonate (EMS) mutagenesis, Benzer's group identified a mutant fly that exhibited learning defects while sensory perception remained intact. In the grand tradition of humorous (and yet remarkably descriptive) naming, the first fly memory mutant was dubbed *dunce* (*dnc*) (Dudai et al., 1976). The next memory mutant isolated through chemical mutagenesis was *rutabaga* (*rut*). The mutation was mapped back to the catalytic subunit of adenylate cyclase rendering it unresponsive to calcium or calmodulin (Levin et al., 1992; Livingstone et al., 1984). Subsequent work identified *amnesiac,* a memory mutant which displayed normal learning immediately after training but given a rest period of 45 minutes would forget four times as quickly as control (Quinn et al., 1979). Further testing through modification of the training paradigm revealed that *amnesiac* was defective in memory retrieval, not memory storage. Intriguingly, in addition to *rutabaga*, both *dunce* and *amnesiac* represent mutations in cAMP signaling (Feany and Quinn, 1995; Qiu et al., 1991; Quinn et al., 1979). While components of the cAMP-signaling cascade were some of the first hits in these forward mutagenic screens, subsequent mutations highlighted other critical processes involved in memory formation. While there

is an extensive body of literature on additional *Drosophila* learning/memory mutants (for review (Skoulakis and Grammenoudi, 2006), I will focus on those which are important to Adf-1 signaling (discussed in greater detail in subsequent sections).

Phases of Memory Formation

There are distinct phases of memory formation identified in *Drosophila* (Figure 1). Short-term memory is defined as memory that lasts for less than an hour and is independent of transcription and translational processes. Middle-term memory (MTM) is defined as lasting between 1-4 hours and is thought to involve translation of already present populations of mRNAs. Different olfactory training paradigms led to the discovery of distinct forms of consolidated memory formation. Massed training in *Drosophila* leads to an anesthesia-resistant memory (ARM) that lasts for up to four days while spaced training leads to long-term memory (LTM) formation that lasts up to seven days. Long-term memory formation is dependent on new protein synthesis. It has been shown that feeding flies cyclohexamide, a protein synthesis inhibitor, either before or after training inhibits LTM (but not ARM suggesting these represent two functionally independent pathways (Skoulakis and Grammenoudi, 2006; Tully et al., 1994).

The Mushroom Body in Learning & Memory:

While the *Drosophila* community was genetically dissecting the important biochemical pathways important in memory, researchers were also beginning to identify which neuronal subsets were critical to these processes. These studies led to the discovery of the importance of the mushroom body (MB) structure in learning and memory

formation (Figure 2). The first evidence came from Heisenberg which observed two *Drosophila* mutants, *dunce* and *rutabaga*, exhibited structural defects in the MB that lead to deficits in performance in the olfactory conditioning assay (Heisenberg et al., 1985). Furthermore, it was found that these memory relevant gene products are preferentially expressed in the MB suggesting that this structure was the site of higher learning in the fruit fly (Nighorn et al., 1991; Qiu and Davis, 1993). Structurally, the mushroom body consists of three types of neurons: α/β, α′/β′ and γ (Crittenden et al., 1998). Chemical ablation of the entire mushroom body through hydroxyurea feeding resulted in flies which were defective in olfactory conditioning (de Belle and Heisenberg, 1994). In fact, disruption of the cAMP signaling cascade specifically in the MB results in disruption of olfactory learning (Connolly et al., 1996). Upon establishing this structure's importance in olfactory learning, future screening for memory mutants used preferential MB expression as a way to find novel mutants (Grotewiel et al., 1998; Skoulakis and Davis, 1996). Despite the obvious importance of the MB in memory, recent studies have elucidated other neurons outside of this structure that are critical components of memory formation (Keene et al., 2006; Waddell et al., 2000; Zhang et al., 2013).

An *in vivo* system to study synaptic plasticity

The *Drosophila* larval motor system has been used extensively as a model system for examining aspects underlying synaptic plasticity (for review (Collins and DiAntonio, 2007). It makes use of the vast array of *Drosophila* genetic tools to tease apart complex pathways involved in development and function of neuronal synapses. These synapses are glutamatergic, like the central synapses in vertebrates, which suggests molecular

mechanisms identified in these synapses are likely to be conserved. Also, the pattern of innervation in this system is elaborate and highly stereotyped (Hartwig et al., 2008; Mauss et al., 2009; Vonhoff and Duch, 2012). Most importantly, the larval motor system demonstrates robust activity-dependent changes and this process of activity-dependent plasticity is thought to be the cellular mechanism underlying long-term synaptic plasticity and memory formation (Davis and Goodman, 1998). In fact, a large number of *Drosophila* learning and memory mutants have corresponding defects in the neuromuscular system (Broadie et al., 1997; Cheng et al., 2001; DeZazzo et al., 2000; Rohrbough et al., 1999; Zhong and Wu, 1991). An additional benefit in using this system in studying synaptic plasticity is that electrophysiological and behavioral outputs can be easily measured to determine functional consequences of any given genetic manipulation.

My study specifically attempts to identify the role of the transcription factor, Adf-1 in dendritic plasticity. I utilize a previously characterized *in vivo* system developed in our laboratory that allows expression of any transgene of interest within a subset of motor neurons expressing membrane-bound GFP (Hartwig et al., 2008). This system takes advantage of the GAL4-UAS system that allows for tissue-specific expression of any transgene of interest (Brand and Perrimon, 1993). The system utilizes activation of a transient GAL4 which labels two subsets of motor neurons (aCC and RP2). Expression from this GAL4 is weak in later stages of larval development, so in order to visualize these neurons, the fly stock includes the following: UAS-Flippase, an actin promoter sequence next to a GAL4 separated by a spacer, and a UAS-cd8 GFP. However, this GAL4 is not expressed from the actin promoter until the spacer sequence (flanked by FRT sites) is removed through activation of the flippase. Once removed, this actin-GAL4

drives robust expression of membrane bound GFP in both the aCC and RP2 motor neurons (which are easily distinguished from one another by branching pattern). Subsequent confocal imaging and semi-automated 3D reconstruction allow detection of changes in dendritic arborization patterns following genetic manipulation (Evers et al., 2005; Schmitt et al., 2004). The dendrites of these motor neurons (aCC and RP2) are morphologically and functionally similar to that of vertebrate dendrites in the following ways: they lack pre-synaptic markers such as synaptotagmin but contain post-synaptic components such as Rdl (subunit of the ionotropic GABA receptor), and are responsive to calcium following neuronal excitation (Hausser et al., 2000; Sanchez-Soriano et al., 2005). It is likely that mechanistic insight gained from studying the dendrites of the RP2 motor neurons will be directly applicable to dendrites of the vertebrate system.

Drosophila memory mutant: *nalyot*

A transposon-based mutagenesis screen led to the isolation of a novel *Drosophila* long-term memory mutant dubbed as *nalyot* (named in honor of one of Pavlov's dogs). Subsequent mapping led to the discovery that the transposable element had landed within the large regulatory intron of the Adf-1 gene resulting in a 2-fold reduction in Adf-1 protein levels (Figure 3A) (DeZazzo et al., 2000). Adf-1 was originally isolated as a transcription factor which bound to the Alcohol dehydrogenase gene, hence its name Alcohol dehydrogenase distal factor -1 (Adf-1). Through DNase I footprinting, it was found that Adf-1 binds to the to -86 to -46 region of the Adh promoter (Heberlein et al., 1985). In embryos, Adf-1 expression coincides with the start of zygotic transcription (45 minutes post-egg laying). Adf-1 protein is localized to the nucleus and it is highly

expressed in the *Drosophila* nervous system, including the adult mushroom body (DeZazzo et al., 2000). This is in contrast to Adh protein expression, which is confined to the larval fat body and highly expressed in 3rd instar larvae and adults (England et al., 1992; Savakis and Ashburner, 1985). Prior to our study, Adh represented the only downstream target of Adf-1 that had been extensively characterized. Previously identified targets of Adf-1 were characterized primarily based on Adf-1 binding to their promoter regions (Han et al., 1998). These targets include the homeobox protein Antennapedia (Antp) and Dopa Decarboxylase (Ddc). Subsequent alignment of these downstream targets led to the first characterization of an Adf-1 binding consensus sequence, a trinucleotide repeat consisting of G C/T C/T (England et al., 1990).

Adf-1 Protein Structure:

The Adf-1 protein structure consists of an amino terminal DNA binding domain, a carboxy terminal protein interaction domain and transactivation domains located throughout the protein (Figure 3B) (Cutler et al., 1998). While it appears that there is no direct Adf-1 homolog in vertebrates based on sequence alone, both the DNA binding domain and protein activation domain have a high degree of homology to the oncoprotein Myb (England et al., 1992). Adf-1 is unique in that it is non-modular in structure, that is Adf-1 activity is dependent on the protein remaining intact, including its DNA binding domain (Cutler et al., 1998). In addition, expression of Adf-1 requires tight regulation as it has been shown that neuronal overexpression of Adf-1 can lead to lethality (DeZazzo et al., 2000).

Adf-1 in Plasticity:

Despite *nalyot* mutants exhibiting profound defects in memory formation, very little else was known about Adf-1's role in synaptic plasticity. It has been observed that Adf-1 hypomorphs exhibit mild pre-synaptic defects at the neuromuscular junction (DeZazzo et al., 2000). In addition, previous work had shown an interaction between Adf-1 and CaMKII, a well-known player in activity-dependent plasticity (discussed below). Based on amino acid sequence, there are two predicted CaMKII phosphorylation sites on Adf-1, Serines 64 and 184. Serine 64 resides in the DNA binding domain while Serine 184 is in the activation domain. Through expression of Adf-1 mutants that altered these Serine residues to an Alanine (either in single copy or as double mutants), they find that CaMKII appears to preferentially phosphorylate Adf-1 on Serine 64. The interaction between enzyme and substrate appears to be transient as evidenced by the inability to co-immunopreciptate Adf-1/CaMKII as a complex (GuptaRoy et al., 2000).

CaMKII:

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a Serine/Threonine kinase with an extensively studied role in synaptic plasticity. It represents one of the most abundant proteins in the brain (Erondu and Kennedy, 1985). In mammals, there are multiple genes encoding related CaMKII proteins: α and β isoforms are expressed highly in the brain while the γ and δ isoforms are ubiquitously expressed. *Drosophila* have only one gene which is alternatively spliced to represent various isoforms (Griffith and Greenspan, 1993). These isoforms make up the CaMKII holoenzyme, which is composed of 12 subunits. The structure of CaMKII consists of an N-terminal catalytic domain, a

regulatory domain and a self-association domain. The regulatory domain alters its conformation upon binding to Ca^{+2}/calmodulin such that the catalytic domain becomes available to phosphorylate potential substrates. In addition, this change in conformation can lead to autophosphorylation on Threonine 286 (in CaMKIIα, corresponds to Threonine 287 in *Drosophila*) that allows the kinase to retain its active conformation even in the absence of calcium and also enhances its affinity for $Ca^{+2/}$calmodulin (Hanson et al., 1989). This autophosphorylation domain is critical to CaMKII function. When a point mutation was introduced to alter the Threonine 286 residue to an Alanine resulting in a CaMKII that could no longer become Ca^{+2}/calmodulin independent, these animals exhibited spatial learning defects (Giese et al., 1998). In addition, CaMKII has been shown to control neuronal excitability through regulation of synaptic proteins. It has been shown that CaMKII docks on NMDA (NR2B) and the voltage-gated potassium channel *ether-a-gogo* DN in *Drosophila* (Griffith et al., 1994; Leonard et al., 2002; Tapia et al., 2012). Additional regulation of CaMKII is achieved through a variety of processes, allowing for tight regulation of its function. For example, negative regulation of CaMKII activity can be achieved through a number of factors such as its binding of α-actinin, interaction with its endogenous inhibitor CamKIIN, binding to the *Drosophila* CASK, and autophosphorylation of T305/306 (corresponds to T306/307 in *Drosophila*) (Chang et al., 1998; Colbran and Soderling, 1990; Hodge et al., 2006; Robison et al., 2005).

CaMKII's Role In Dendrites

CaMKII is a critical component in maintenance of dendritic arbors (Wu and Cline, 1998). It has been shown that active CaMKII holoenzymes composed of CaMKIIα/β isoforms,

lead to filopodial growth and formation of new dendritic spines in hippocampal slice culture (Jourdain et al., 2003) and increase in dendritic filopodia in *Drosophila* neurons (Andersen et al., 2005). Despite both isoforms having affects on dendritic morphology, the α and β isoforms of CaMKII appear to regulate dendritic plasticity through distinct mechanisms. CaMKIIβ is localized to the actin cytoskeleton making it uniquely poised to alter dynamic processes that change dendritic structure (Shen et al., 2000). The β isoforms bundle F-actin and have important roles in the maintenance of synaptic structures (Okamoto et al., 2007). Furthermore, either increasing activity of decreasing activity of CaMKIIβ leads to corresponding changes in neurite growth (Fink et al., 2003). A more recent study has shown a novel way CamKIIβ can affect dendritic plasticity. The study found that dendrite growth can be regulated by CamKIIβ phosphorylation of centrosome-associated proteins (Puram et al., 2011).

While CaMKIIα does not appear to have a direct interaction with actin, it has been shown that in a variety of cell types it plays an important role in neurite growth and differentiation (Donai et al., 2000; Goshima et al., 1993; Kutcher et al., 2003; Sogawa et al., 2000). In addition to the different isoforms, the phosphorylation status of CaMKII's autoinhibitory region is important to its role in dendrites. The calcium- independent form of CaMKII (T286D) has been shown to lead to an increase in spine density and size in hippocampal slice culture (Yamagata et al., 2009).

CaMKII in *Drosophila* learning and memory:

CaMKII mutants exhibit defects in an associative learning assay known as courtship conditioning. This assay involves placing males with recently mated females.

These females will reject the male's attempts at copulation and subsequently these males will learn to avoid initiating courtship behaviors. The trained males are then placed with receptive virgin females and observed for whether they have suppression of courtship behaviors (Tompkins et al., 1983). Transgenic expression of an inhibitory peptide that targets the autophosphorylation site on CaMKII results in a defect in courtship suppression. These mutant males immediately attempt to mate with virgin females, having not learned from their prior training experience (Broughton et al., 2003; Griffith et al., 1993). One drawback of this inhibitory peptide approach is that it alters activity of both calcium-independent /calcium-dependent forms of CaMKII. As mammalian studies had shown, the calcium-independent CaMKII appears to act as a 'molecular switch' to memory formation. To address the relevance of this autophosphorylation event, Threonine 287 was mutated and assayed for behavioral defects. Pan-neuronal expression of transgenic forms of the calcium-independent form (T87D) resulted in increased lethality (Park et al., 2002). What few adult flies that successfully eclosed exhibited morphological defects such as failure of wing expansion. At the larval stage these animals were sluggish and exhibited defects in neuronal excitability (all phenotypes that are seen in Adf-1 mutants, discussed later). However, if transgenic expression of T87D is limited to a subset of cholinergic neurons and expressed only after eclosion, these animals exhibit enhanced courtship suppression (Mehren and Griffith, 2004). This represented the first study that showed behavioral enhancement with the calcium-independent form of CaMKII. In congruence with mammalian studies, it appears that Ca^{+2} independent CaMKII is essential for memory formation but the affect it exhibits is

both dose-dependent and neuron-specific (Bach et al., 1995; Silva et al., 1992; Tan and Liang, 1996).

Cell Adhesion Molecules in Memory

Cell adhesion molecules are a class of molecules, which include integrins, and neuronal cell adhesion molecules (NCAMs). FasII is the homolog to the vertebrate NCAM (Cunningham et al., 1987) and Aplysia apCAM (Mayford et al., 1992). A member of the Ig superfamily, it has a conserved structure consisting of two fibronectin domains and five Ig domains. Both FasII and NCAM have multiple isoforms. NCAM has three major isoforms: NCAM-120 is bound to the membrane through a glycophosphatidylinositol linkage, and NCAM 140 and 180 represent transmembrane forms of the protein. The *Drosophila* NCAM homolog, FasII, has four isoforms. The two transmembrane forms are distinguished by either the presence or absence of a PEST sequence (FasII A PEST+, PEST-), FasII C is GPI-linked and FasII B is poorly characterized (Lin et al., 1994). While it is currently unknown what the biological significance of this PEST sequence is in FasII function, it has been shown in other systems to target proteins for rapid proteolysis (Rechsteiner and Rogers, 1996). Of particular note, the transmembrane forms of FasII are essential for synaptic development suggesting intracellular signaling contribute to its function in this process (Beck et al., 2012).

Previous work has shown that mutants in FasII have defects in fasciculation, defasiculation and growth cone guidance (Grenningloh et al., 1991; Lin et al., 1994; Lin and Goodman, 1994). In a series of papers from Corey Goodman's lab, FasII was shown

to be a critical component of activity-dependent synaptic plasticity at the neuromuscular junction (NMJ). Additionally, it suggested that FasII levels must be down regulated in order to increase neuronal growth. A ~50% reduction in FasII levels observed in FasII mutants leads to a synaptic overgrowth phenotype (Schuster et al., 1996a). In the case of increased neuronal activity or an increase in cAMP signaling it was observed that FasII levels decreased which then corresponded to an increase in synaptic size (Schuster et al., 1996a). Subsequent studies showed that when *Drosophila* larvae are subjected to conditions which force them to crawl more; this leads to an experience-dependent increase in active zones and bouton number and a decrease in FasII levels at the NMJ (Sigrist et al., 2003). It appears that in the process of activity-dependent plasticity, FasII levels need to reduced in order to achieve synaptic outgrowth.

FasII/ NCAM in memory

FasII mutants exhibit defects in short-term memory formation (Cheng et al., 2001). FasII is highly expressed within the adult mushroom body; however, its expression is not uniform and is predominately within the α/β lobes with modest expression in the γ lobes (Cheng et al., 2001; Crittenden et al., 1998). FasII induction in the FasII memory mutants just 1-2 hours prior to training restores memory to that of control animals and this process can be quickly reversed. Taken together this points to a role of FasII that is non-developmental in origin.

Additionally, a number of mammalian studies have elucidated the importance of NCAM in cognitive function (Becker et al., 1996; Doyle et al., 1992; Muller et al., 1996; Murphy et al., 1996; O'Connell et al., 1997; Ronn et al., 1995). In mice, knock-outs of

NCAM lead to a spatial learning defect as assayed by the Morris Water Maze and defects in axonal pathfinding were observed in the hippocampus (Cremer et al., 2000; Cremer et al., 1994). Also, the addition of antibodies against NCAM into hippocampal slices interferes with induction of long-term potentiation (LTP), a mechanism thought to correlate with long-term changes at the synapse (Luthl et al., 1994).

Integrins in Memory

Integrins are heterodimeric cell adhesion receptors that aid in crosstalk between the cell and surrounding extracellular matrix. They are involved in a variety of cellular processes ranging from cell proliferation, transcription, and organization of the cytoskeleton (Berrier and Yamada, 2007). These cell adhesion molecules are involved not only in the development of synapses but also in synaptic plasticity. A series of studies in mammalian systems have implicated integrins in mediation of synaptic plasticity through calcium and NMDA receptor signaling (Bernard-Trifilo et al., 2005; Lin et al., 2003). Additionally, integrins have been shown to be involved in modulation of dendritic spines through CaMKII signaling (Shi and Ethell, 2006).

In *Drosophila*, the memory mutant *volado* was isolated in an enhancer detector screen looking for lines that were preferentially expressed within the mushroom body. This screen lead to the identification of an α- integrin dubbed *volado* (translation roughly corresponds to Chilean for 'forgetful'). Furthermore, *volado* mutants have a short-term memory defect in either the homozygous or heterozygous condition, that is, it has a dominant effect on memory formation (like other memory mutants *dnc* and *rut*) (Grotewiel et al., 1998). Volado mutants exhibit an overgrowth phenotype at the

neuromuscular junction suggesting they are critically involved in inhibition of developing synapses. Additionally, these mutants exhibited defects in functional plasticity at the NMJ (Rohrbough et al., 2000). Taken together, it appears that cell adhesion molecules such as FasII/NCAM and integrins are critical mediators in synaptic development and plasticity underlying complex behavioral processes such as memory formation.

Local translation:

Over 60 years ago, it was first postulated that protein synthesis was critical to formation of lasting memories. It was nearly a decade later before the discovery of protein synthesis inhibitors allowed for neuroscientists to test this theory. We first gained insight into the importance of protein synthesis when work done by Louis and Josefa Flexner noted that injection of the protein synthesis inhibitor puromycin directly into the brain of mice resulted in memory defects (Flexner et al., 1963). A large body of work since then has cemented this idea that there is a critical period of new protein synthesis shortly after training to establish long-lasting memories (Sutton and Schuman, 2006). From the perspective of cell biological processes, the question arose of how total protein synthesis could be sequestered to relevant synapses. How do we achieve synapse-specific changes during the process of learning and memory? The idea that proteins could be translated outside of the nucleus and that this mechanism could lead to a synapse-specific response was not considered a possibility until research done by Steward and Levy in the 1980s. Through electron microscopy, they observed the existence of polyribosomes within the distal dendrites of granular neurons (Steward and Levy, 1982). Why would there be components of the protein synthesis machinery outside of the cell body unless

there was translation that needed to occur in a temporo-spatial manner? After these initial anatomical observations, biochemical analysis showed that radioactively labeled amino acids could be rapidly incorporated into new proteins in hippocampal CA1 pyramidal cell dendrites (Feig and Lipton, 1993). The speed at which this incorporation was observed suggested that these proteins were being translated from a population of RNAs that were already present in this location, as transport from the nucleus would take far longer. Furthermore, it was observed that certain mRNAs such as those encoding microtubule-associated protein 2 (MAP2) and the α-subunit of CaMKII were localized to the dendrites (Bruckenstein et al., 1990; Burgin et al., 1990; Garner et al., 1988; Kleiman et al., 1990). How do these mRNAs find themselves localized to these regions of the neuron?

The first observation of mobile RNA granules was done by microinjection of the myelin basic protein (MBP) RNA in oligodendrocytes. This led to the observation that ribonucleoproteins actively moved along the cytoskeleton (Ainger et al., 1993). The addition of SYTO 14 (a nucleic acid dye which fluoresces upon binding) showed RNA granules moving into the dendrites. Isolation of these granules showed that they contain components of the translational machinery, suggesting that these granules represented potential pools of RNA that could be translated upon reaching their destination (Knowles et al., 1996). It was later shown that these RNA structures were mobilized due to changes in neuronal activity, in agreement with the notion that these mobile RNA granules could be critical in synaptic plasticity (Kang and Schuman, 1996). In the case of the CaMKIIα RNA, depolarization resulted in selective movement towards the dendrites (Rook et al., 2000). Once localized, CaMKII mRNAs are translated in response to

processes such as exposure to Brain-derived neurotrophic factor (BDNF) (Schratt et al., 2004), and activation of NMDA receptors (Scheetz et al., 2000).

Lastly, many attempts have been made to elucidate components of these ribonucleoprotein (RNP) complexes and have yielded different results owing to the heterogeneous nature of RNPs. Studies have identified a variety of transcripts and proteins including: mRNAs encoding for αCaMKII, Arc and β actin, and proteins such as the polyribosome-associated protein FMRP (whose loss of function leads to Fragile X Syndrome) and Staufen (Elvira et al., 2006; Kanai et al., 2004).

Staufen:

Staufen is a double stranded-RNA binding protein that is involved in RNA localization, translation and mRNA decay (Park et al., 2013; Tang et al., 2001; Wickham et al., 1999; Yamagata et al., 2009). In *Drosophila*, Staufen has been primarily studied in the context of embryonic development and localization of mRNAs *bicoid* and *oskar* (Ephrussi et al., 1991; Kim-Ha et al., 1991; Schupbach and Wieschaus, 1986). The *Drosophila* Staufen protein contains five double-stranded RNA binding domains (dsRBD). Of these domains, only dsRBD2 and dsRBD5 do not appear to bind to dsRNAs *in vitro*. However, RBD2 and RBD5 have been shown to be important in microtubule-localization and depression of *oskar* mRNA through protein-protein interactions, respectively (Micklem et al., 2000). Staufen binding to RNA targets appears to be largely nonspecific; however, it has been shown that binding to *bicoid* mRNA depends largely on secondary stem loop structures in the 3'UTR of the transcript (Ferrandon et al., 1994; Wickham et al., 1999). In addition to *oskar* and *bicoid*, Staufen is required for the proper

localization of *prospero* mRNA in neuroblasts (Li et al., 1997). Prospero is a homeobox protein that is important in determining cell fate of neuronal precursors and proper routing of axons during development (Doe et al., 1991; Vaessin et al., 1991). While proper *osk* and *bic* localization is dependent on microtubules, *prospero* localization requires actin microfilaments (Broadus and Doe, 1997). It appears these differences in transport mechanisms are at least in part through protein-protein interactions. For example, Miranda interacts with dsRBD5 of Staufen to mediate its interaction with *prospero* mRNA (Fuerstenberg et al., 1998; Schuldt et al., 1998; Shen et al., 1998)

There are two Staufen genes (Staufen1 and Staufen2) in mammals which share 51% identify based on amino acid sequence (Furic et al., 2008). Both Staufens bind dsRNA through dsRBD3 and dsRBD4 (Luo et al., 2002; Wickham et al., 1999). And while both Stau1 and Stau2 are found in RNA granules within dendrites, they do not appear to co-localize (Duchaine et al., 2002; Kiebler et al., 1999; Tang et al., 2001). Furthermore, in HEK293T cells it has been shown that Stau1 and Stau2 containing granules consist of distinct populations of RNAs with some only minor overlap between them (Furic et al., 2008).

Staufen is a critical component of synaptic plasticity across model systems. *Drosophila* Staufen mutants exhibit defects in long-term memory formation (Dubnau et al., 2003). Furthermore, down regulation of mammalian Staufen leads to defects in dendritic spine morphology and synaptic function (Goetze et al., 2006; Lebeau et al., 2011; Lebeau et al., 2008; Vessey et al., 2008). Also the amount of RNA present in RNA granules is directly affected by Stau2 levels and neuronal activity (Mikl et al., 2011).

One possible mechanism by which Staufen effects synaptic plasticity could be due to its transport of CaMKII mRNAs in response to changes in neuronal activity (Jeong et al., 2007; Krichevsky and Kosik, 2001). Mutating the dendritic targeting element of the CaMKIIα mRNA results in complete loss of CaMKII targeting to the dendrites, loss of the protein at the post-synaptic density (PSD) and corresponding defects in long-term memory (Miller et al., 2002).

Purpose:

Adf-1 mutants exhibit profound defects in long-term memory formation. To determine if Adf-1 functions in the process of activity-dependent plasticity, a process underlying memory formation, we turn to an *in vivo* system to measure Adf-1 effect on post-synaptic (dendritic) plasticity. We also attempt to elucidate the functional significance of the phosphorylation of Adf-1 by CaMKII. Finally, through ChIP-sequencing from larval brain tissue, we identify novel transcriptional targets of Adf-1 that are critical to its function in dendritic plasticity.

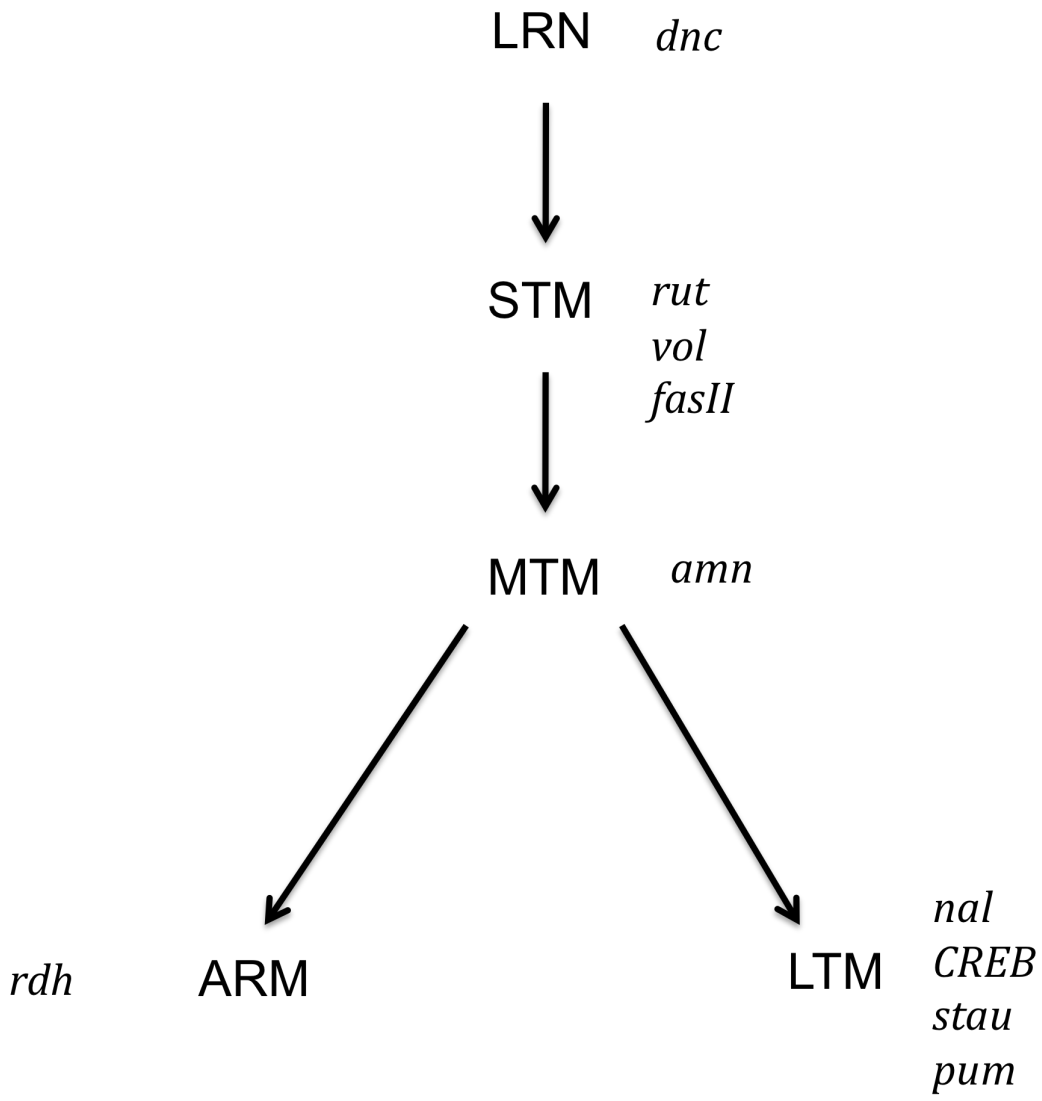

Figure 1: Distinct Phases of Learning & Memory in *Drosophila*

Figure 1: Phases of memory formation and corresponding memory mutants. Dunce mutants exhibit defects in learning. Rutabaga and the cell adhesion molecules FasII and Volado exhibit defects in short-term memory formation. Amnesiac mutants disrupt middle-term memory. Anesthesia resistant memory is defective in flies with mutations in Radish. Long-term memory formation is inhibited in CREB, Adf-1 (*nalyot*), Staufen and Pumilio mutants.

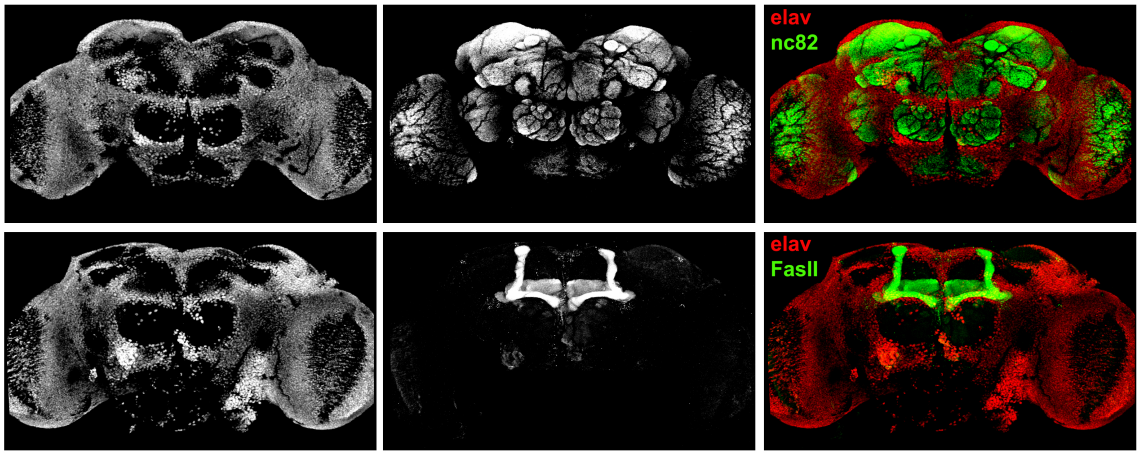

Figure 2: *Drosophila* Adult Brain Structure

Figure 2: *Drosophila* Adult Brain Structure: Adult brain stained with pan neuronal marker elav and presynaptic marker bruchpilot. Additional staining by fasII that marks the mushroom body (site of higher learning).

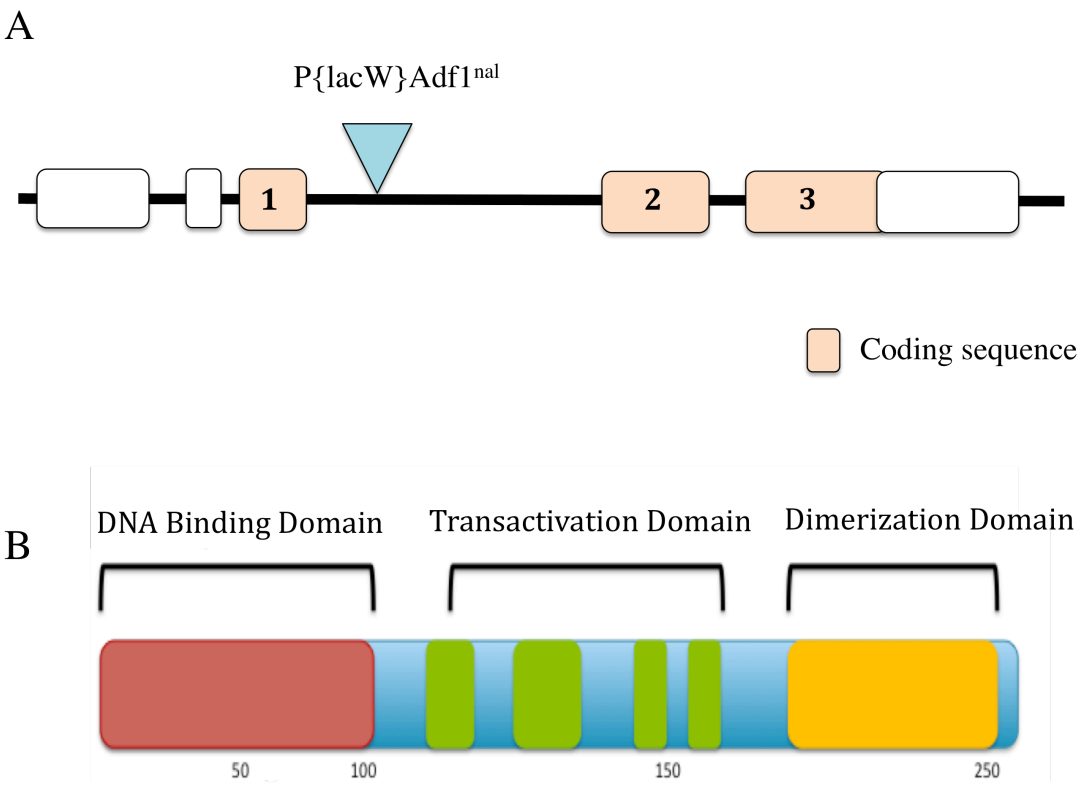

Figure 3: Adf-1 Protein and Gene Structure

Figure 3: Adf-1 Protein and Gene Structure: A) Adf-1 gene region. Insertion of transposable element into the large intron results in an Adf-1 hypomorph (*nalyot*). B) Protein structure of Adf-1: The Adf-1 protein is 34kDA in size and consists of a DNA binding domain, a dimerzation domain and transactivation domains located throughout the protein.

Chapter 2

The *Drosophila* transcription factor Adf-1(*nalyot*) regulates dendrite growth by controlling FasII and Staufen expression downstream of CaMKII and neural activity

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Summary

Memory deficits in *Drosophila nalyot* mutants suggest that the Myb-family transcription factor Adf-1 regulates neuronal plasticity. Using *in vivo* 3D reconstruction, I show that Adf-1 is required cell-autonomously for dendritic development and activity-dependent plasticity downstream of CaMKII. Adf-1 inhibition reduces dendrite growth and neuronal excitability and results in motor deficits and altered transcriptional profiles. Surprisingly, comparative ChIP-Seq analysis of Adf-1, RNA PolymeraseII and histone modifications in Kc cells shows that Adf-1 binding correlates positively with high Pol II pausing indices and negatively with active chromatin marks such as H3K4me3 and H3K27ac. Consistently, expression of Adf-1 targets Staufen and FasII, identified through larval brain ChIP-Seq for Adf-1, is negatively regulated by Adf-1 and manipulations of these genes predictably modify dendrite growth. Our results imply mechanistic interactions between transcriptional and local translational machinery in neurons and suggest that CaMKII, Adf-1, FasII and Staufen influence crucial aspects of memory formation by regulating dendritic plasticity.

Introduction

Normal development and plasticity of neuronal dendrites is indispensible for basal nervous system output as well as behavioral adaptations in the brain such as memory formation (Cline, 2001; Tavosanis). Recent studies suggest that pathophysiological changes in dendrite development and function are likely to underlie neurological disorders such as autism spectrum disorders, Alzheimer's disease and schizophrenia (Penzes et al.; van Spronsen and Hoogenraad). Since dendrites are the sites for signal integration, changes in the pattern of connectivity of dendrites have profound effects on information processing in any given neuronal circuit (Yuste, 2011). As a result, molecular mechanisms that regulate dendrite architecture are likely to be fundamental components of the overall cellular apparatus that regulates activity-dependent plasticity and behavioral adaptation. Given the requirement for new protein synthesis during long-term changes in neurons, transcription and local translation are the two key processes that are expected to regulate changes in dendrite growth and function (Bramham and Wells, 2007; Steward and Worley, 2002; Sutton and Schuman, 2006).

New gene transcription is an integral part of protein synthesis-dependent forms of neuronal plasticity (Sanyal and Ramaswami, 2006; West and Greenberg). A number of transcription factors, including CREB, CREST, Fos, Jun, MEF-2 and NFAT, respond to changes in neural activity and calcium to activate transcriptional programs that regulate pre- and post-synaptic plasticity (Aizawa et al., 2004; Chrivia et al., 1993). This response, often induced by signaling proteins such as the mitogen-activated protein kinase (MAPK) and calcium-calmodulin kinase (CaMK), includes post-translational modifications,

transcriptional activation, nuclear translocation and recruitment of chromatin modifying factors (Burgoyne, 2007; West and Greenberg; West et al., 2002). Neuron-specific gene expression programs that are regulated by these transcription factors are thought to orchestrate long-lasting changes in the structure, function and connectivity of relevant neurons. Several studies have also demonstrated the importance of rapid, synapse-specific translational activation in protein synthesis-dependent plasticity (Aakalu et al., 2001; Jakawich et al.; Ju et al., 2004; Kang and Schuman, 1996; Martin et al., 1997; Miller et al., 2002; Steward, 1987). It is generally believed, that local translation serves to restrict activity-dependent changes in neurons to relevant synapses (Frey and Morris, 1997; Martin et al., 1997). Such synaptic specificity is determined by conserved RNA-binding proteins that are involved in the transport and translational regulation of dendritic and axonally targeted mRNAs (Barbee et al., 2006; Bramham and Wells, 2007; Krichevsky and Kosik, 2001; Martin and Zukin, 2006; Steward and Worley, 2001; Sutton and Schuman, 2006; Udagawa et al.). In spite of intense research, one area that has been explored sparsely is interplay between activity-dependent transcriptional in nuclei and the machinery that regulates translation of sequestered mRNA at synapses, especially in dendritic plasticity.

Molecular regulation of dendritic plasticity can be studied in identifiable *Drosophila* motor neurons (Hartwig et al., 2008; Mauss et al., 2009). These neurons are glutamatergic, display activity-dependent plasticity and possess stereotypic dendritic arbors. High throughput methods to visualize and measure the architecture of these dendrites has become feasible due to the development of genetic methods of labeling these neurons, improved imaging and semi-automated methods to reconstruct dendrites in

3D (Hartwig et al., 2008; Schmitt et al., 2004). A further advantage to using motor neurons as a model to study dendrite growth is that their output - motor activity - is easy to measure and correlate with cellular changes. Finally, patch-clamp protocols have also been optimized to examine membrane properties of the soma in these neurons that might include contributions from dendritically localized currents (Choi et al., 2004; Hartwig et al., 2008). In this report we use these techniques to explore cellular functions for the Myb family transcription factor Adf-1, mutations in which lead to long-term memory deficits in *Drosophila*. We find that Adf-1 controls the growth of RP2 dendrites downstream of neural activity and CaMKII. To discover transcriptional targets of Adf-1 in neurons, we performed ChIP-Sequencing on larval brains to identify promoters that bind Adf-1 *in vivo*. We examine two genes, the neural cell adhesion molecule FasII, and the RNA binding protein Staufen for their transcriptional response to Adf-1 activity and their ability to modify dendrite growth. We identify a negative impact of Adf-1 on the transcriptional regulation of FasII and Staufen, and a strong correlation between Adf-1 binding at promoters with RNA Pol II pausing and the relative absence of marks of active chromatin. Together, our results suggest that Adf-1 plays an important role in controlling the development and plasticity of neuronal dendrites by modulating two key processes, cell adhesion and mRNA transport.

Results

Adf-1 is expressed in motor neurons in the Drosophila larval nerve cord

We used the *Drosophila* larval motor neuron RP2 as a model to examine Adf-1 dependent regulation of dendrite development and plasticity since this neuron displays activity-dependent changes in growth (Hartwig et al., 2008). We first tested whether Adf-1 is expressed endogenously in these neurons. To test for Adf-1 expression, we generated affinity purified polyclonal antibodies against two Adf-1 derived peptides (see experimental methods). These antibodies detected both basal and transgenically over-expressed Adf-1 in the nuclei of larval motor neurons (Figure 1D, E and F; see insets for dorsal midline motor neurons RP2 and aCC). Wild type FLAG-tagged Adf-1, when expressed transgenically using a synthetic GAL4 that is only active in RP2 and aCC neurons in a mosaic fashion (a system that we have described previously (Hartwig et al., 2008)), is elevated selectively in the nuclei of motor neurons where GAL4 activity is present (compare GAL4 driven GFP expression with Adf-1 and transgenic FLAG expression in Figures 1D-F). Anti-Adf-1 antibodies also detected a band of the predicted size (~ 30 kDa) on western blots of proteins extracted from larval and adult brains (Figure 1G) that was increased in intensity when wild type Adf-1 is over-expressed pan-neuronally, and reduced in Adf-1 hypomorphic *nalyot* mutants. Further, chromatin immuno-fluorescence experiments showed that this antibody is also capable of detecting Adf-1 that is bound to DNA in larval salivary gland polytene chromosomes consistent with its known function as a DNA binding transcription factor (Figure 1H-J) (England et al., 1990; Heberlein et al., 1985). Nervous system expression of Adf-1 was also evident

from the expression pattern of a new Adf-1-GAL4 enhancer trap line which revealed extensive neuronal staining in addition to some non-neuronal cells in the ventral nerve cord (Figure 1A-C; compare Elav staining of post-mitotic neuronal nuclei with GFP expression driven by the Adf-1-GAL4 line; see also adult expression in supplementary Figure 1 A-F). We generated this line by replacing a lacZ enhancer trap P-element (P{PZ}Adf-1^{01349}) inserted in an the first exon 408 base pairs downstream of the transcription start site with a P{GawB} element inserted close to the *futsch* gene (C380-GAL4; (Sanyal, 2009)) using a transposase mediated P-element switching technique described previously (Sepp and Auld, 1999) (supplementary Figure 1 G and H). Since this P-element, and the original lacZ enhancer trap, are inserted in an exon, they result in recessively lethal null alleles. Interestingly, lethality in these alleles could be rescued by adding back wild type Adf-1 in the nervous system using *elav*^{C155}-GAL4 driven expression from the UAS-Adf-1::FLAG transgene shown in Figure 1E. This is in contrast to the original homozygous viable *nalyot* allele described previously (DeZazzo et al., 2000) in which a P-element transposon is inserted in an intron 639 base pairs downstream of the transcription start site thereby generating a hypomorphic allele (supplementary Figure 1 H). Taken together, these data clearly demonstrate that Adf-1 is an essential gene that is expressed in the *Drosophila* nervous system.

Inhibition of Adf-1 in the RP2 motor neuron leads to severely reduced dendrite growth and excitability

nalyot mutants have been shown to result in marginally smaller pre-synaptic termini at larval synapses with fewer boutons (DeZazzo et al., 2000). Following our earlier observation that transcription factors such as AP-1 affect both pre- and post-

synaptic growth (Hartwig et al., 2008; Vonhoff et al.), we asked whether loss of Adf-1 affects dendrite growth in larval motor neurons such as the RP2 motor neuron. We first examined the effect of a mild reduction in Adf-1 levels in a *nalyot* heterozygous animal. Dendrites of optically isolated RP2 neurons labeled with mcd8::GFP were reconstructed using a semi-automated plugin developed for AMIRA (see experimental procedures for details; mcd8::GFP colocalizes with dendritic markers such as DenMark, supplementary Figure 2A-C (Sanchez-Soriano et al., 2005) (Evers et al., 2005; Schmitt et al., 2004; Vonhoff et al.). These reconstructions revealed a 50% reduction in both the number of dendritic branches and total dendrite length in *nal* heterozygotes as compared to controls (compare reconstructed dendrite arbors in Figure1 B and C; Figure 1E and F present volumetric Sholl analysis and quantification of dendrite branches and length; supplementary Figure 2D shows branch order analysis). Multiple attempts to excise this P-element or the Gal4 enhancer trap did not result in any verifiable null allele for Adf-1 owing perhaps to the close proximity of this gene to the second chromosome centromere. For this reason, we were also unable to generate a recombinant of the *nalyot* allele with an FRT containing insertion on 2R and carry out MARCM based mosaic analysis of Adf-1. In spite of these limitations, we attempted to determine the cell-autonomous origin of this phenotype, by targeting expression of a modified Adf-1 protein (in which 18 amino acids in the putative trans-activating domain have been replaced with 5 alanine residues) to RP2 neurons using our synthetic GAL4 driver line (supplementary Figure 1H; experimental procedures). Expression of this Adf-1-dominant negative transgene (Adf-1 DN) resulted in a more severe loss of dendrites (compare Figure 1B and D) and a dramatic reduction in dendritic complexity (Figure 1E), branch number and length

(Figure 1F) as compared to *nalyot* heterozygous animals. Whole-cell patch clamp measurements from RP2 motor neurons expressing Adf-1 DN revealed healthy neurons with robust input resistance and resting membrane potentials albeit with lower cell capacitance (likely due to reduced dendritic membrane; supplementary Figure 2 E-G). However, these cells were largely refractory to the injection of depolarizing current which typically elicited robust action potentials from control RP2 neurons (Figure 2G, H). The Adf-1 DN transgene likely leads to inhibition of endogenous Adf-1 since: *a)* phenotypes resulting from the expression of Adf-1 DN match those from a genetic reduction of Adf-1 (*nalyot* mutants), *b)* Adf-1 DN phenotypes are similar to those resulting from expression of a [S64/184A] version of Adf-1 (see next section and Figure 3A-E), *c)* pulsed expression of Adf-1 DN in the adult brain influences expression of a validated Adf-1 target gene, Alcohol dehydrogenase (ADH) in a manner similar to that in *nalyot* mutants (Figure 2A, note that Adf-1 inhibition increases ADH expression, see later for Adf-1's effect on gene expression), *d)* Adf-1 DN affects expression of two Adf-1 targets FasII and Staufen similar to a [S64/184A] Adf-1 (see later, Figure 7A), *e)* Adf-1 has been shown to function as a dimer (Cutler et al., 1998), hence a truncated Adf-1 that retains a dimerization domain is likely to function as a dominant-negative, and, *f)* chronic pan-neuronal expression of Adf-1 DN results in lethality similar to Adf-1 null homozygotes. Taken together, results in this section suggest that Adf-1 activity is required in motor neurons for normal dendrite growth and complexity.

Adf-1 controls dendrite growth downstream of CaMKII signaling

Adf-1 is an atypical Myb-family transcription factor that is non-modular, contains both DNA and protein binding domains and has a "distributed" trans-activation domain

(Cutler et al., 1998; England et al., 1990; Heberlein et al., 1985). While Adf-1 is known to regulate ADH transcription (Heberlein et al., 1985) and is involved in memory formation, virtually nothing is known about upstream regulatory elements that control Adf-1 activity. To determine whether Adf-1 activity might be regulated by phosphorylation we carried out an *in silico* scan of the Adf-1 coding sequence for potential phosphorylation sites and relevant kinases using NetPhosK (Blom et al., 2004). This scan highlighted two Serine residues, Ser-64 and Ser-184 that are putative CaMKII targets. Indeed, a previous study had shown that both endogenous and exogenous *Drosophila* CaMKII could phosphorylate purified recombinant Adf-1 with a preference for Ser-64 (GuptaRoy et al., 2000). Based on these findings and the widely appreciated role for CaMKII in dendritic plasticity across species (Gaudilliere et al., 2004; Lee et al., 2009b; Miller et al., 2002; Rongo and Kaplan, 1999; Vonhoff et al.; Wu and Cline, 1998; Zou and Cline, 1999), we tested the idea that CaMKII-dependent phosphorylation of Ser-64/184 regulates Adf-1 activity, and thereby dendrite growth, in RP2 motor neurons.

In order to test the important of this CaMKII phosphorylation site on Adf-1 function, we constructed two UAS-driven, Adf-1 transgenes, one in which Ser-64/184 were mutated to Alanine (Adf-1-[S64/184A], a phospho-null substitution) and another in which the residues were changed to Aspartate (Adf-1-[S64/184D], a phospho-mimic substitution). We expressed these transgenes in RP2 neurons using our synthetic GAL4 either singly or in combination with reagents that increase or inhibit CaMKII (Jin et al., 1998). Our results, summarized in Figure 3A-C, show that mutating Ser-64/184 to Ala leads to a dramatic reduction in dendritic complexity, number of dendritic branches and overall dendrite length. Since this phenotype resembles both *nalyot* mutants and Adf-1

DN it suggests a crucial role for Ser-64/184 phosphorylation in normal Adf-1 function. Consistent with the idea that CaMKII is the kinase responsible for this phosphorylation (GuptaRoy et al., 2000), expression of the Adf-1 [S64/184A] transgene completely abrogates the stimulatory effect of a constitutively active calcium-independent CaMKII transgene (CaMKII-T287D) on dendrite growth (Figure 3A-C) (Jin et al., 1998; Vonhoff et al.). Of note also is the observation that expression of CaMKII[T287D] results in a significant increase in overall dendrite length, but not the number of branches (Figure 3C). Volumetric Sholl analysis indicates, however, that the overall pattern of branching changed such that there were a greater number of branches closer to the neuronal soma (Figure 3B; the physiological basis for this is unclear, although it might reflect a region of higher pre-synaptic connectivity). A similar shift occurred when we expressed the Asp substituted Adf-1 (Adf-1[S64/A84D]) although both overall dendrite branch number and length remained unchanged as compared to controls (Figure 3D, E). Significantly, expression of Adf-1[S64/184D] significantly increased both branch number and total dendrite length in a CaMKII inhibited background (a CaMKII peptide inhibitor; ala (Jin et al., 1998)), which results in a strong decrease in dendrite growth (Figure 3D, E). In sum, these results support the importance of the two putative CaMKII target sites in Adf-1in the regulation of Adf-1 activity and dendrite growth.

Adf-1 regulates activity-dependent developmental plasticity of dendrites in RP2 motor neurons

As in other species, elevated neuronal activity increases dendrite growth while silencing neurons leads to a reduction in dendrite growth in *Drosophila* motor neurons

(Hartwig et al., 2008; Vonhoff et al.). Since both CaMKII and a number of plasticity-related transcription factors function downstream to changes in neural activity (Aizawa et al., 2004; Colbran and Brown, 2004; Corty et al., 2009; Flavell et al., 2006; Gaudilliere et al., 2004; Inagaki et al., 2000; Lee et al., 2009b; Ouyang et al., 1997; Redmond et al., 2002; Sanyal et al., 2002; Shalizi et al., 2006; Vaillant et al., 2002; Vonhoff et al.; Wayman et al., 2006), we tested whether Adf-1 also belongs in this category. Elevating neuronal firing in RP2 motor neurons through the co-expression of two dominant-negative voltage gated potassium channels Eag and Shaker (EKI) resulted in increased dendritic complexity near the soma (Figure 3F and G) and increased dendrite branch number (Figure 3H). In this background, expression of Adf-1[S64/184A] completely inhibited dendrite growth (Figure 3F, G, H) suggesting that normal Adf-1 activity is necessary for activity-dependent growth of RP2 dendrites. Conversely, silencing RP2 neurons by expressing the inward rectifying $K_{ir}2.1$ channel strongly reduced dendrite growth as expected (Figure 3G, I, J). Consistent with a role downstream of neural activity, Adf-1[S64/184D] expression in an activity-inhibited background was able to modestly rescue dendrite growth such that both dendrite branch number and total length were significantly greater than in $K_{ir}2.1$. Thus, Adf-1[S64/184D] was only able to increase dendrite growth in a background where neural activity, and by extension CaMKII and Adf-1 activity, were severely limiting. Overall, these results suggest that normal Adf-1 phosphorylation downstream of CaMKII is required for activity-dependent changes in dendrite growth.

Adf-1 inhibition in motor neurons impairs sensory-motor transmission

Our results indicate that normal Adf-1 activity is required in motor neurons for the development and activity-dependent plasticity of dendrites downstream of CaMKII signaling. Patch-clamp experiments shown in Figure 2 also reveal that strong Adf-1 inhibition results in reduced excitability and altered membrane properties in these motor neurons. We next asked whether these changes affect synaptic excitation of motor neurons and their output at the neuro-muscular junction (NMJ). For these experiments we used the Adf-1[S64/184A] transgene as pan-neuronal expression of Adf-1 DN is lethal. Results shown in Figure 4 suggest that synaptic transmission from these motor neurons at the neuro-muscular junction is normal, consistent with the previous report (DeZazzo et al., 2000) (Figure 4A). Both EJP and mEJP amplitude are unchanged as are mEJP frequency and quantal content of transmitter release (not shown). This indicates that dendrite phenotypes observed in these neurons do not affect neurotransmission at the NMJ. Basic membrane properties of these neurons were also not affected by the expression of Adf-1[S64/184A] since cell capacitance, input resistance, resting membrane potential and the threshold for action potential firing were all comparable to control neurons (Figure 4C). Next, we tested whether synaptic stimulation of motor neurons is altered following Adf-1 inhibition. Since precise pre-synaptic partners for larval motor neurons are currently unknown, we made use of an assay where high-frequency (10Hz) stimulation of the sensory afferent results in recruitment of motor units in the contralateral segment (Dasari and Cooper, 2004) in a frequency and amplitude-dependent manner (Figure 4B shows the recording configuration). As reported previously, when a sensory afferent was stimulated, we invariably observed motor neuron firing in the contralateral segment that continued beyond the stimulus duration (motor

neuron firing was monitored indirectly by recording muscle EPSPs from muscle 6; 10 independent animals were tested) (Figure 4D). By contrast, animals in which Adf-1[S64/184A] was expressed in a motor neuron enriched manner using the *futsch*^{C380}-GAL4 driver often failed to display motor neuron firing under these conditions, such that we were not able to elicit motor neuron firing in more than 50% animals (Figure 4D). Since *futsch*^{C380}-GAL4 also expresses in sensory neurons (Sanyal and Ramaswami, 2006), we limited expression to motor neurons by suppressing GAL4 expression in cholinergic neurons with the Cha-GAL80 transgene (Hartwig et al., 2008; Salvaterra and Kitamoto, 2001). Even under these conditions, we observe the same deficiency in sensory-motor transmission, suggesting that inhibition of Adf-1 in motor neurons is sufficient to cause this transmission defect (Figure 4E). These results are consistent with phenotypic consequences that are expected from a reduction in dendrite branching (reduced pre-synaptic input) and alteration in excitability and membrane properties at the soma. They also suggest that locomotor behavior might be seriously impaired following Adf-1 inhibition in motor neurons, a prediction we test in the next section.

Developmental perturbation of Adf-1 in motor neurons alters locomotor behavior

Dendrite growth phenotypes in larval motor neurons that result from inhibition of Adf-1 activity are likely to cause aberrant motor behavior especially in light of deficient sensory-motor transmission (Figure 4). To directly test this possibility, we measured larval crawling in control and Adf-1 inhibited genotypes. As shown in Figure 5A and B, expression of the Adf-1[S64/184A] mutant transgene in motor neurons through the *futsch*^{C380}-GAL4 driver (Sanyal and Ramaswami, 2006) results in reduced larval

locomotion (Figure 5B). Since we observe aberrant development of motor neuron dendrites upon Adf-1 inhibition, we next asked whether locomotor phenotypes in these animals derive from developmental abnormalities. To this end we turned to adult flies that allow us to inhibit Adf-1 after developmental changes in the nervous system are largely completed. We first confirmed that Adf-1 is expressed in adult motor neurons in the thoracic-abdominal ganglion using a marker for motor neurons, the transcription factor Zfh1 (Layden et al., 2006). We find that both Adf-1 and the *futsch*^{C380}-GAL4 are expressed in these motor neurons and co-localize with Zfh1 (supplementary Figure 4A-F). In order to inhibit Adf-1 in adult motor neurons, we expressed the Adf-1 [S64/184A] transgene with the *futsch*^{C380}-GAL4 driver. When reared continuously at 25^{o}C, expression of this transgene resulted in widespread adult lethality with few escaper animals that display striking defects in locomotion. Interestingly, *nalyot* mutants also displayed sluggish locomotion as compared to age-matched controls, an observation consistent with a less severe loss of Adf-1 in these mutants. To circumvent lethality, and focus on the motor neuron derived phenotypes of Adf-1, we tested multiple transgenic lines with different genomic insertion sites to isolate ones with weaker GAL4 induced transgene expression. As shown in Figure 5C, we identified two lines that showed weak and moderate transgene expression as compared to the original "strong" line when combined with the *elav*^{C155}-GAL4 driver (western analysis on larval brain protein extracts; transgenic expression is detected by probing for FLAG). The weak line, when crossed to *futsch*^{C380}-GAL4 and reared at 25^{o}C, resulted in adults that displayed prominent locomotor phenotypes in the Buridan's assay (Freeman et al.; Gotz, 1980; Strauss and Heisenberg, 1993) (supplementary Figure 4G and H; see experimental procedures for

details). Similarly, the strong line, when crossed to C380-GAL4 and grown at 18^{o}C, also resulted in similar locomotor defects (Figure 5D and E). Interestingly, these phenotypes could be completely rescued by preventing GAL4 expression during development using the TARGET system (McGuire et al., 2004) (Figure 5F and G). Release of GAL80-mediated inhibition after eclosion in the strong line (by shifting from 18^{o}C to 28^{o}C for a week), produced very mild phenotypes (supplementary Figure 4I and J). Together, these results demonstrate that perturbation of Adf-1 in motor neurons during development, but not acutely post-development, results in strong locomotor phenotypes. This is consistent with the dendrite development and sensory-motor transmission phenotypes that we observed in larvae and suggests that Adf-1 is required for the normal development, function and output from motor neurons.

Potential neuronal targets of Adf-1 identified through genome-wide ChIP-Seq analysis

Our results thus far demonstrate that Adf-1 functions downstream to neural activity and CaMKII signaling to regulate dendrite development and excitability in motor neurons. In order to identify novel genes whose expression is regulated by Adf-1, we carried out chromatin immuno-precipitation (ChIP) from dissected larval brains using anti-Adf-1 antibodies followed by sequencing (ChIP-Seq) (see experimental procedures for details). We identified robust binding of Adf-1 to the ADH enhancer as described previously (supplementary Figure 5A). Further analysis showed, that as expected for a transcription factor, more than 50% of Adf-1 binding sites occur within 200 base pairs of transcription start sites (TSS) (Figure 6A, B; see Table 1 for a list of genes that bind Adf-1 close to gene TSS). These binding sites were used to calculate a primary Adf-1 binding

consensus sequence (Figure 6C) (Machanick and Bailey). Interestingly, this primary consensus sequence [ACGG/CCGG/ACA/TGC/AG] derived from whole genome ChIP-Seq analysis contains the core of a recently reported high-affinity Adf-1 binding consensus [GT/CGG/AC] (Lang and Juan), and is dissimilar to the binding sequence reported previously $[GC/TC/T]_{4-5}$ (Heberlein et al., 1985). More than 80% of the genes that bound Adf-1 close to the TSS in the brain also fell into a network of proteins (Figure 6F) created using GeneMania on the basis of published reports of genetic and physical interactions among *Drosophila* proteins (Guruharsha et al.; Mostafavi et al., 2008). Moreover, gene ontology analysis revealed a high degree of enrichment for genes with neuronal functions (Figure 6G, supplementary Figure 5B) as can be expected for a transcription factor with strong neural roles.

We had noticed previously that Adf-1 inhibition in the brain resulted in increased transcription of ADH (Figure 2A). In order to derive a mechanistic understanding of Adf-1-dependent gene expression, we carried out additional Adf-1 ChIP-Seq experiment in Kc cells. This allowed us to generate a data set that could be compared with a library of ChIP-Seq data for RNA Polymerase II (Pol II) binding and the distribution of covalent histone modifications. Kc cell ChIP-Seq with anti-Adf-1 antibodies resulted in a larger number of binding peaks that, however, showed a similar profile of binding as compared to the brain ChIP-Seq experiment (supplementary Figure 5E, F). Comparison to Pol II binding sites and active marks of chromatin also showed that Adf-1 is enriched at promoters that show a higher Pol II pausing index (Figure 6E, H). Visual inspection of specific genomic sites suggest a negative correlation between Adf-1 binding and H3K4me3 and H3K27ac (representative gene tracks are shown in Figure 6D). To

determine the general significance of this observation, we arranged all TSSs in the genome in descending order based on Adf-1 levels and we examined the distribution of Pol II, H3K4me3 and H3K27ac (Figure 6E). Genes with high levels of Adf-1 (Group 1 genes) contain Pol II at their TSS but they have low levels of H3K4me3 and H3K27ac, suggesting low transcriptional activity. On the other hand, genes with low Adf-1 have similar amount of Pol II but higher levels of these two histone modifications (Figure 6E). The presence of Pol II at the TSSs of both groups of genes with different transcriptional activity suggest a possible role for Adf-1 in transcription pausing. To further dissect the role of Adf-1 in gene expression, we measured the pausing index of Group 1 and Group 2 genes. Figure 6H shows that Adf-1-bound genes have a high pausing index, supporting a role for this protein in promoter-proximal pausing.

Does Adf-1 regulate a subset of genes whose mRNA is highly enriched in dendrites? Although a data set for dendritically enriched mRNAs is not available for *Drosophila*, a recent study has reported a set of 2550 mRNAs that are enriched in either axons or dendrites in the synaptic neuropil of area CA1 in the adult rat hippocampus. Comparison of the Adf-1 binding genes in the larval brain with this data set shows that 91 genes (including L1 CAM and Stau2) are shared between them (supplementary Figure 5G) – a group that might represent Adf-1 regulated genes that are especially relevant to dendrite development.

Adf-1 controls FasII and Staufen expression to regulate dendrite growth

We tested a number of genes that were identified as binding Adf-1 close to the TSS as potential Adf-1 targets in the brain, and confirmed Adf-1-dependent regulation of

FasII, a *Drosophila* homolog of NCAM (Neural cell adhesion molecule) (Lin et al., 1994). The pattern of regulation followed the same pattern as for ADH in that Adf-1 inhibition resulted in increased FasII mRNA and protein in adult heads (Figure 7A; supplementary Figure 6C). Adf-1 binding to the FasII enhancer region in Kc cells also followed the same general pattern and correlated with paused Pol II and minimal occupancy for H3K4me3 and H3K27ac (supplementary Figure 5A). Since we determined FasII to be a direct target of Adf-1 in the brain, we asked whether FasII functions downstream of Adf-1 to regulate dendrite growth in RP2 motor neurons in a predictable manner. If Adf-1 inhibits FasII expression, then we would predict: *a)* reduced dendrite growth following over-expression of FasII in RP2 neurons, and, *b)* suppression of Adf-1 inhibition-dependent dendrite growth phenotypes by FasII knock down. Results shown in Figure 7A-F support these predictions. Thus, over-expression of full-length FasII (using a transgene that expresses the FasIIA-PEST+ sequence in RP2 neurons results in reduced dendritic complexity (Figure 7A, B) and reduced dendritic branching and total length (Figure 7C). Conversely, RNAi-mediated FasII knockdown in an Adf-1 inhibited background (expression of Adf-1[S64/184A]) partially rescued dendritic complexity, branch number and total length as compared to Adf-1 inhibition alone (Figure 7A, D, E). Note that as compared to control neurons, both over-expression and knockdown of FasII resulted in fewer dendrites. This is consistent with prior observations (Schuster et al., 1996b)that have suggested an optimum requirement for FasII in neuronal growth, where both increased and decreased levels of FasII result in less growth.

In our search for genes that are potentially regulated by Adf-1 in the brain, we also noticed that Adf-1 perturbations affected mRNA levels of the RNA-binding and

transporting protein Staufen. Although we did not detect Adf-1 binding close to the Staufen gene region, Adf-1 inhibition increased Staufen expression (Figure 7G) perhaps through an indirect mechanism. Phenotypically, Staufen over-expression using an "EP" insertion (we verified Staufen over-expression from this line using antibody staining, data not shown) resulted in reduced dendritic complexity, branch number and length (Figure 7H, I, J). Additionally, RNAi-mediated Staufen knockdown partially rescued dendrite growth phenotypes resulting from Adf-1 inhibition in RP2 neurons (Figure 7H, K, L) as compared to Adf-1 inhibition alone. Thus, similar to our results with FasII, we found that too much or too little Staufen impairs dendrite growth, and that reduced dendrite growth due to Adf-1 inhibition requires increase in Staufen expression. Since both FasII and Staufen have been implicated in memory formation and in axonal or dendrite development (Barbee et al., 2006; Beck et al., 2012; Cheng et al., 2001; Dubnau et al., 2003; Kiebler et al., 1999; Kristiansen et al., 2005; Miyashita et al.; Schuster et al., 1996a; Tang et al., 2001; Vessey et al., 2008), our results support a model in which FasII and Staufen are two target genes that function downstream to Adf-1 in the regulation of behavioral and cellular forms of activity-dependent plasticity (Figure 8).

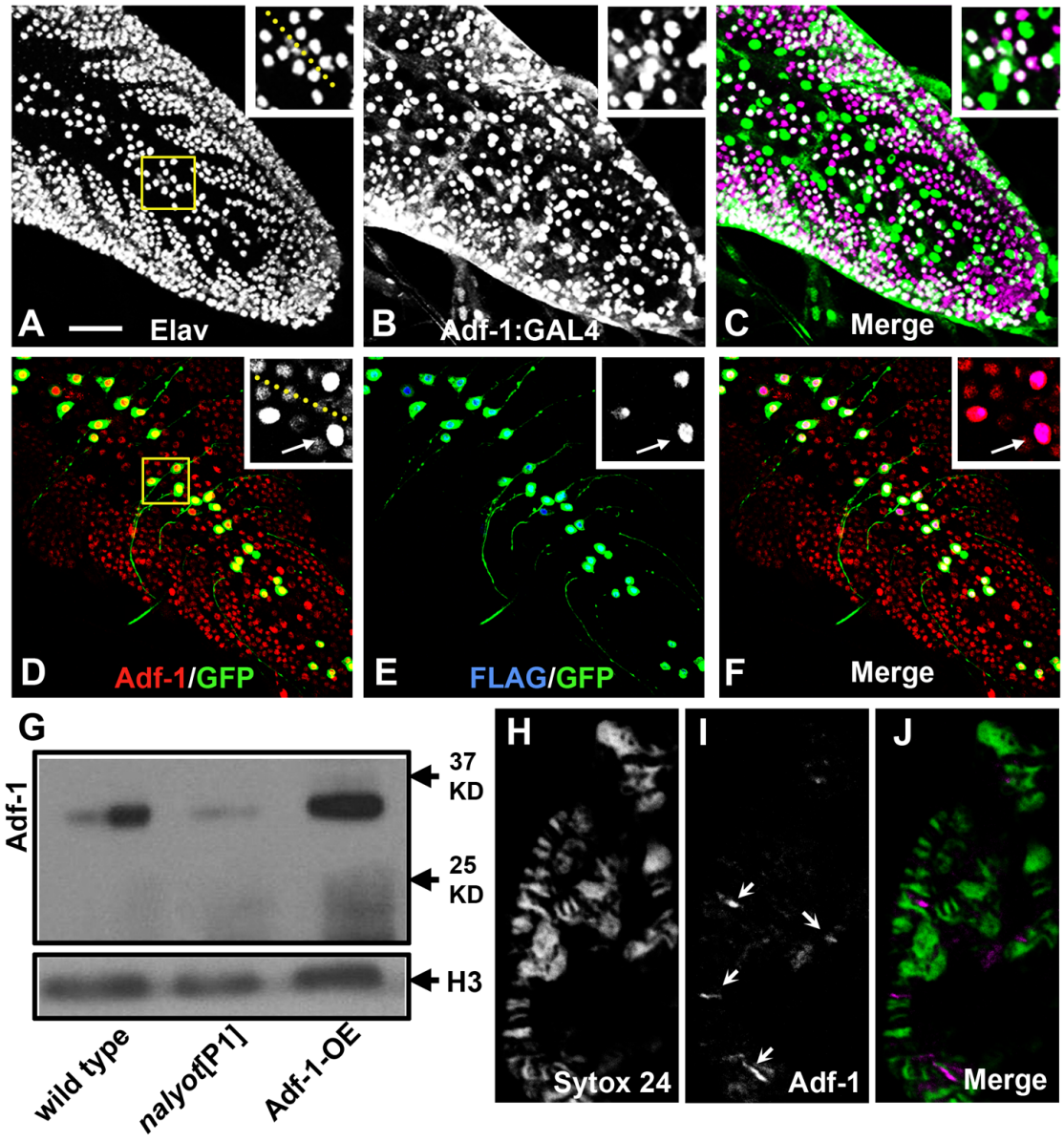

A
Elav
B
Adf-1:GAL4
C
Merge
D
Adf-1/GFP
E
FLAG/GFP
F
Merge
G
Adf-1
37 KD
25 KD
H3
wild type
nalyot[P1]
Adf-1-OE
H
Sytox 24
I
Adf-1
J
Merge

Figure 1

Figure 1: Adf-1 is expressed in larval motor neurons. A, B, C) An Adf-1 enhancer trap line (Adf-1-GAL4) expresses *b*-Galactosidase from a UAS-nls-lacZ reporter in larval neurons in the larval nerve cord (B), double-labeled with anti-Elav to mark all post-mitotic neuronal nuclei (A). Dorso-medial motor neurons also express Adf-1 including RP2 motor neurons (shown in inset and merged images in (C)). (D, E, F) Over-expression of a FLAG tagged wild type Adf-1 using our synthetic GAL4 system is also detectable using staining for the FLAG epitope, mCD8::GFP or increased Adf-1 staining. Inset shows close-up of neuronal nuclei that show expression of FLAG, GFP and elevated levels of Adf-1 driven by GAL4. The larval nerve cord is stained for Adf-1 as a reference. (G) Western blot of protein extracted from adult brain tissue probed using our peptide antibodies to Adf-1. The previously described nal^{P1} allele shows reduced expression as expected, while transgenic over-expression of Adf-1 increases Adf-1 protein levels. Anti-Histone H3 is used as a loading control. (H, I, J) Polytene chromosome immuno-fluorescence shows Adf-1 staining at discrete regions (I). Sytox 24 is used as a general DNA stain to reveal the banding pattern in a standard polytene chromosome preparation (H). See also supplementary Figure 1.

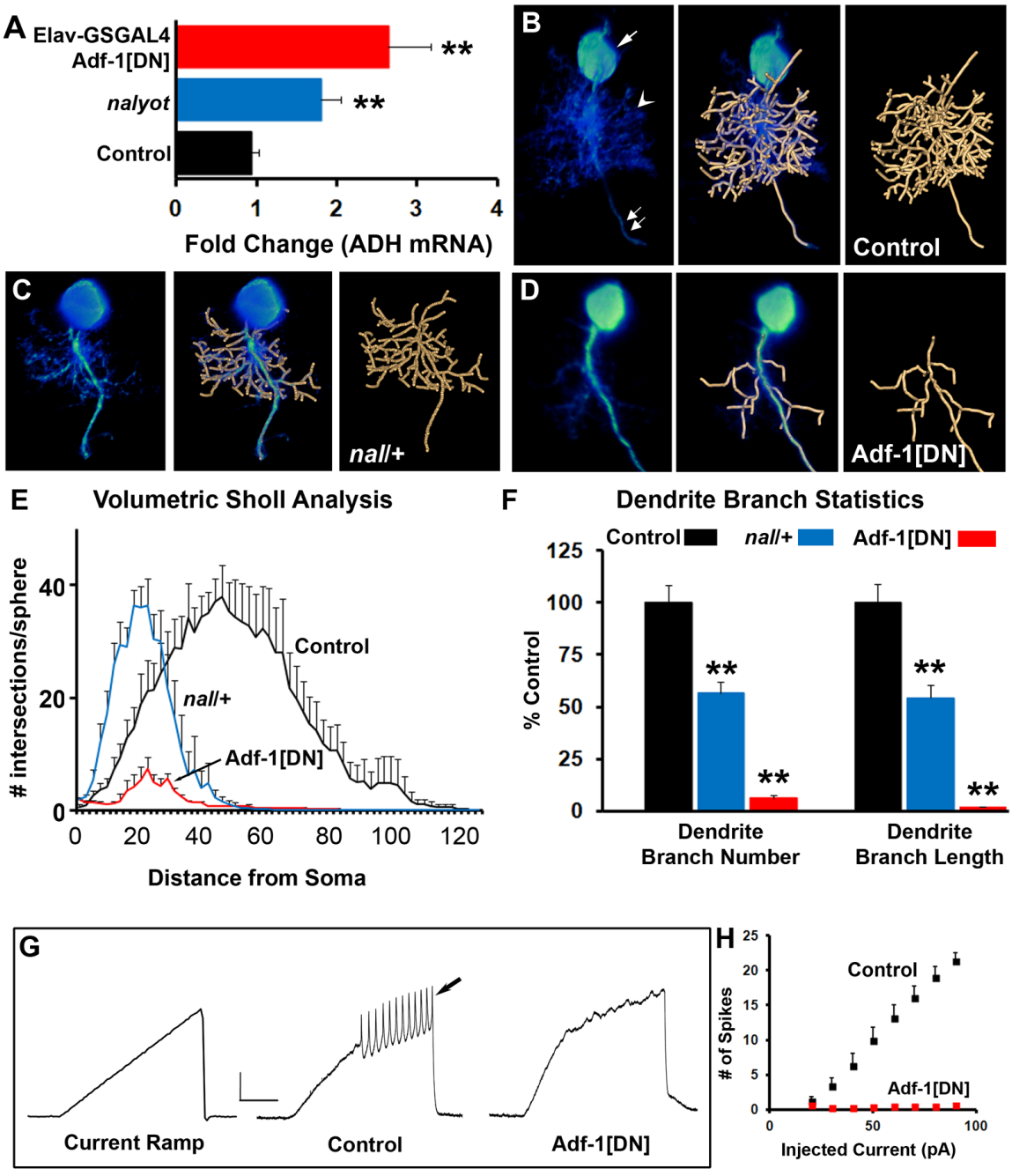

A
Elav-GSGAL4
Adf-1[DN]
nalyot
Control
**
**
0
1
2
3
4
Fold Change (ADH mRNA)
B
Control
C
nal/+
D
Adf-1[DN]
E
Volumetric Sholl Analysis
intersections/sphere
40
20
0
Control
nal/+
Adf-1[DN]
0
20
40
60
80
100
120
Distance from Soma
F
Dendrite Branch Statistics
Control
nal/+
Adf-1[DN]
% Control
125
100
75
50
25
0
**
**
**
**
Dendrite
Branch Number
Dendrite
Branch Length
G
Current Ramp
Control
Adf-1[DN]
H
# of Spikes
25
20
15
10
5
0
Control
Adf-1[DN]
0
50
100
Injected Current (pA)

Figure 2

Figure 2: Normal dendrite growth in larval RP2 motor neurons requires Adf-1. (A) qRT-PCR from adult brains showing the effect of Adf-1 inhibition on the expression of the target gene Alcohol Dehydrogenase (ADH). (B, C, D) 3D reconstruction of RP2 neuron dendrite branches *in vivo* using a semi-automated AMIRA based method in control (n=9) (B), *nalyot* heterozygotes (n=5) (C) and animals expressing a dominant-negative Adf-1 transgene selectively in RP2 neurons (n=5) (D). (E) Volumetric Sholl analysis shows strong reduction in dendrite complexity that results from Adf-1 inhibition. (F) Both number of dendrite branches and total dendrite length are reduced in *nalyot* heterozygotes and animals expressing an Adf-1 dominant-negative transgene in RP2 neurons (double asterisks denote $p<0.01$). (G) Whole-cell patch clamp recordings from control RP2 neurons and those expressing the Adf-1 DN transgene. Current ramps elicit action potentials in control neurons (arrow) whereas Adf-1 inhibited neurons remain silent. (H) Quantification of spike frequency with increasing current injection. See also supplementary Figure 2.

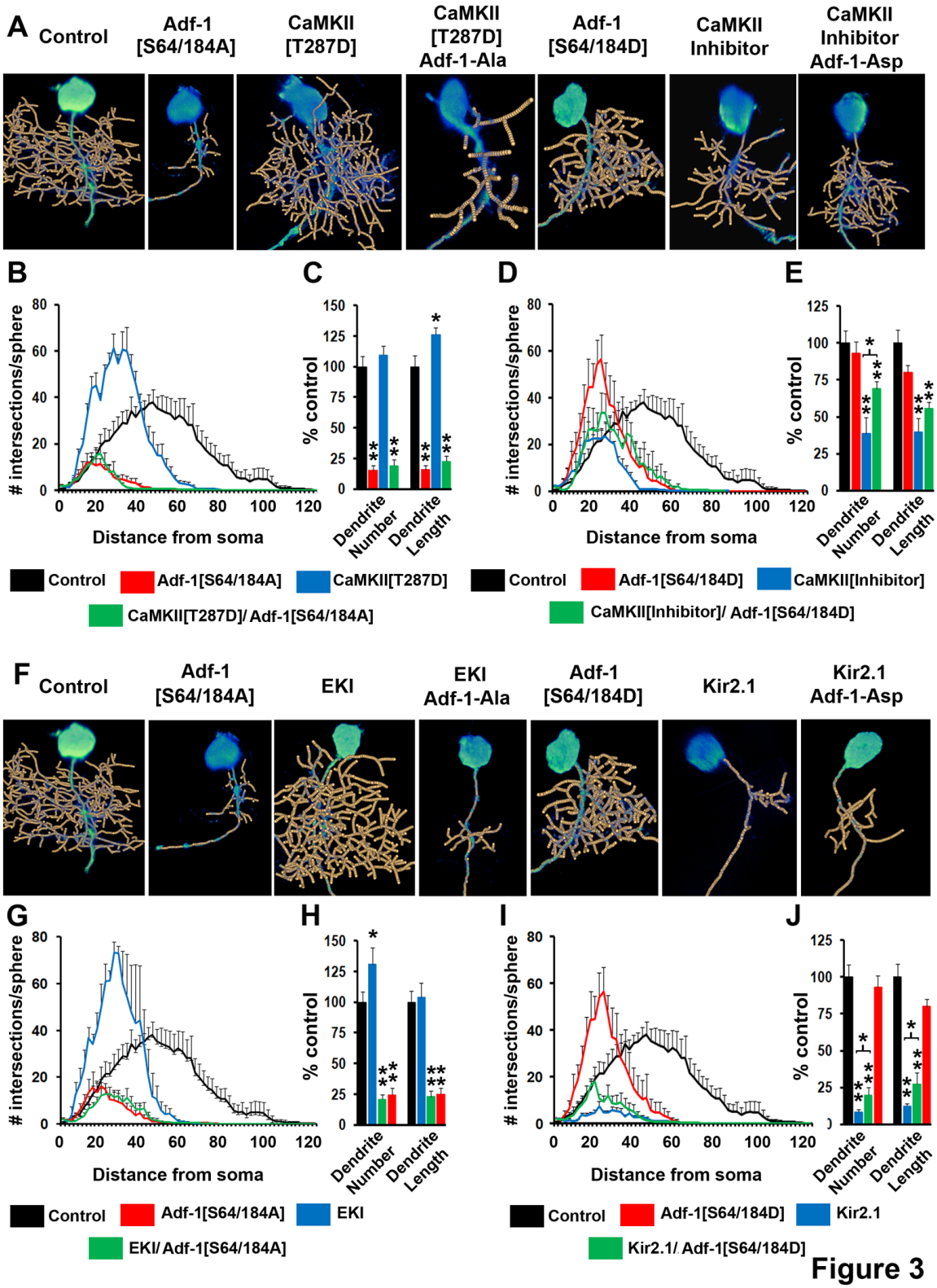

A
Control
Adf-1 [S64/184A]
CaMKII [T287D]
CaMKII [T287D] Adf-1-Ala
Adf-1 [S64/184D]
CaMKII Inhibitor
CaMKII Inhibitor Adf-1-Asp
B
C
D
E
intersections/sphere
Distance from soma
% control
Dendrite Number
Dendrite Length
Control
Adf-1[S64/184A]
CaMKII[T287D]
CaMKII[T287D]/ Adf-1[S64/184A]
Control
Adf-1[S64/184D]
CaMKII[Inhibitor]
CaMKII[Inhibitor]/ Adf-1[S64/184D]
F
Control
Adf-1 [S64/184A]
EKI
EKI Adf-1-Ala
Adf-1 [S64/184D]
Kir2.1
Kir2.1 Adf-1-Asp
G
H
I
J
intersections/sphere
Distance from soma
% control
Dendrite Number
Dendrite Length
Control
Adf-1[S64/184A]
EKI
EKI/ Adf-1[S64/184A]
Control
Adf-1[S64/184D]
Kir2.1
Kir2.1/ Adf-1[S64/184D]

Figure 3

Figure 3: Adf-1 functions downstream to CaMKII signaling and neural activity to regulate dendrite growth in RP2 motor neurons. (A) Representative RP2 dendrite reconstructions for Adf-1 and CaMKII genetic interactions. (B, C) Volumetric Sholl analysis and measurement of dendrite branch number and total dendrite length for CaMKII[T287D], Adf-1[S64/184A] and their combination, as compared to control neurons. (D, E) Volumetric Sholl analysis and measurements of dendrite branch number and length for CaMKII-peptide inhibitor, Adf-1[S64/184D] and their combination as compared to control neurons. Expression of Adf-1[S64/184D] significantly increases dendrite branch number in a CaMKII inhibited background (single asterisks denote $p<0.05$ for pair wise comparison after Bonferroni correction). (F) Representative RP2 dendrite reconstructions to test interaction between Adf-1 and changes in neuronal activity. (G, H) Volumetric Sholl analysis and measurement of dendrite branch number and length following increase in neural activity and Adf-1 inhibition. (I, J) Volumetric Sholl analysis and measurement of dendrite branch number and length following neuronal silencing and Adf-1 activation. Adf-1[S64/184D] increases both dendrite number and length in an activity silenced background (single asterisk indicates $p<0.05$ for pair wise comparison after Bonferroni correction). Double asterisks denote $p<0.01$. See also supplementary Figure 3.

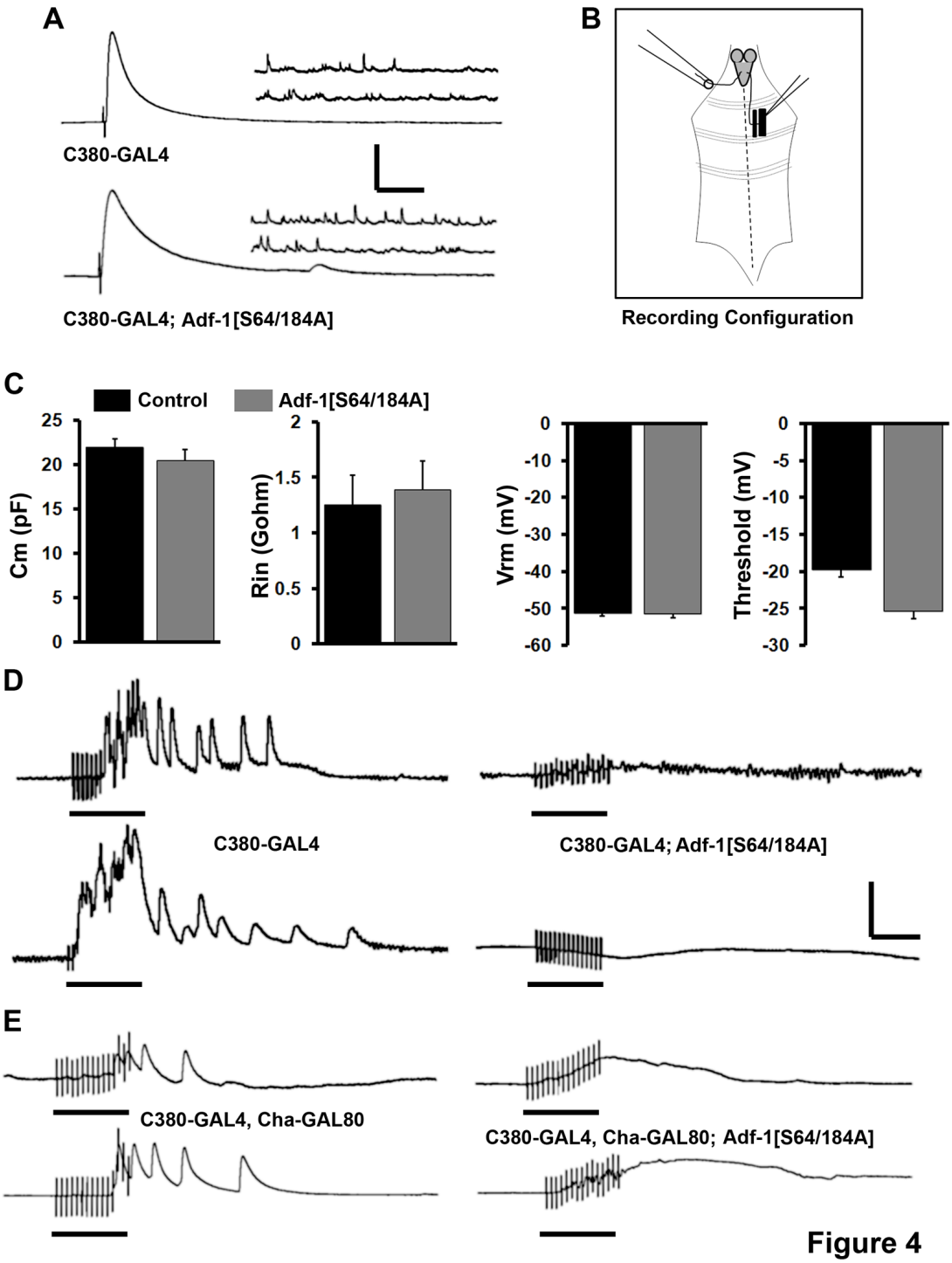

A
C380-GAL4
C380-GAL4; Adf-1[S64/184A]
B
Recording Configuration
C
Control
Adf-1[S64/184A]
Cm (pF)
Rin (Gohm)
Vrm (mV)
Threshold (mV)
D
C380-GAL4
C380-GAL4; Adf-1[S64/184A]
E
C380-GAL4, Cha-GAL80
C380-GAL4, Cha-GAL80; Adf-1[S64/184A]

Figure 4

Figure 4: Adf-1-mediated dendrite phenotypes affect sensori-motor transmission in larvae. (A) Neuro-muscular synaptic transmission (both evoked and spontaneous release) is unaffected following Adf-1 inhibition in motor neurons as compared to control neuromuscular junctions. (B) Schematic of recording configuration for testing sensori-motor transmission in larvae. (C) Membrane capacitance, input resistance, resting membrane potential and threshold of firing are comparable in control neurons and those expressing Adf-1[S64/184A] as measured using whole-cell patch clamp. (D) Representative muscle recordings reflecting motor neuron firing following stimulation of contralateral sensory afferents at 10 Hz for 1.5 seconds. Traces are shown from control animals (*futsch*^{C380}-GAL4/*w*^{1118}) and animals expressing Adf-1[S64/184A] from the *futsch*^{C380}-GAL4 driver. (E) Representative traces of muscle recordings as in (D) from animals that selectively express Adf-1[S64/184A] in glutamatergic motor neurons (*futsch*^{C380}-GAL4; Cha-GAL80; UAS-Adf-1[S64/184A] as compared to appropriate genetic controls. Vertical scale bar = 10 mV; Horizontal scale = 50 ms (EJP) and 500 ms (mEJP) in (A) and 1 sec in (D) and (E). Black bars under traces denote the duration of sensory stimulation.

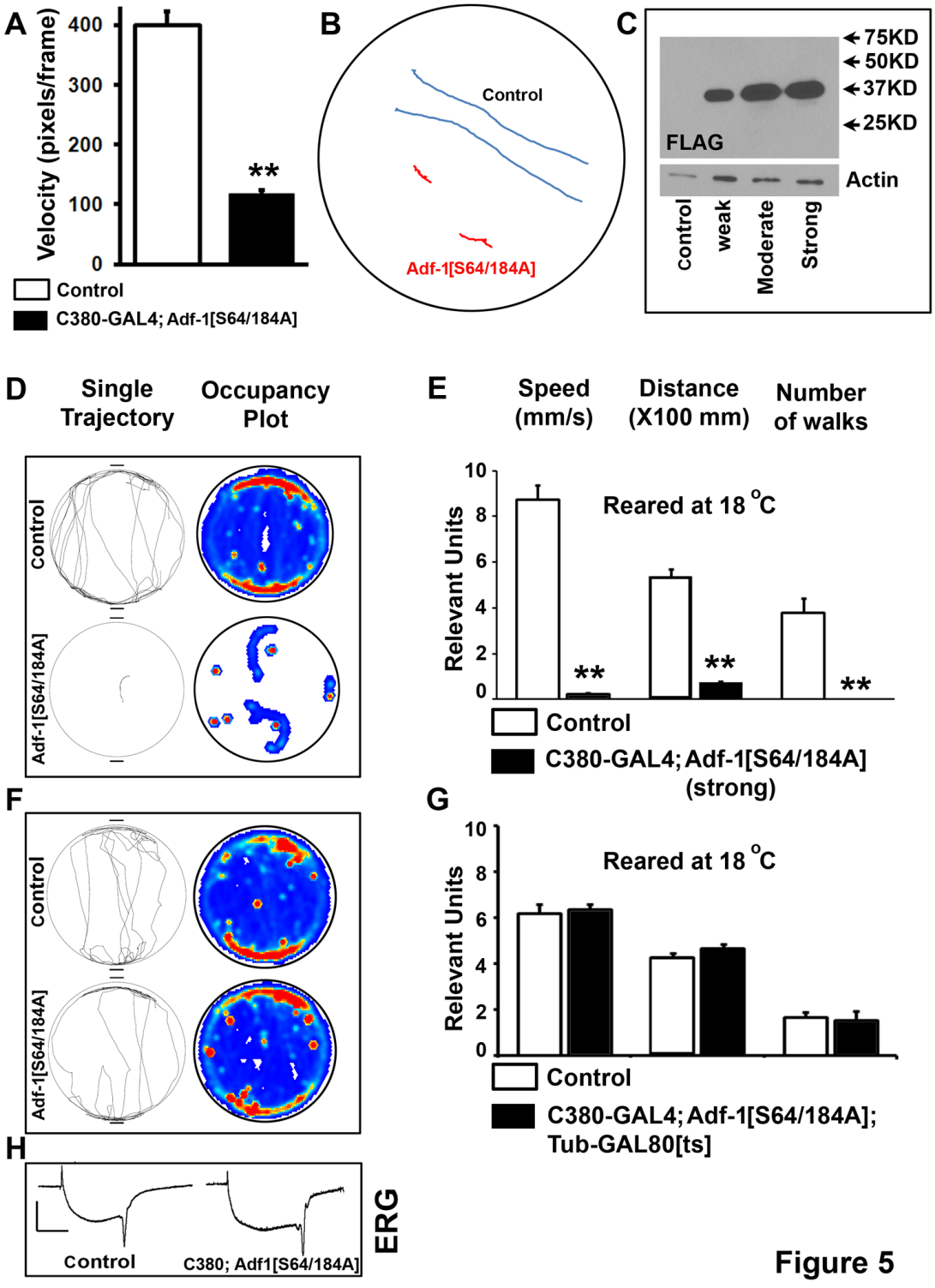

A
Velocity (pixels/frame)
400
300
200
100
0
**
Control
C380-GAL4; Adf-1[S64/184A]
B
Control
Adf-1[S64/184A]
C
75KD
50KD
37KD
25KD
FLAG
Actin
Control
weak
Moderate
Strong
D
Single Trajectory
Occupancy Plot
Control
Adf-1[S64/184A]
E
Speed (mm/s)
Distance (X100 mm)
Number of walks
Reared at 18 °C
Relevant Units
10
8
6
4
2
0
**
**
**
Control
C380-GAL4; Adf-1[S64/184A] (strong)
F
Control
Adf-1[S64/184A]
G
Reared at 18 °C
Relevant Units
10
8
6
4
2
0
Control
C380-GAL4; Adf-1[S64/184A]; Tub-GAL80[ts]
H
Control
C380; Adf1[S64/184A]
ERG

Figure 5

Figure 5: Behavioral consequences of Adf-1 inhibition in motor neurons. (A) Expression of Adf-1[S64/184A] in larval motor neurons using the *futsch*^{C380}-GAL4 driver leads to a dramatic reduction in larval locomotion. Average velocities are reduced almost by 4-fold. (B) Two representative traces of crawling larvae from control (blue) and Adf-1[S64/184A] animals (red) over a 3 minute recording period. (C) Western blot of protein from larval brains in which different Adf-1[S64/184A] transgenic lines are used to express FLAG tagged Adf-1[S64/184A] protein. (D, E) Representative individual fly tracks (Cremer et al.) and cumulative occupancy plots of controls compared to animals in which strong expression of Adf-1[S64/184A] is driven from the *futsch*^{C380}-GAL4 driver at 18^{o}C. (F, G) The strongly impaired locomotor behavior shown above is dependent on expression of the transgene and is not observed when a temperature-sensitive GAL80 is used to represses GAL4 activity at 18^{o}C. Figure shows representative individual tracks, cumulative occupancy plots (F) and quantification of locomotor parameters (G). (H) Representative electroretinogram traces from control and animals strongly expressing Adf-1[S64/184A] in all neurons. Double asterisks denote $p<0.01$. See also supplementary Figure 4 and movie M1 and M2.

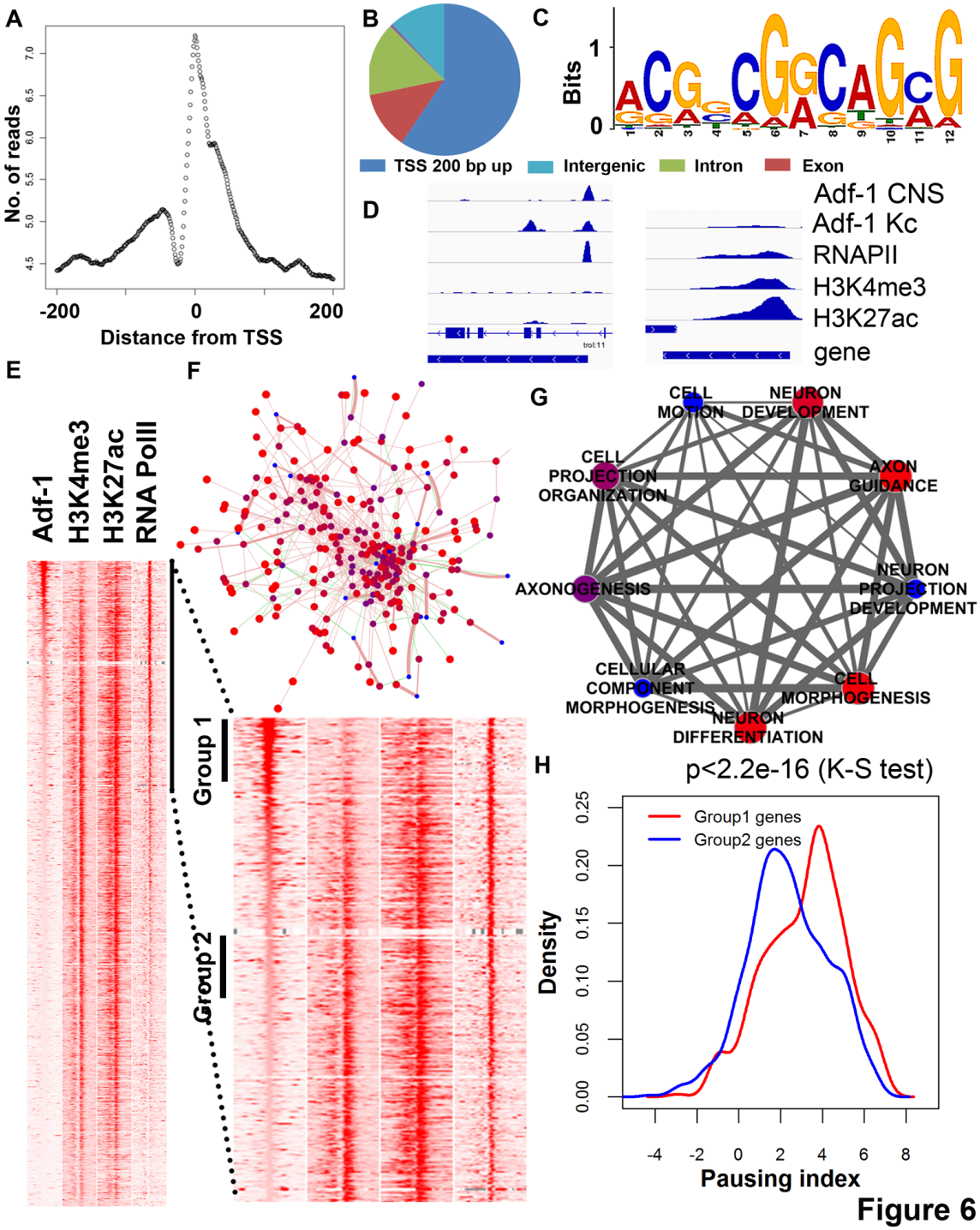
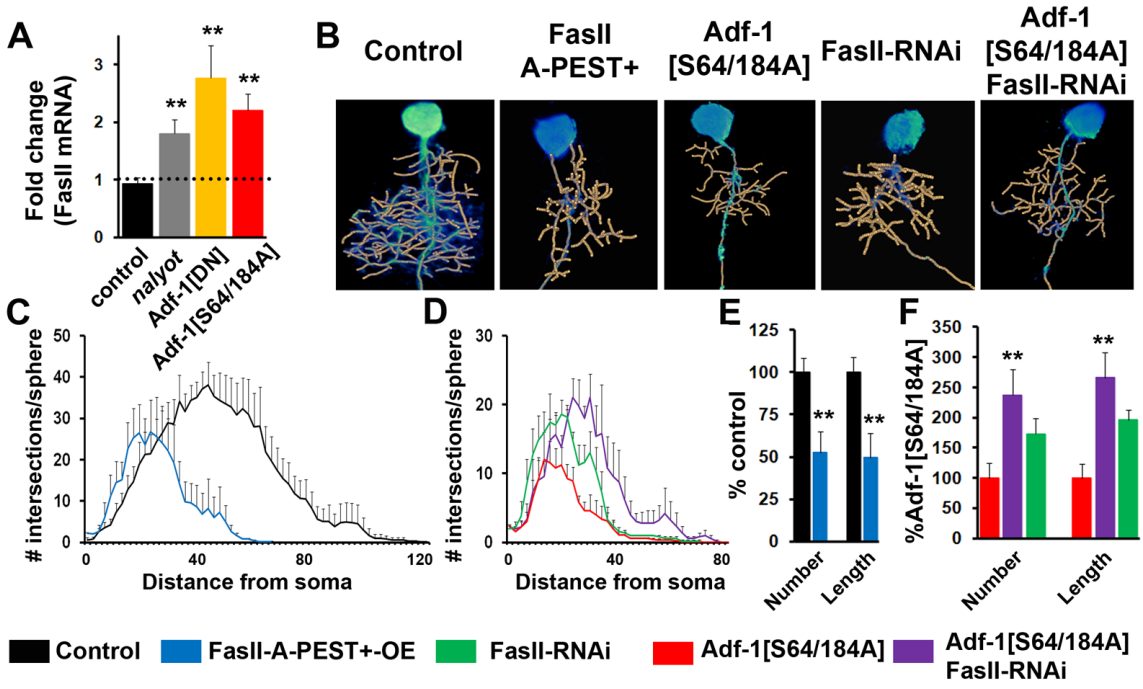

A
No. of reads
Distance from TSS
-200
0
200
B
TSS 200 bp up
Intergenic
Intron
Exon
C
Bits
D
Adf-1 CNS
Adf-1 Kc
RNAPII
H3K4me3
H3K27ac
gene
E
Adf-1
H3K4me3
H3K27ac
RNA PolII
Group 1
Group 2
F
G
CELL MOTION
NEURON DEVELOPMENT
CELL PROJECTION ORGANIZATION
AXON GUIDANCE
AXONOGENESIS
NEURON PROJECTION DEVELOPMENT
CELLULAR COMPONENT MORPHOGENESIS
CELL MORPHOGENESIS
NEURON DIFFERENTIATION
H
p<2.2e-16 (K-S test)
Group1 genes
Group2 genes
Density
Pausing index

Figure 6

Figure 6: Adf-1 transcriptional targets in the brain identified by ChIP-Seq analysis. (A) Adf-1 DNA binding peaks in the brain are enriched close to the transcription start site (TSS). (B) Distribution of Adf-1 binding peaks within the genome from brain ChIP-Seq. (C) Primary consensus Adf-1 binding sequence derived from brain and Kc cell ChIP-Seq experiments using MEME-ChIP. (D) Two representative gene tracks comparing Adf-1 occupancy in brain and Kc cells to RNA Pol II, H3K4me3 and H3K27ac. (E) Heatmaps showing chromatin features around TSSs with different Adf-1 levels. Each panel represents 2 kb upstream and downstream of the TSSs. The sites are ordered by intensity of Adf-1 at TSSs. Part of the left panel is enlarged to allow detailed visualization. Group 1 and Group 2 TSSs were selected for examination of pausing index in Figure 6H. (F) Protein interaction network of genes that contain Adf-1 within 200 base pairs of their TSS in larval brains created using GeneMania and published genetic and physical interaction studies in *Drosophila*. Sphere color and size reflect GeneMania scores (red and larger are higher scores), green lines denote genetic interactions and pink lines denote physical interactions. Line thickness denotes interaction strength. 82% of input genes show physical interaction, while 18% have known genetic interaction. (G) Clustering of highly significant neuron-related Gene Ontology (GO) terms represented by genes that bind Adf-1 close to the TSS. Size and color of spheres represents p-values, line thickness denotes the number of shared genes between two GO terms. (H) Group 1 genes (that have a higher enrichment of Adf-1) have a higher pausing index as compared to Group 2 genes (that are less enriched for Adf-1 binding). (H) Distribution of genes in Groups 1 and 2 with respect to pausing index. See also supplementary Figure 5 and Table 1.


A
Fold change (FasII mRNA)
control
nalyot
Adf-1[DN]
Adf-1[S64/184A]
B
Control
FasII A-PEST+
Adf-1 [S64/184A]
FasII-RNAi
Adf-1 [S64/184A] FasII-RNAi
C
intersections/sphere
Distance from soma
D
intersections/sphere
Distance from soma
E
% control
Number
Length
F
%Adf-1[S64/184A]
Number
Length
Control
FasII-A-PEST+-OE
FasII-RNAi
Adf-1[S64/184A]
Adf-1[S64/184A] FasII-RNAi

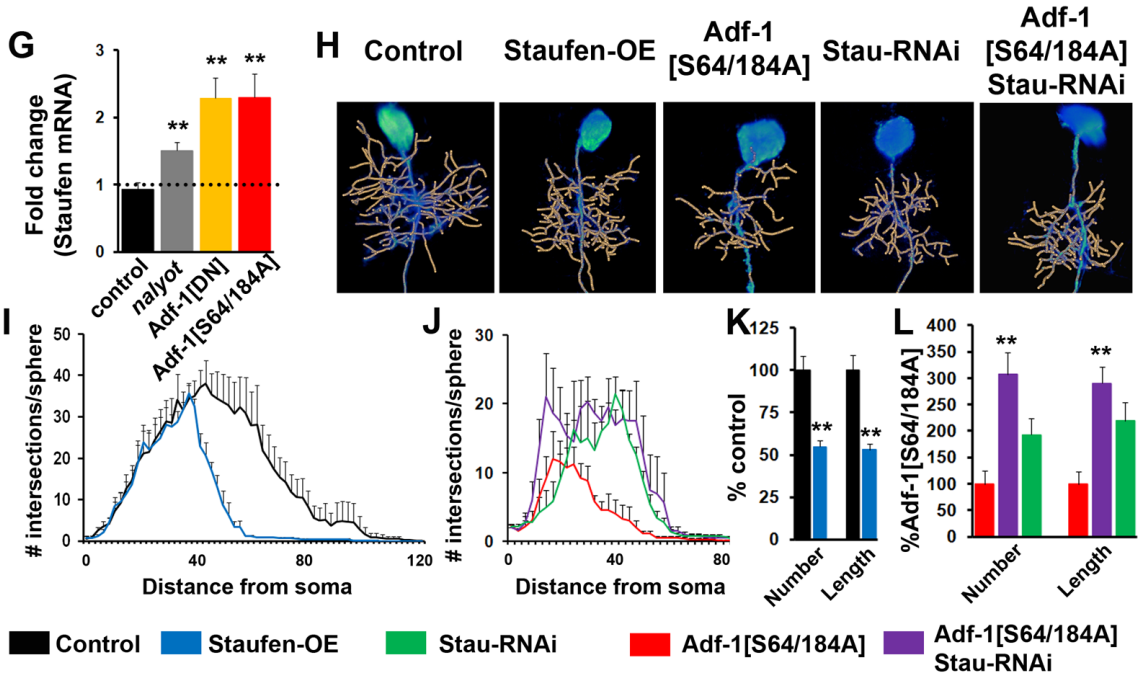

G
Fold change (Staufen mRNA)
control
nalyot
Adf-1[DN]
Adf-1[S64/184A]
H
Control
Staufen-OE
Adf-1 [S64/184A]
Stau-RNAi
Adf-1 [S64/184A] Stau-RNAi
I
intersections/sphere
Distance from soma
J
intersections/sphere
Distance from soma
K
% control
Number
Length
L
%Adf-1[S64/184A]
Number
Length
Control
Staufen-OE
Stau-RNAi
Adf-1[S64/184A]
Adf-1[S64/184A] Stau-RNAi

Figure 7

Figure 7: Adf-1 regulates RP2 dendrite growth by controlling FasII and Staufen expression. (A) qRT-PCR analysis from adult brains showing Adf-1-dependent regulation of FasII. (B) Representative images of 3D reconstructed RP2 neurons from genotypes that modify Adf-1 and FasII. (C) Volumetric Sholl analysis of FasII over-expressing neurons as compared to control. (D) Volumetric Sholl analysis to test genetic interaction between Adf-1 and FasII. (E) Quantification of dendrite branch number and total dendrite length in FasII over-expressing neurons. (F) Quantification of dendrite branch number and length for Adf-1-FasII interaction. (G) qRT-PCR analysis of Staufen expression from adult brains following Adf-1 perturbation. (H) Representative RP2 neuron dendrite reconstructions from genotypes that modify Adf-1 and Staufen. (I) Volumetric Sholl analysis of Staufen over-expressing neurons as compared to control. (J) Volumetric Sholl analysis to test genetic interaction between Adf-1 and Staufen. (K) Quantification of dendrite number and length in Staufen over-expressing neurons. (L) Quantification of dendrite branch number and length for Adf-1-Stau interactions. Double asterisks denote $p<0.01$. See also supplementary Figure 6.

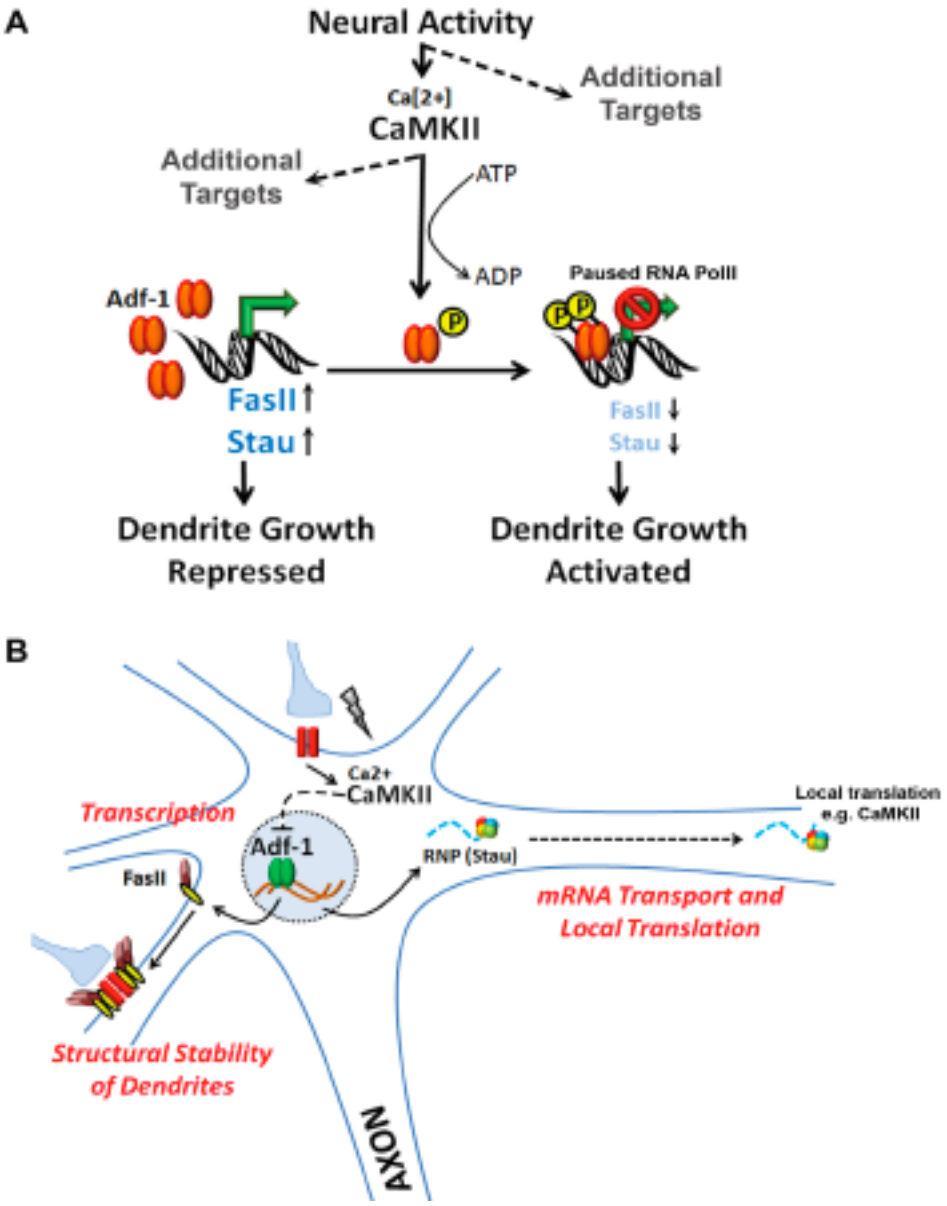
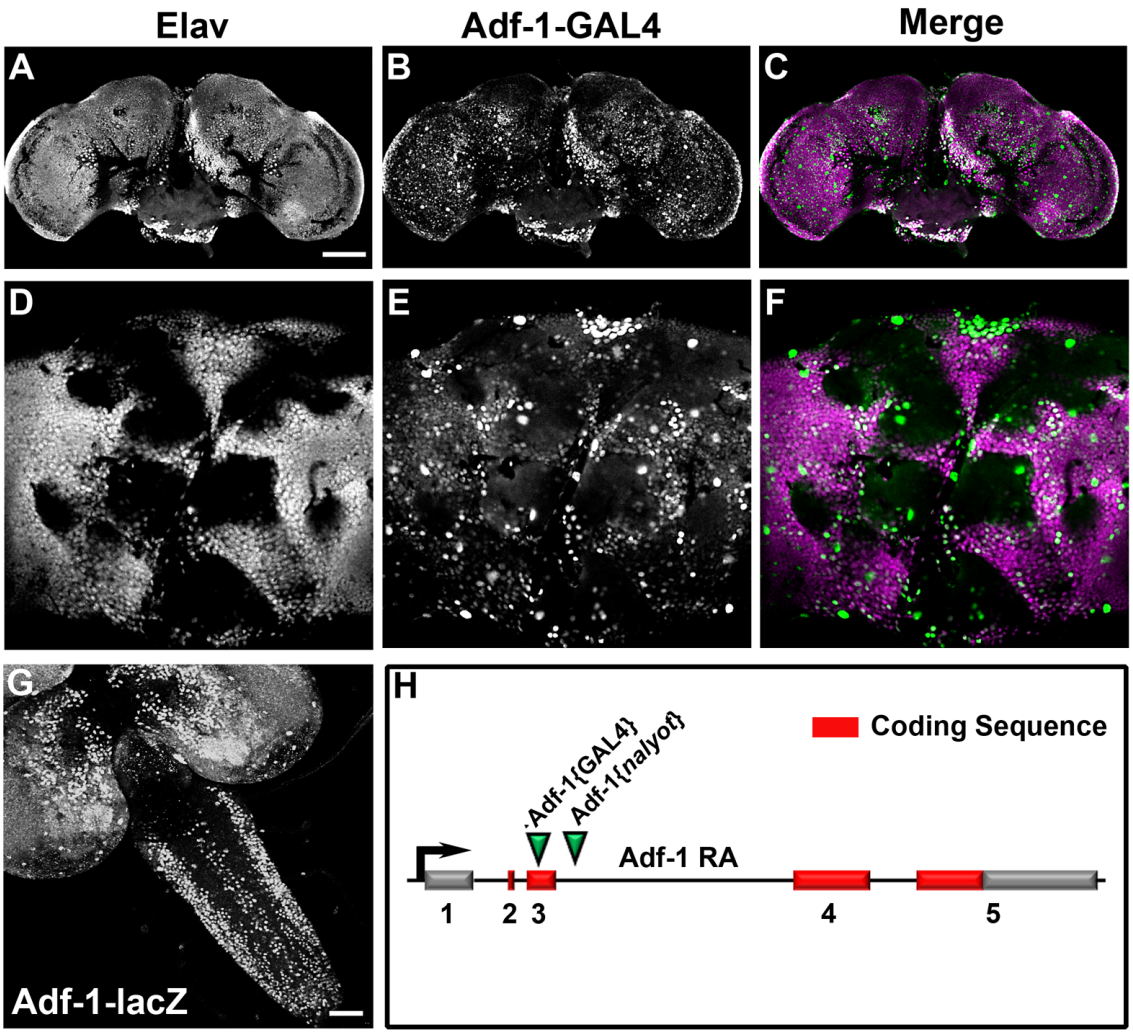

A
Neural Activity
Ca[2+]
CaMKII
Additional Targets
Additional Targets
ATP
ADP
Adf-1
Paused RNA PolII
FasII ↑
Stau ↑
FasII ↓
Stau ↓
Dendrite Growth Repressed
Dendrite Growth Activated
B
Ca2+
CaMKII
Transcription
Adf-1
FasII
RNP (Stau)
Local translation e.g. CaMKII
mRNA Transport and Local Translation
Structural Stability of Dendrites
AXON

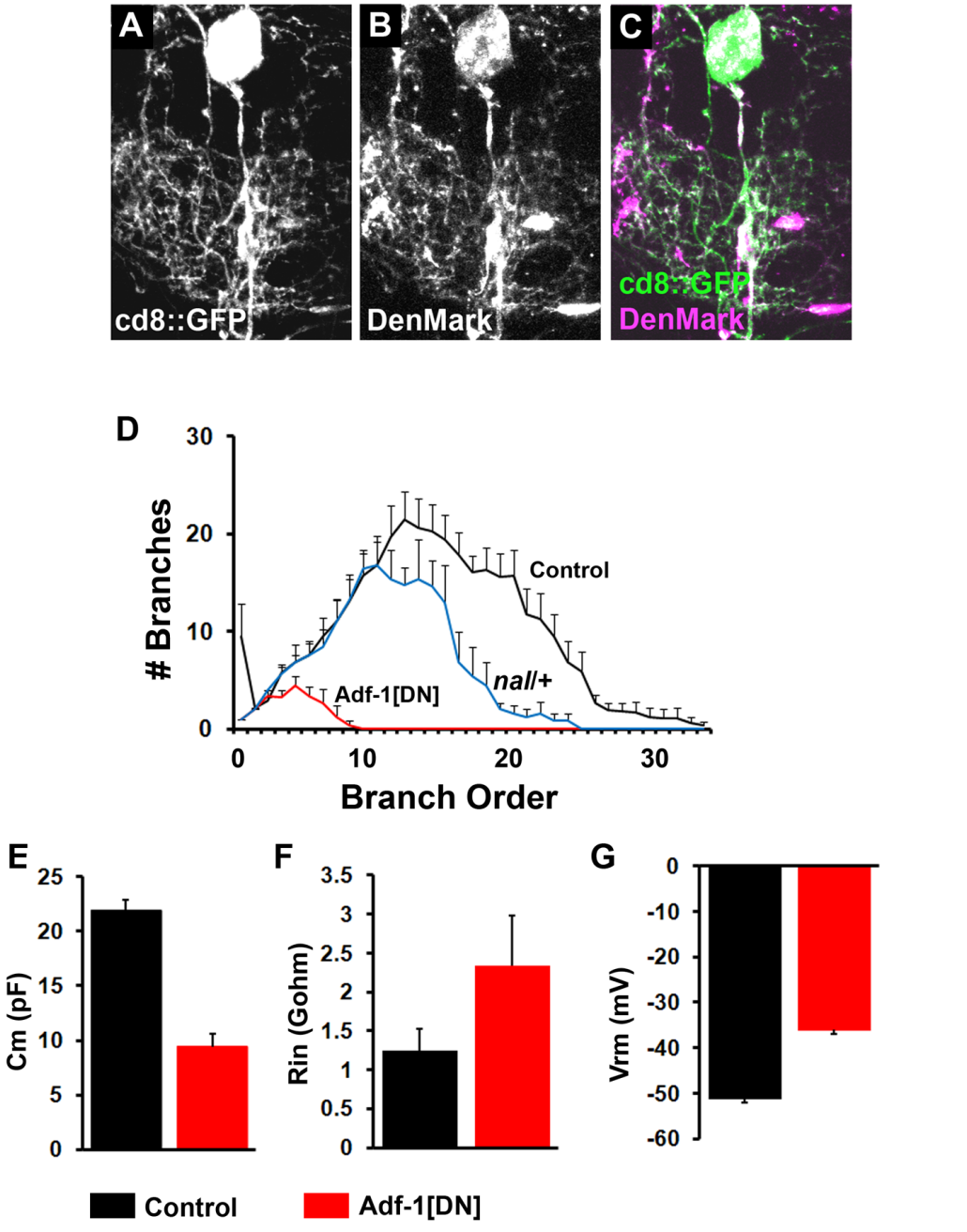
Figure 8

Figure 8. Regulation of dendrite plasticity in *Drosophila* by the transcription factorAdf-1. (A) Model of Adf-1-dependent regulation of dendrite growth. Adf-1 functions downstream of neuronal activity and CaMKII signaling. Adf-1 binding at promoters of genes such as FasII negatively regulates their transcription thereby optimizing their expression to suit demands for dendrite growth. (B) By regulating genes such as FasII and Staufen, Adf-1might be in a position to couple mechanisms underlying transcription and local translation in neurons during growth and plasticity of dendrites.



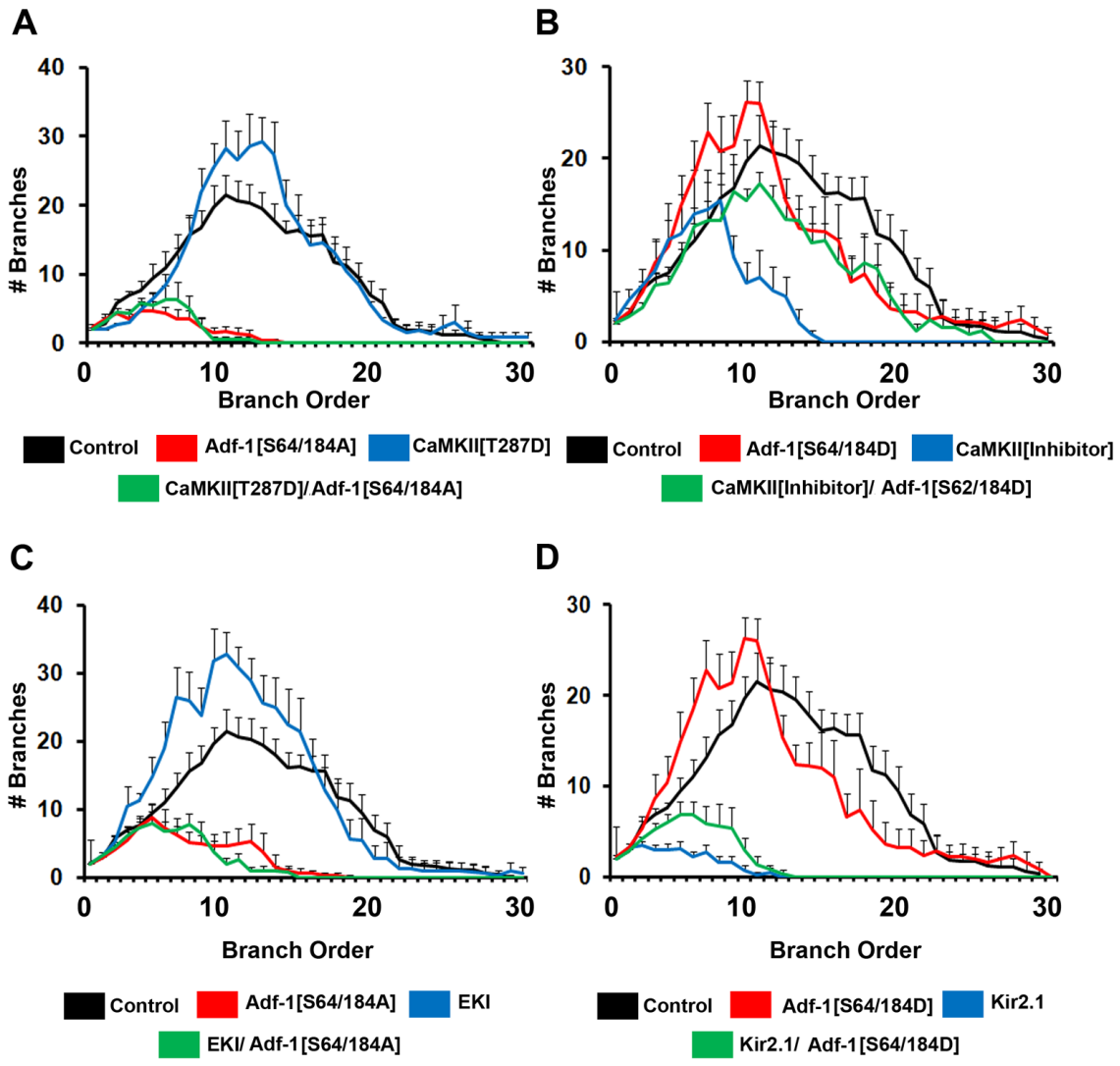
Supplementary Figure 1

Figure S1 – related to Figure 1: Adf-1 staining in the adult brain, expression of an Adf-1 enhancer trap line in the larval brain and schematic of transposon insertions in the Adf-1 gene. (A, B, C) Adult brain whole mounts stained for Elav and nuclearLacZ driven by the Adf-1-GAL4 line. (D, E, F) Close up of the central brain region. (G) Larval brain stained for LacZ in the original LacZ enhancer trap line. (H) Schematic of the Adf-1 gene region showing sites of insertion for the pGawB enhancer trap and the P[ry^{+}] *nalyot* P-element transposons.



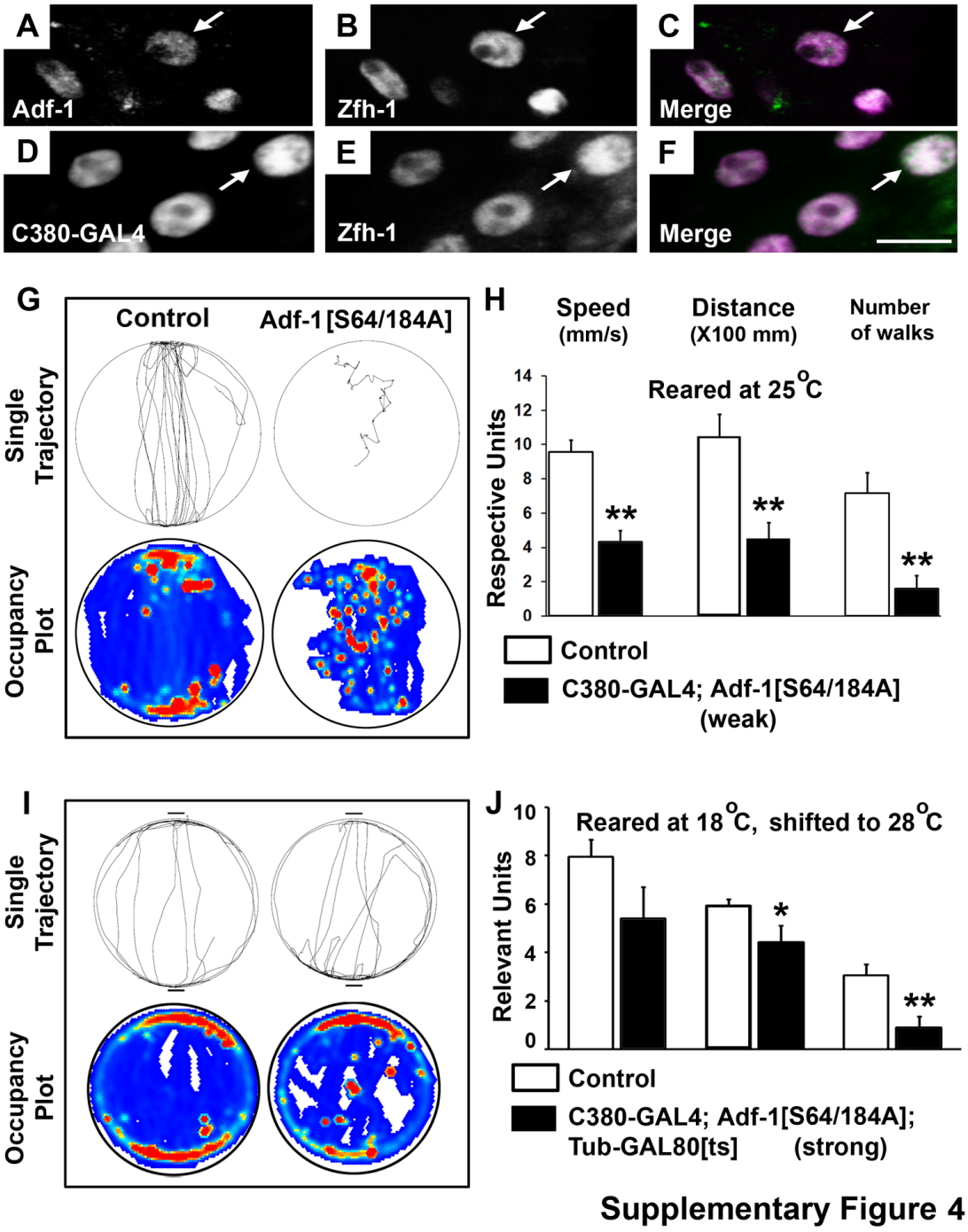
Supplementary Figure 2

Figure S2 – related to Figure 2: Adf-1 inhibition reduced dendrite growth in RP2 motor neurons. (A, B, C) Expression of the dendritic marker DenMark in RP2 Motor Neurons labels dendritic structures that are also more easily observed and imaged with mCD8::GFP. (D) Branch order analysis of *nalyot* heterozygotes and animals expressing an Adf-1DN transgene as compared to controls. (E, F, G) Average capacitance (E), input resistance and resting membrane potential from control RP2 neurons and those expressing Adf-1 DN.



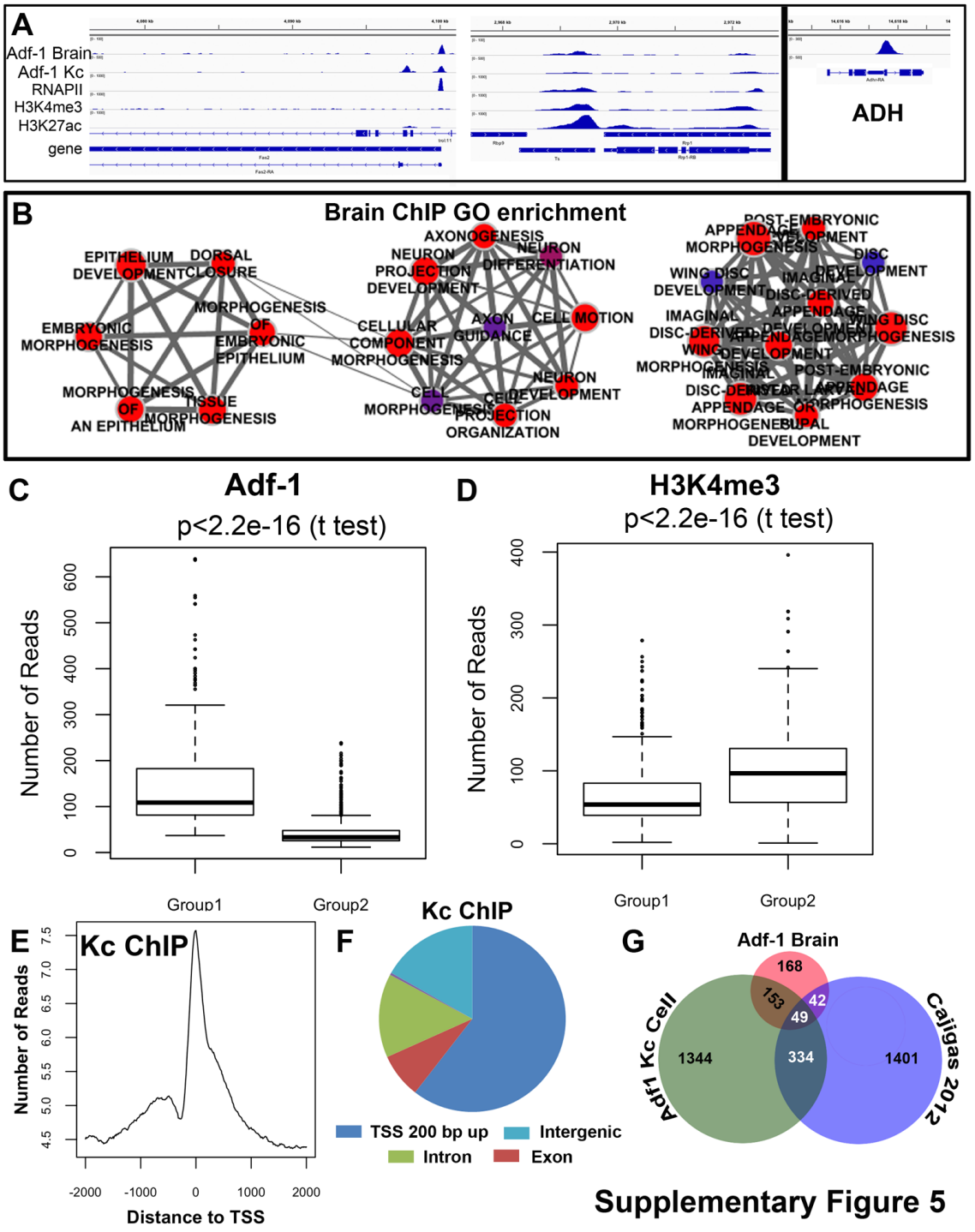
Supplementary Figure 3

Figure S3 – related to Figure 3: Adf-1 functions downstream of CaMKII signaling and neural activity to control dendrite growth. (A, B) Branch order analysis in experiments to test genetic interaction between CaMKII and Adf-1. (C, D) Branch order analysis in experiments that test Adf-1 function downstream of activity manipulations in RP2 motor neurons.



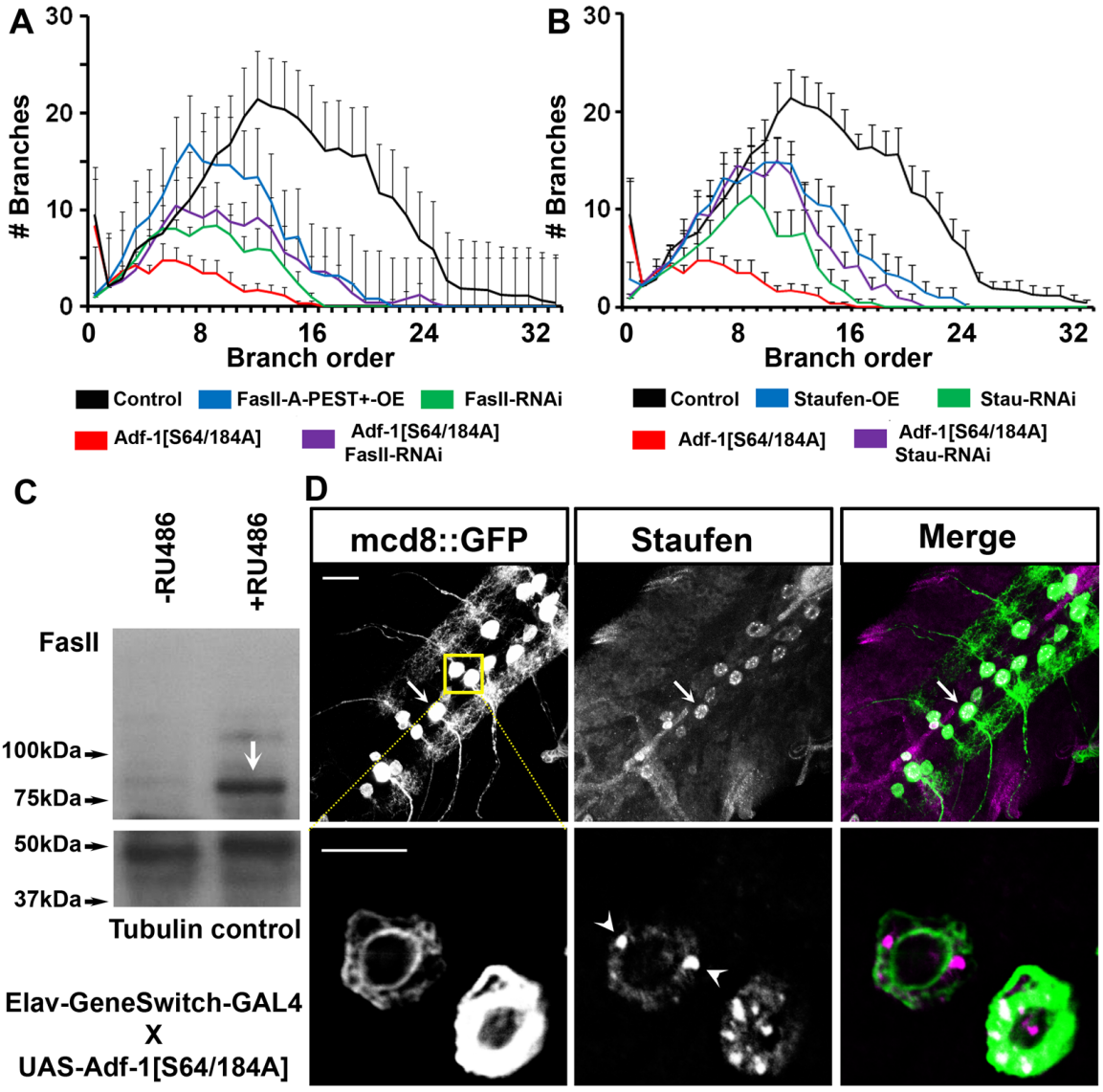
Supplementary Figure 4

Figure S4 – related to Figure 5: Motor phenotypes resulting from Adf-1 inhibition are predominantly developmental in origin. (A, B, C) Motor neurons in the thoracic ganglion of adult flies identified with anti-Zfh-1(B) staining also express Adf-1 (A). (D, E, F) The *futsch*^{C380}-GAL4 line expresses in these motor neurons. GAL4 expression is visualized with GFP (D) and (E) represents anti-Zfh-1 staining. (G) Walking traces for single flies and occupancy plots shows severely reduced locomotor behavior when the weak Adf-1[S64/184A] transgenic line is used to express the Ala substituted Adf-1 in motor neurons using the *futsch*^{C380}-GAL4 line. (H) Statistical evaluation of locomotor parameters in the above genotypes. (I, J) GAL80[ts] containing animals that are reared at 18^{o}C, but then shifted to 28^{o}C post eclosion for 4 days show significant locomotor defects as compared to control animals that are, however, mild as compared to chronic inhibition of Adf-1. Double asterisks denote $p<0.01$, and single asterisk denotes $p<0.05$.



Supplementary Figure 5

Figure S5 – related to Figure 6: Analysis of Adf-1 binding sites from brain and Kc cell ChIP-Seq experiments. (A) Gene track of FasII showing Adf-1 occupancy, compared to RNA Pol II, H3K4me3 and H3K27ac – an additional gene is shown as a control gene with less Adf-1 occupancy. Box shows Adf-1 binding at the ADH gene locus in brains. (B) Three significant GO clusters for Adf-1 binding genes in the brain. (C, D) Box plot of Adf-1 (C) or H3K4me3 (D) levels at TSSs of genes in Group 1 or Group 2 genes from figure 6H. (E) Clustering of Adf-1 occupancy close to the TSS in Kc cells. (F) Distribution of genome-wide Adf-1 binding in Kc cells. (G) Venn diagram representing overlap between genes that bind Adf-1 in brains and Kc cells compared to dendrite-enriched mRNAs in the rodent brain.



Supplementary Figure 6

Figure S6 – related to Figure 7: Adf-1 controls FasII and Staufen expression to regulate dendrite growth. (A, B) Branch order analysis for Adf-1-FasII (A) and Adf-1-Staufen (B) interaction. (C) Western blot from adult brain showing increased expression of FasII (arrow) 18 hours after induction of Adf-1[S64/184A] in neurons with RU486. Tubulin is used as a loading control. (D) Ventral nerve cords of an EP insertion upstream of Staufen crossed to the RN2-flipout tester GAL4 line stained for mcd8::GFP and Staufen. Bottom row shows close up of RP2 neurons. Arrowheads mark Staufen-positive granules. Scale bar = 50mM (top row) and 20 mM (bottom row).

Chapter 3

Materials & Methods

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Drosophila stocks, rearing and genetics

Drosophila strains were raised on standard media in a 25^{0}C incubator throughout life (unless otherwise noted). *nalyot* mutants (DeZazzo et al., 2000), UAS-FasIIA-RNAi and UAS-FasII A PEST+ UAS-CaMKII [T287D] and UAS-CaMKII ala peptide inhibitor (Jin et al., 1998), *futsch*^{C380}-GAL4 (Budnik et al., 1996; Sanyal, 2009), Cha-GAL4 and Cha-GAL80 (Kitamoto, 2002), UAS-DenMark ,UAS-K_{ir}2.1 (Baines et al., 2001), UAS-SH and UAS-eag that together make up EKI (Broughton et al., 2004; Hartwig et al., 2008; Mosca et al., 2005), Tub-GAL80^{ts} flies that are part of the TARGET system (McGuire et al., 2003) and the RU486 sensitive *elav*-GeneSwitch system (Osterwalder et al., 2001) have all been described previously. In order to visualize dendritic structure in individual RP2 motor neurons, we made use of the RN2 flipout line previously characterized by our laboratory (Hartwig et al., 2008). The Adf-1 DN line was generated by replacing 18 amino acids in the transactivation domain (aa131-148: D I F A Q P F N G S A T T S A Q A L) with 5 Alanine residues using standard cloning procedures and then inserting this deletion into the pUASt vector. The Adf-1 Gal4 line was generated by transposase mediated P-element replacement of the lacZ enhancer trap P{PZ}Adf1^{01349} (Sepp and Auld, 1999). The Adf-1 [S64/184] and Adf-1 [S64/184D] substituted lines were generated by site-directed mutagenesis of the wild type Adf-1 cDNA (obtained from the Drosophila Genomics Resource Center) and cloning into the Gateway compatible pTWF vector using p-ENTR/D as the entry vector (Invitrogen). Staufen EP and staufen RNAi (TRiP collection at Harvard) were obtained from the Bloomington Stock Center. Other fly stocks used are part of the Sanyal laboratory stock collection.

Antibody generation, immunohistochemistry and western blotting

Rabbit anti-Adf-1 polyclonal antibodies were generated by Alpha diagnostics against two KLH tagged antigenic peptides GSQSANQVADPSQQ and GKDQKPYFYEPPLK and affinity purified against these immobilized peptides. Larval immuno-histochemistry was carried out following established protocols (Sanyal and Ramaswami, 2006). Animals were fixed in 4% paraformadehyde followed by washing in phosphate buffered saline (PBS). Preparations were incubated in primary antibody in PBS with 0.1% Triton X-100 (PBT) overnight at 4^{o}C, then washed in PBT followed by incubation in specific Alexa-fluorophore conjugated secondary antibody (Molecular Probes, Invitrogen). After final washes in PBT, brains were dissected out and mounted on poly-Lysine coated slides in Vectashield (Vector Labs.).

Antibodies:

Antibody dilutions for IHC are as follows:

rabbit anti-Adf-1(1:100)

rabbit anti-lacZ (Abcam 1:1000);

rabbit/mouse anti-GFP (Invitrogen 1:1000)

mouse anti-Elav (DSHB, Iowa 1:10)

rabbit anti-Staufen (1:500, (St Johnston et al., 1991)

guinea pig anti-Zfh-1 (1:100, (Postigo et al., 1999)).

Antibody dilutions for Western blot are as follows:

rabbit anti-Adf1 antibody (1:5000)

rabbit anti-H3 antibody (Abcam 1:10000)

mouse anti-actin (Millipore, 1:50,0000)

mouse anti-FLAG (Sigma, 1:1000).

Microscopy and 3D reconstructions

Dissected and fixed larval nerve chords were imaged using a Zeiss 510 confocal microscope with the 40X oil-immersion lens as previously described (Hartwig et al., 2008). Briefly, our synthetic GAL4 line reliably marks two subsets of motor neurons, RP2 and aCC. These neurons are easily distinguishable from one another based on their branching patterns. Only single optically isolated RP2 motor neurons were chosen for imaging so that contributions from neighboring dendrites would not confound the 3D reconstruction. Z-spacing was set such that it matched the pixel size in the x-y plane to generate cubic voxels. Dendrites were reconstructed using a custom semi-automated AMIRA (4.1.1) plug-in as previously described (Evers et al.; Vonhoff et al.). Quantitative measures of dendrite growth were analyzed and plotted using Microsoft Excel.

Electrophysiology

Whole cell tight seal recordings were performed on motoneuron RP2 (MNISN1s) using external (mM: 118 NaCl, 2 NaOH, 2 KCl, 4 $MgCl_2$, 4 $CaCl_2$, 40 sucrose, 5 trehalose, 5 HEPES; pH 7.1; osmolality 305 mmol kg−1) and internal salines (mM 130 K-gluconate, 2 NaCl,10 HEPES, 1 EGTA, 2 $MgCl_2$, 0.1 $CaCl_2$, 10 KOH; pH 7.2;

osmolality 285 mmol kg−1) as described previously (Choi et al., 2004; Freeman et al.; Sandstrom, 2008). RP2 somata were visualized with the synthetic GAL4 line (Hartwig et al., 2008) driving membrane-bound GFP.

For recording, the CNS was removed from the larva, immobilized on a small chip of coverslip coated with poly-DL-ornithine (Sigma), and placed in a recording chamber under continuous superfusion. The sheath was softened and removed using a small-bore pipette filled with 0.1% collagenase type XIV (Sigma). Neurons were visualized with a 63× water-immersion objective, using Nomarski optics and GFP fluorescence (Olympus BX51WI; Olympus America, Center Valley, PA). Whole-cell recordings were performed with an AxoPatch 200B controlled by pClamp 8.2. Electrodes were fabricated to 5–10 MΩ from thick-walled capillary glass (World Precision Instruments, Sarasota, FL) on a vertical puller (Narishige PP-830, Narashige International USA, East Meadow, NY). F/I curves were measured in current clamp at −60 mV, while input resistance was measured from the slope of the I–V relation in voltage clamp at a holding potential of −70 mV. Larval neuromuscular recordings were carried using HL 3 external saline (in mM: NaCl 70, KCl 5, $MgCl_2$ 20, $CaCl_2$ 1, $NaHCO_3$ 10, Sucrose 115, Trehalose 5, BES 5; pH 7.2) as described previously. Intracellular recording electrodes with tip resistances between 25 and 50 MW were filled with 3 M KCl. Only those recordings were used where the resting membrane potential was more polarized than -60 mV and the muscle input resistance was greater than 10 MW. Muscle 6 (VL3) in abdominal segment A2 was used for all recordings. For sensori-motor transmission, the axon bundle contra-lateral to the recording segment was cut close to the muscle entry point and introduced into a stimulating electrode and stimulated at 10 Hz for 1.5 seconds (Dasari and Cooper, 2004).

Behavior:

Larval Locomotion:

Larvae were placed on Sylgard plates moistened with tap water to prevent the animals from drying out. Larval locomotion was measured by videotaping individual larvae with a webcam for a period of 5 minutes. Their motions were tracked using a Spot Tracker plugin in Image J.

Buridan's Assay:

Flies were aged 3-5 days had their wings clipped off close to their thorax and allowed to recover for three days prior to behavioral analysis. Single flies were placed onto an arena (10 cm in diameter) with the only visual cues being two black bars on opposite ends of the arena. At the edge of the arena is a moat (2 cm in width of water surrounding the platform). Wild-type flies will normally respond by repetitively walking between the two visual cues. This motor behavior was videotaped for 5 minutes. Locomotor tracks were analyzed using custom designed software in the statistical package *R*.

ChIP-Seq analysis and determination of molecular networks

ChIP was performed with 500 hand-dissected larval brains or ~4×10^7 Kc167 cells. Cells were cross-linked with 1% formaldehyde for 10 min at room temperature. Nuclear lysates were sonicated to generate 200-1000 bp DNA fragments. ChIP was then performed with 6 μL of *Drosophila* α-Adf-1 antibody. Libraries were prepared using the

Illumina TruSeq DNA Sample Preparation Kit. Fragments in the 200-300 bp ranged were selected and sequenced in an Illumina HiSeq sequencer at the HudsonAlpha Institute for Biotechnology. Sequences were aligned to *Drosophila* dm3 using Bowtie, and binding intensity files (.wig) were generated using MACS. In addition to the *Drosophila* Adf-1 data obtained in this study, we used several datasets obtained from public sources including Pol II ChIP-chip (modENCODE_328) and ChIP-seq data sets for H3K4me3 and H3K27ac (GSE36374). To build heatmaps, values for each ChIP-seq dataset were extracted for the 2000 bp region around TSSs of genes in dm3 annotation using custom R scripts (available upon request) and heatmap graphs were created using TreeView. Regions were sorted according to the intensity of Adf-1 signal at TSSs. Group1 TSSs were selected to represent genes associated with Adf-1. Group 2 TSSs were selected to represent genes with low intensity of Adf-1. Group 1 and Group 2 TSSs have approximately the same level of RNA polymerase to eliminate the effect of Pol II intensity when calculating pausing indices. The pausing index of genes was calculated using ChIP-chip data sets of Pol II in Kc cells obtained from modENCODE. Pol II levels at TSSs (P_{TSS}) were calculated as the mean enrichment of Pol II in the 200 bp region around each TSS. Pol II in the gene body (P_{body}) was calculated as the mean enrichment of Pol II from +200 bp to the end of the gene. The pausing index is defined as the difference between P_{TSS} and P_{body}. ‘

Network analysis of genes that bind Adf-1 close to their promoters in the larval brain was carried out using GeneMANIA as an executable plugin within Cytoscape (Montojo et al.; Mostafavi et al., 2008). Cluster analysis of gene ontology terms was carried out using BiNGO (Maere et al., 2005) and enrichment map plugin for Cytoscape.

RU-486 Preparation and Feeding

RU486 (Sigma M8046) was reconstituted in EtOH. Animals were starved overnight at 25^{o}C. After starvation, animals were transferred to vials containing a Whatman filter disc saturated with either a 2% sucrose solution with either RU486 to final concentration of 1mM or a 2% sucrose solution with EtOH (unfed controls). Flies were left on this food for 24 hours and then transferred to standard corn meal fly food for 6 hours to recover.

RNA extraction

Heads were removed by flash freezing whole animals collected in eppendorf tubes and placing the tubes in a dry ice/ethanol bath for approximately two to three minutes. Samples were vortexed virgoursly until all the animals heads were removed from their body (about one minute of vortexing). Heads were placed on a clean fly pad for sorting. Heads were placed in TriZOL and homogenized with a motorized pestle. RNA was extracted from adult heads using the standard TRIzol protocol.

cDNA synthesis

Following isopropanol precipitation and quantification, RNA was reverse transcribed with the Applied Biosystems High Capacity cDNA Reverse Transcription Kit. Samples are quantified using the nanodrop.

qRT-PCR:

Quantitative PCR was carried out using Thermo Scientific Maxima Sybr Green kit and reactions were run on the Roche Lightcycler 480. Each reaction was run in triplicate and each genotype was tested a minimum of three times. Samples were normalized to the housekeeping gene rp49.

Primers:

Primer sequences obtained from published data: *fasIIA*, *rp49* (LaLonde et al., 2006). Other primer sequences were designed using the Roche Universal ProbeLibrary assay design center and are as follows:

adh-(F 5' attttcgttgccggtctg, R 5'cgaggatcaccaggttcttc)

staufen (F 5'agtcttcagcagcaacagca, R 5'gattcagacgtgcgtggag).

Statistical methods

Student's t-test was used for pairwise comparisons. One-way ANOVA was used for multiple comparisons, followed by Bonferroni post-hoc testing.

Concluding Remarks and Future Directions

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Discussion:

Our study reveals novel functions for the Myb-family transcription factor Adf-1 in regulation of dendrite growth and plasticity and, by connecting Adf-1 with CaMKII signaling and transcriptional regulation of FasII and Staufen, provides a cellular basis for

long-term memory defects seen in *nalyot* mutants. We find that reduction of Adf-1 in RP2 motor neurons, either genetically (*nalyot*), through the expression of a truncated Adf-1 protein (Adf-1 DN) or through the expression of a mutant Adf-1 that alters two CaMKII targeted Serines to Alanine (Adf-1[S64/184A]), leads to a reduction in dendritic complexity. Adf-1 inhibition also completely attenuates dendrite growth either through activation of CaMKII or increased neural activity. Conversely, under conditions of chronic silencing or CaMKII inhibition – both of which severely restrict dendrite growth – expression of an "activated" Adf-1 ([S64/184D] – Serine residues mutated to Aspartate) partially rescues dendrite growth. Since Adf-1 is not the sole transcription factor that regulates dendritic plasticity, it is to be expected that Adf-1-dependent rescue of dendrite growth will be partial. Thus, Adf-1 is necessary, but not wholly sufficient for dendrite development and activity- and CaMKII-dependent plasticity. It is known that a number of transcription factors such as Fos, Creb, Mef2 and NFAT (Flavell et al., 2006; Hartwig et al., 2008; Schwartz et al., 2009; Shalizi et al., 2006; Vonhoff et al.) regulate dendrite development and further studies are needed to determine the relative importance of Adf-1 in relation to these factors. It is also possible that another kinase might phosphorylate Adf-1 on these Serines as well other amino acid residues. Nonetheless, the strength of dendrite growth phenotypes following Adf-1 perturbation underscores the importance of this transcription factor as well as these phosphorylation events in dendrite growth.

Motor neuron inhibition of Adf-1 leads to discernible negative outcomes in the membrane properties of neurons. Whole-cell recordings shown in Figure 2 document a loss in excitability in Adf-1 inhibited RP2 neurons in response to injection of depolarizing current. We speculate that this is due to a significant reduction in the

thickness of the primary neurite that makes it difficult for the current to spread from the electrode to the spike initiating zone. Synaptic stimulation of motor neurons achieved by high frequency triggering of contra-lateral sensory afferents is also strongly attenuated when Adf-1 function is selectively impaired in motor neurons. It is likely that a reduction in the number and branching of dendrites limits the number of pre-synaptic inputs on to these motor neurons. Consequently, synaptic release from putative pre-synaptic neurons is unable to reliably elicit action potentials in downstream motor neurons. However, such deafferentation is difficult to measure directly in the compact neuropil of the larval nerve cord, especially in the absence of reliable retrograde trans-synaptic tracers. Ideally, if precise pre-synaptic partners of these motor neurons were known, then a one-to-one correlation between pre-synaptic transmitter release, resulting post-synaptic potentials and action potential generation could be established. Alternatively, synaptic stimulation of motor neurons might also be impaired due to the reduced diameter of dendrites that would increase axial resistance, and reduce the spread of synaptic currents to the spike initiating zone thereby reducing the transfer function (Gray and Weeks, 2003). One possibility in the future might be to investigate this in the adult giant fiber system where pre- and post-synaptic partners are known and a role for CaMKII has recently been described. Animals with motor neuron inhibition of Adf-1 also show strong deficits in motor behavior that are developmental in origin, consistent with a developmental requirement for Adf-1 in memory formation (DeZazzo et al., 2000). We think that a reduction in excitability of motor neurons and, therefore, a loss in the efficacy of sensori-motor integration, provides the most parsimonious explanation for the behavioral deficits seen in Adf-1 mutants and in animals with a motor neuron-specific knock down of Adf-1.

Previous studies have attempted to identify genes that are regulated by transcription factors important in the process of synaptic plasticity both in *Drosophila* and in the mammalian brain (Etter et al., 2005; McClung and Nestler, 2003). However, it has generally been difficult to mechanistically correlate the transcription factor in question with putative target genes owing part to "output noise" and the difficulty in assigning transcription factor binding to target genes based on canonical binding sites (Etter et al., 2005; Fisher et al.). Recent work suggests the transcriptional state of a gene can only be accurately derived from transcription factor binding correlated with gene-specific chromatin states in a cell-specific manner. Based on this observation, we carried out ChIP-Seq analysis of Adf-1 binding sites in the larval brain, so that we may identify target genes. Our results confirm that Adf-1 binds close to the transcription start site of many genes in the brain. Clustering based on gene ontology reveals a tight collection of GO terms that relate to neuronal function and development consistent with neural functions for Adf-1. Additionally, the primary consensus motif for Adf-1 binding derived from our ChIP-Seq studies matches a recent analysis of Adf-1 binding sites. The importance of carrying out ChIP-Seq in a tissue-specific manner is also underscored by the observation that an equivalent ChIP-Seq analysis carried out in *Drosophila* Kc cells reveals Adf-1 binding sites that display a minor overlap with the brain data set suggesting tissue-specific gene regulation by Adf-1.

We were surprised to find Adf-1 inhibition led to increased expression of Alcohol dehydrogenase (ADH) – a result that is contrary to the proposed function as a transcriptional activator. Molecularly, Adf-1 is an unusual transcription factor since it does not have a modular architecture (Cutler et al., 1998), a feature that might suggest

unknown complexity in its function. Consistent with the effect on ADH, inhibition of Adf-1 increased expression of FasII mRNA (a direct Adf-1 target gene) and Staufen mRNA. Interestingly, comparison of Adf-1 bound promoters with RNA Pol II distribution in the promoter and gene body, and active chromatin marks such as H3K4me3 and H3K27ac in Kc cells suggested a pattern in which Adf-1 bound genes correlated positively with higher proximal pausing indices for RNA Pol II and negatively with H3K4me3 and H3K27ac. The biological relevance of the correlation between RNA Pol II pausing and Adf-1 binding is currently unknown. Also, underscoring the idea of tissue-specific roles for Adf-1, this relationship needs to be further evaluated in larval brains but unfortunately that data set is currently unavailable.

To obtain further mechanistic insight into Adf-1 dependent regulation of dendrite development, we examined two genes that are targets of Adf-1 in the brain. FasII, the *Drosophila* homolog of vertebrate neural cell adhesion molecule (NCAM), binds Adf-1 close to its transcription start site. Similar to ADH, FasII mRNA expression in the brain is increased following Adf-1 inhibition (*nalyot* mutants and following expression of either Adf-1 DN or Adf-1[S64/184A]). Consistent with this finding, knock down of FasII using a FasII-RNAi transgene rescues dendrite growth phenotypes that result from the expression of Adf-1[S64/184A]. Conversely, over-expression of full length FasII containing a PEST domain results in reduced complexity (Figure 7B, C, E). As we do not have any reason to believe that Adf-1 regulation of FasII is isoform specific, we find the FasII- PEST- transgenes exhibit similar reductions in complexity (data not shown). These results suggest Adf-1 activity in motor neurons regulates dendrite growth and development by modulating the expression of FasII. It is unsurprising that either over-

expression or knock-down of FasII results in dendritic defects. An optimum requirement for FasII is also reminiscent of FasII-dependent pre-synaptic growth regulation that has been described at larval motor synapses. Either too much or too little FasII results in reduced dendrite growth and complexity. This suggests that signaling mechanisms that are either proximal or distal to FasII/NCAM might also be conserved during post-synaptic growth. Since essential roles for NCAMs in neurite outgrowth have been reported (see (Corty et al., 2009; Schmid and Maness, 2008) for reviews), our findings implicate a conserved mechanism downstream of Adf-1 in dendrite growth.

Adf-1 also regulates Staufen expression in the brain, but since we did not detect direct binding of Adf-1 to Staufen *cis*-regulatory regions, we favor the idea that this regulation is indirect. Nonetheless, the observation that Adf-1 can modulate expression of a key regulator of mRNA transport in dendrites is intriguing. It might suggest a crucial interplay between nuclear transcription and mechanisms that govern local mRNA transport, sequestration and translation in dendrites. Staufen positive RNP granules in these larval neurons have been shown to contain regulatory components of yeast P-granules (Barbee et al., 2006) and we speculate that part of the mechanism by which Adf-1 regulates dendrite growth is by controlling Staufen levels. In support of this idea, knocking down Staufen levels in a background of Adf-1 inhibition rescues dendrite growth phenotypes. It seems likely that there needs to be a tight regulation between transcription and local translation during neuronal plasticity. While other studies have revealed how the local translational machinery can impact transcription, our results indicate a complementary pathway by which transcription factors can impact RNP granules that could affect local translation in dendrites. Since it has been shown that

Staufen transports αCaMKII mRNAs, future studies should address whether Adf-1 affects Staufen's movement in RNA granules and whether this ultimately affects local translation of CaMKII. Also, it appears that Adf-1 might interact with other components of the RNA translational machinery. Adf-1 has been shown to very strongly to the promoter of FMR1 (data not shown). This observation is of interest as Staufen has been shown to both co-localize and genetically interact with FMRP (Bolduc et al., 2008; Dubnau et al., 2003).

Adf-1's Role in the Mushroom Body

As the original *nalyot* flies exhibited defects in long-term memory formation, the neuronal roles we have elucidated in motor neurons need to be recapitulated in the higher center learning in *Drosophila,* the mushroom body. We expect mechanisms of cellular plasticity to be conserved between the RP2 motor neuron and mushroom body and have preliminary evidence to suggest that Adf-1 [S64/184A] expression within the larval MB results in defects in learning (Figure 1). Additionally, driving expression (OK107-Gal4, 201Y-Gal4) of the 'strong' Adf-1 [S64/184A] transgene within the MB results in pupal lethality (data not shown) suggesting Adf-1 is important in these structures. Future studies should be aimed at expression of Adf-1 [S64/184A] and Adf-1 [S64/184D] in the adult mushroom body to determine if these perturbations alone could lead to effects of memory. Ongoing projects within the laboratory are attempting to generate single mushroom body clones utilizing MARCM (mosaic analysis with a repressible cell marker) to elucidate if inhibition of Adf-1 leads to defects in MB morphology (Lee and Luo, 2001).

Additional Targets of Adf-1

There were numerous potential downstream targets of Adf-1 identified in the neuronal ChIP-seq that have yet to verified. This includes genes involved in axon guidance (Sema1a), memory formation (Dunce, Rutabaga), and components of the cytoskeleton ((Actin) see Figure 2A). Our work has shown that inhibition of Adf-1 results in dramatic loss of dendritic complexity in the RP2 motoneuron. Unsurprisingly, preliminary evidence shows these same mutants have corresponding decreases in actin transcription (Figure 2B), suggesting Adf-1 could directly control actin dynamics contributing to dendrite formation. Further study of Adf-1 in this process need to be investigated. Other downstream targets of Adf-1 should be explored in greater depth to determine the breadth of interaction between this transcription factor and other critical components of synaptic plasticity.

Ethanol Sensitivity & Memory Formation:

It has been noted that a variety of *Drosophila* memory mutants exhibit sensitivity to ethanol. A transposon-based screen looking to identify flies that had an increase in ethanol sensitivity led to the identification of *cheapdate* (*chpd*), which is an allele of the memory mutant *amnesiac*. Previous studies had also identified a possible link between cAMP signaling and ethanol tolerance in mammals (Diamond and Gordon, 1997). Further analysis found that the memory mutant *rut* and the major catalytic subunit of PKA, DCO exhibited increased ethanol sensitivity underscoring the importance of cAMP signaling in both memory and alcohol tolerance (Lane and Kalderon, 1993). These

ethanol phenotypes in both *chpd* and *rut* were rescued by activation of adenylate cyclase (AC) through the application of forskolin (Moore et al., 1998). In addition, reduction of FasII also leads to an increased sensitivity to ethanol; however, so does over expression of the transmembrane forms of FasII. This suggests that either the other forms of FasII are required in ethanol tolerance or a perturbation of levels of FasII in either direction negatively affects these ethanol behaviors (Cheng et al., 2001). Given that our Adf-1 is both a factor that is involved in memory formation and regulation of the Alcohol Dehydrogenase gene, future studies could be aimed at elucidating possible cross talk between these two processes.

Future work on Adf-1 phosphorylation by CaMKII

Though this work has underscored the importance of phosphorylation of Adf-1 by CaMKII, we have yet to explore what this phosphorylation event does in relation Adf-1's affinity for DNA. Ser64 of Adf-1 resides within the DNA-binding domain which would lead us to postulate that CaMKII phosphorylation alters its affinity for binding DNA. Future studies would need to determine if altered forms of Adf-1 (Ala/Asp substitutions) changed its affinity for the promoter regions of target genes. Additionally, we have never detected Adf-1 expression outside of the nucleus and conversely, CaMKII has never been shown to localize within the nucleus. Previous work had attempted to co-IP Adf-1 and CaMKII together and had failed, leading the authors to conclude that this interaction might be transient (GuptaRoy et al., 2000). This study had also observed that CaMKII phosphorylation only occurred on Ser64 while our studies focused on characterization of both Ser64/184 residues. Future studies with single mutations on Ser64 or 184 would

need to be investigated in our system to determine if one or both of these Serines are contributing to the phenotypes we have observed.

Summary

This work has shown that the transcription factor Adf-1 acts downstream of CaMKII and activity-dependent mechanisms to modulate dendritic plasticity. Additionally, we find that Adf-1 regulates the actions of genes involved in synaptic growth (FasII) and local translation (Staufen). Our results suggest that the coordinate action of these genes, all of which have been implicated in plasticity and memory formation, function together to modulate dendrite growth during development and plasticity through interplay between transcription, cell adhesion and local translation. Future studies will be required to explore other transcriptional targets of Adf-1, investigate the molecular basis for transcriptional regulation by Adf-1, and test whether cooperation between transcription and local translation is conserved in vertebrates.

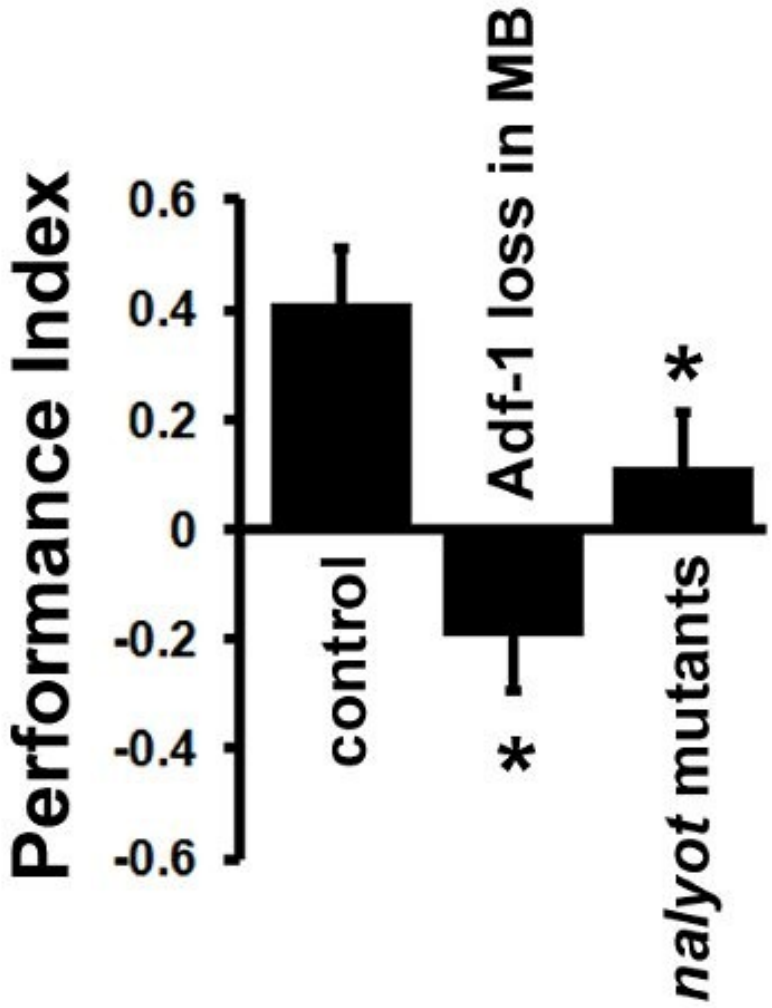
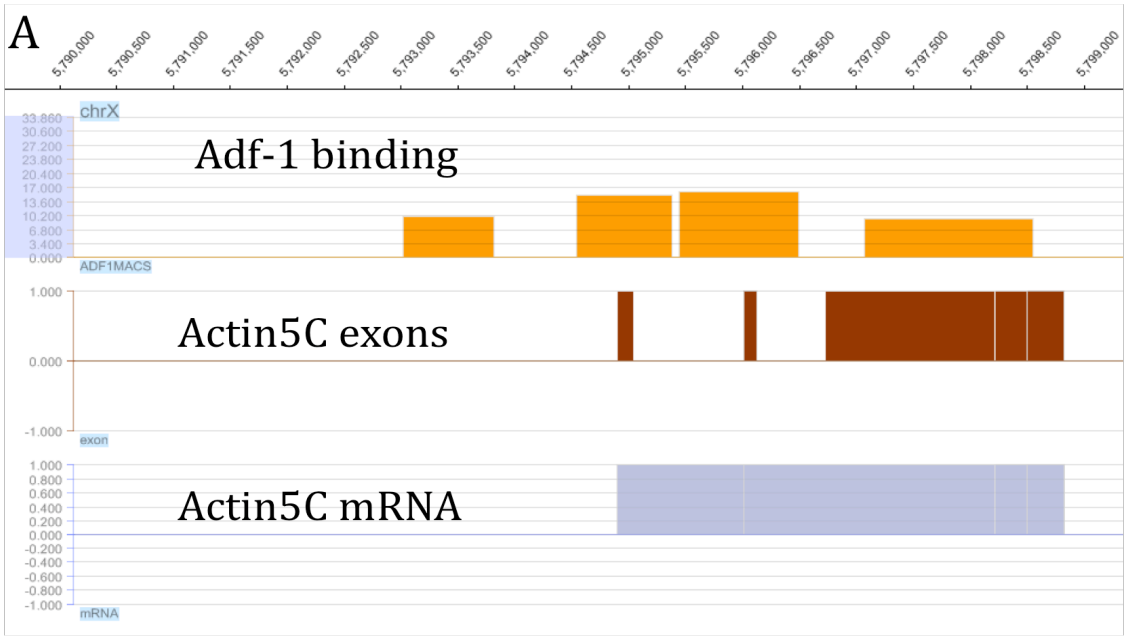


Figure 1: Perturbation of Adf-1 expression in the Mushroom Body

Figure 1: Perturbation of Adf-1 expression in the mushroom body leads to larval learning defects: As compared to control, Adf-1 [64/184Ala] expression in the

mushroom body (OK107-Gal40 leads to a dramatic learning defect. In addition, *nalyot* (Adf-1 hypomorph) animals exhibit learning defects in this paradigm.



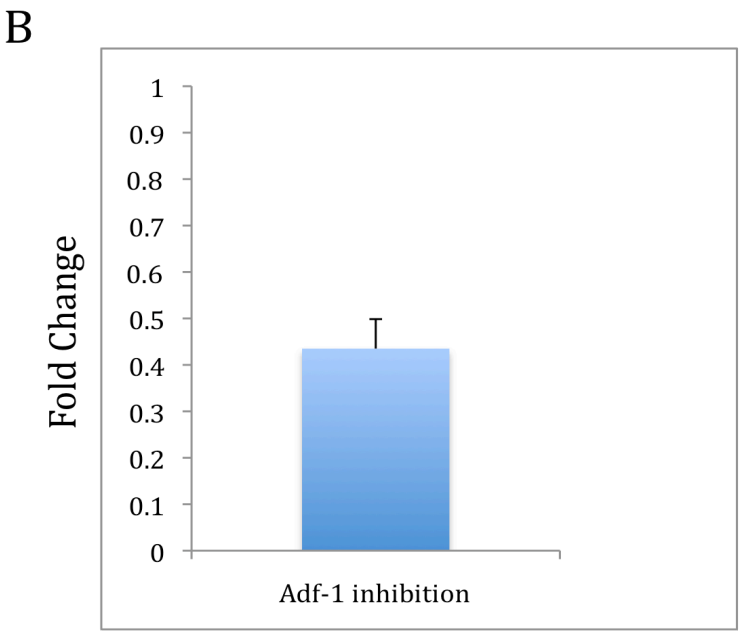


Figure 2 Adf-1's role in Actin5c transcription

Figure 2: Adf-1's role in Actin5c transcription. A: Adf-1 binding was detected at the promoter of *actin5c* in neuronal tissue. B: Inhibition of Adf-1 following neuronal expression of the Adf-1[S64/184Ala] transgene leads to decrease in *actin5c* transcript levels as detected by qRT-PCR.

Appendix

Behavioral and electrophysiological outcomes of tissue-specific *Smn* knockdown in *Drosophila melanogaster*

Christina Timmerman and Subhabrata Sanyal

(paper published in *Brain Research*, December 2012)

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ABSTRACT

Severe reductions in Survival Motor Neuron 1 (SMN1) protein in humans leads to Spinal Muscular Atrophy (SMA), a debilitating childhood disease that leads to progressive impairment of the neuro-muscular system. Although previous studies have attempted to identify the tissue(s) in which SMN1 loss most critically leads to disease, tissue-specific functions for this widely expressed protein still remain unclear. Here, we have leveraged RNA interference methods to manipulate SMN function selectively in *Drosophila* neurons or muscles followed by behavioral and electrophysiological analysis. High resolution measurement of motor performance shows profound alterations in locomotor patterns following pan-neuronal knockdown of SMN. Further, locomotor phenotypes can be elicited by SMN knockdown exclusively in motor neurons, supporting previous demonstrations of motor neuron-specific SMN function in mouse models. Electrophysiologically, SMN modulation in muscles reveals significant but modest alterations in spontaneous synaptic events, but otherwise normal synaptic transmission, quantal release and trans-synaptic homeostatic compensation at the larval neuro-muscular junction. Neuronal SMN knockdown does not alter baseline synaptic transmission, high frequency synaptic depletion and recovery and acute homeostatic compensation. However, chronic glutamate receptor-dependent developmental homeostasis at the neuro-muscular junction is strongly attenuated following reduction of SMN in neurons.

Together, these results support a distributed model of SMN function with distinct neuron-specific roles that are likely to be compromised following global loss of SMN in patients. Our results are complimentary to and in broad agreement with recent mouse studies that suggest a strong necessity for SMN in neurons but recommend a multi-tissue approach in the treatment of SMA.

INTRODUCTION

Spinal Muscular Atrophy (SMA) is a severely debilitating childhood disorder that leads to progressive muscle weakness resulting from a loss or degeneration of motor neuron innervation. It is a relatively common autosomal recessive disorder and can affect up to 1 in 6000 babies (Lefebvre et al., 1995; Pearn, 1978; Sleigh et al., 2011) SMA results predominantly from mutations in the *Survival Motor Neuron 1* gene (*SMN1*). Severity is related to the copy number and activity of a near identical homolog in humans, *SMN2* (Coovert et al., 1997). The *SMN2* gene carries a C-to-T transition leading to aberrant splicing in 80-90% of the *SMN2* transcript and loss of exon 7 (*SMND7*) resulting in a protein with reduced stability (Lorson and Androphy, 2000; Lorson et al., 1999; Monani et al., 1999). The overall reduction of the SMN protein leads to severe neuromuscular degeneration including the loss of motor neuron cell bodies over time (Balabanian et al., 2007; Jablonka et al., 2000; Monani et al., 2000). SMN is a highly conserved ubiquitously expressed protein that is required critically for normal mRNA splicing since it affects the biogenesis of U snRNP particles across species (small nuclear ribonucleoprotein) (Fischer et al., 1997; Kroiss et al., 2008; Lee et al., 2009a; Liu et al., 1997; Meister et al., 2001; Pellizzoni et al., 2002). Complete loss of SMN is cell lethal, therefore, SMA is essentially a disorder that results from reduced SMN availability (Feldkotter et al., 2002; McAndrew et al., 1997) Several mouse models of SMA also

suggest that full-length SMN is required for cell viability, and that disease severity is closely linked to the dosage of *SMN* gene product. Thus, total loss of *Smn* leads to early lethality (Frugier et al., 2000), whereas introduction of two copies of the human *SMN2* gene results in normal birth followed by progressive decline in neuromuscular and motor function and subsequent death by post-natal day 7 (PND 7). Introduction of four copies of *SMN2*, however, leads to complete rescue (Hsieh-Li et al., 2000; Monani et al., 2000) Interestingly, introduction of *SMND7* – the mutant form of human SMN missing exon 7 - in an $Smn^{-/-}$; $SMN2^{+/+}$ background provides additional rescue, such that animals now die between PND 6 and 13 (Le et al., 2005). Given that SMN is required in all cells, why its reduction leads to specific neuromuscular symptoms or whether SMA symptoms arise from SMN reduction in motor neurons or muscle (or even other tissues), still remain outstanding questions, though recent experiments indicate prominent neuronal functions (Park, 2010; Gogliotti, 2012; (Gavrilina et al., 2008; Martinez et al., 2012).

Since SMN is a highly conserved protein, invertebrate models such as *C. elegans* and *Drosophila* have also been used to understand molecular functions for SMN and to more rapidly identify networks of genes within which SMN functions (Chan, 2003; Rajendra, 2007; Chang, 2008; Dimitriadi, 2010; Sen, 2011; Briese, 2009; Sleigh, 2011). These studies have revealed remarkable similarities in SMN function in the regulation of U snRNP biogenesis (Kroiss, 2008; Shpargel, 2009; Cauchi, 2008; Lee, 2009; Cauchi, 2010) and NMJ development and physiology (Chan, 2003; Chang, 2008; Rajendra, 2007; Shpargel, 2009; Cauchi, 2008; Praveen, 2012). While a number of these studies have used classical loss-of-function alleles in *Drosophila Smn*, more recent work has made use of gene-targeted RNAi methodologies, that can be used to knock down, but not deplete

SMN function in selected tissues in conjunction with the versatile GAL4-UAS system (Brand, 1993; Chang, 2008). Thus, RNAi based knockdown is a closer approximation to the situation in SMA patients, and also permits experiments in which tissue-specific reduction in SMN can be achieved *in vivo* in an otherwise normal animal. If it is true that different tissues have different thresholds for susceptibility to reduction in SMN function, then selective, and relatively mild, knockdown of SMN might uncover fundamentally conserved tissue-specific functions for SMN (Grice et al., 2011). Here, we use this strategy to test neuronal versus muscle-specific functions for SMN in *Drosophila* in the regulation of motor behavior and synaptic physiology (Figure 1). Our results highlight both neuronal and muscle functions for *Drosophila Smn*, similar to mouse studies, but with stronger neuronal outcomes. But more significantly, they also point to a very precise role for SMN in the regulation of homeostatic mechanisms that maintain parity at the NMJ. These findings are especially relevant in the context of putative developmental roles for SMN in mammals and suggest a unique function for SMN in the homeostatically driven development of mature motor neurons (Foust, 2010; Hammond, 2010; Sleigh, 2011; Gogliatti, 2012; Martinez, 2012). Behavioral and synaptic phenotypes described in this report should also provide powerful platforms for the functional testing of other *Smn*-interacting genes that have been discovered through genetic and biochemical screening approaches (Dimitriadi, 2010; Sen, 2011).

RESULTS

Smn perturbation in neurons or muscle causes behavioral deficits

Gross locomotor defects in *Drosophila Smn* mutants and in mutants for the fly homolog of *Gemin3* – a DEAD-box RNA helicase complexed with SMN, have been reported previously (Chan, 2003; Shpargel, 2009; Cauchi, 2008; Praveen, 2012). Since *Smn* mutants are often pupal and adult lethal, these studies revealed abnormal locomotion predominantly at larval stages and showed that systemic add-back of SMN significantly rescued these motor phenotypes. Consequently, a relationship between normal SMN function and motor physiology has been established in *Drosophila*, similar to human patients and other mammalian model systems (Chan, 2003; Cauchi, 2008; Shpargel, 2009). However, it is currently unclear whether locomotor phenotypes in *Smn* mutants arise from a loss of SMN in neurons or muscle. In this study, we attempted to answer this question using the GAL4-UAS (Brand and Perrimon, 1993) method of tissue-targeted transgene expression and recently available reagents to either over-express the full length wild type SMN gene product or knockdown *Smn* function using RNA interference (Chang, 2008; Sen, 2011). We chose to use these RNAi reagents since their efficacy in knocking down endogenous levels of SMN in neurons and muscles, as well as their potency in generating neuro-muscular defects have been well characterized (Chang, 2008), and a reduction in SMN, rather than its completely removal, more closely approximates the situation in SMA. Since RNAi mediated knockdown of *Smn* does not lead to lethality in most cases, we were also able to measure adult locomotor behavior at a resolution much higher than possible for larvae. Such measurements allow for a

thorough analysis of locomotor parameters that will also be useful as screening tools in future genetic interaction experiments.

Pan-neuronal reduction of SMN using the *elav*^{C155}-GAL4 driver results in significant changes to the normal locomotor patterns of flies in the Buridan's assay (Gotz, 1980). In this assay, flies are allowed to walk between two diametrically placed landmarks on a circular arena while their movement is recorded in real time followed by off-line analysis (Freeman, 2012; Neuser, 2008). In general, our results suggest that neuronal loss of SMN leads to slower walking speed, less overall activity and a larger number of pauses between bouts of walking as compared to genetic controls containing the GAL4 line (Figure 2A). These phenotypes are seen when *Smn* is knocked down using either of the two RNAi lines directed to the C- or N-terminal portion of the *Drosophila Smn* mRNA (Chang, 2008) confirming the specificity of these phenotypes. Neuronal over-expression of SMN, however, does not lead to any discernible defects in locomotion. Single fly tracks and cumulative occupancy plots (heat map coded to show regions of highest occupancy during the performance of the behavior in red) also demonstrate the extent of disruption of locomotor activity in neuronal *Smn* knockdown animals (Figure 2B).

Changes in SMN levels in muscle do not produce strong locomotor phenotypes in adults (Figure 2C and D). Although muscle-specific roles for SMN have been recognized in both mammalian and *Drosophila* models (Chan, 2003; Rajendra, 2007; Chang, 2008; Sen, 2011; Dachs, 2011; Lee, 2011), RNAi mediated knockdown or over-expression of wild type SMN does not result in appreciable locomotor defects. Mild changes are seen in the number of pauses when SMN is either more or less than control levels, suggesting

that these are likely to result from non-specific effects. Single representative fly tracks as well as occupancy plots further confirm that changes of SMN in muscle tissue do not affect locomotor performance in this assay. Overall, results presented in Figure 2 show the presence of profound locomotor deficits in adult animals following selective knockdown of *Smn* in neurons. These findings suggest that in *Drosophila* motor phenotypes previously observed in *Smn* mutants are likely to be due to loss of SMN in neurons.

Knockdown of *Smn* in glutamatergic neurons, but not cholinergic neurons, leads to behavioral deficits

Since pan-neuronal reduction in SMN resulted in locomotor deficits, we asked whether SMN is preferentially required in a subset of neurons for normal motor behavior. Given that motor neurons are strongly implicated in SMA, we first tested if *Smn* knockdown in adult motor neurons produces behavioral phenotypes. *Drosophila* motor neurons are glutamatergic. Therefore, we used the VGlut-GAL4 line (OK371) to either over-express or knockdown *Smn* in all glutamatergic neurons (note that there many non-motor glutamatergic neurons in the *Drosophila* nervous system that are also targeted by OK371-GAL4) (Mahr and Aberle, 2006). OK371-GAL4 driven *Smn* RNAi resulted in phenotypes very reminiscent of pan-neuronal *Smn* knockdown (Figure 3A and B). While the average speed of walking was not significantly altered, total distance moved and overall activity were significantly reduced, and number of pauses elevated, following *Smn* knockdown in the glutamatergic nervous system. These phenotypes were not observed following over-expression of *Smn* in glutamatergic neurons. Single fly tracks

and occupancy plots also confirmed aberrant locomotor behavior in the knockdown animals, though these were not as strong as those following pan-neuronal knockdown of *Smn*. By contrast, *Smn* perturbation in cholinergic neurons did not have any effect on locomotion at all (Figure 3C and D). Thus, when *Smn* was over-expressed or knocked down with the Cha-GAL4 driver (Salvaterra and Kitamoto, 2001), none of the parameters were altered significantly and single fly tracks and occupancy plots reiterated normal locomotor behavior in all genotypes tested. Since by all accounts, Cha-GAL4 is a strong driver and has elicited phenotypes in many previous studies including those that have used RNAi mediated knockdown (for examples see (Lima, 2005; Sakai, 2009; Kitamoto, 2001; Ghosh and Feany, 2004)), we do not favor the idea that the absence of phenotypes is a consequence of weak expression of the SMN-RNAi construct. Instead, these experiments suggest to us that glutamatergic motor neurons are likely to be a prominent locus for *Smn*-dependent locomotor phenotypes in *Drosophila*.

Neuronal perturbation of *Smn* does not alter baseline and high-frequency synaptic transmission

Analysis of adult behavior shows that reduced SMN in motor neurons results in behavioral deficits in a visuo-motor assay. We wondered whether we could detect a possible basis for these motor phenotypes rooted in synaptic physiology. Since the larval neuro-muscular system is simpler and experimentally more accessible than in the adult, we used this model synapse to test our idea. Baseline synaptic transmission was measured at the muscle 6/7 motor synapse in abdominal segment A2 in 1 mM external calcium as reported previously (Freeman, 2011; Sen, 2011). *elav*^{C155}-GAL4 was used to drive

expression of full-length *Smn* or RNAi constructs as described in previous sections. Interestingly, we did not observe any meaningful change in synaptic transmission under these conditions (Figure 4A and B). Mean evoked junction potential (EJP) amplitude, mini evoked junction potential (mEJP) amplitude and the quantal content of release remained unaltered in all genotypes tested, barring a modest increase in mEJP amplitude following over-expression of *Smn*. These data indicate that normal parameters of synaptic transmission are not affected by *Smn* perturbation in neurons, although a 50% reduction in the growth of this synapse and the number of synaptic boutons under these conditions of *Smn* knockdown have been reported previously (Chang, 2008).

We next tested whether *Smn* knockdown impairs synaptic transmission when the system is stressed during high-frequency stimulation under conditions that maximize quantal content of release. We drove the N4 RNAi line with *elav*^{C155}-GAL4 since this should result in the strongest pre-synaptic knockdown of *Smn*. Motor neurons innervating muscle 6/7 were stimulated at 10 Hz for a duration of 10 minutes in an external calcium concentration of 10 mM. Under these conditions more synaptic vesicles are released than can be recovered, leading to a depletion of the readily releasable pool of synaptic vesicles and a rapid decline in the size of the EJP that reflects fewer vesicles being released per action potential. Following this rapid decline, the synapse recovers partially and reaches a stable transmission rate due to vesicle recruitment from the reserve pool of vesicles. After a transition from 10 Hz stimulation to 0.1 Hz stimulation, the synapse recovers almost immediately to pre-stimulus levels since the balance between vesicle fusion and vesicle retrieval is restored. This assay, therefore, examines mechanisms underlying rapid recruitment of distinct vesicle pools and synaptic vesicle fusion and recycling (Dickman

et al., 2005; Seabrooke and Stewart, 2011). When challenged with high-frequency stimulation *Smn* knockdown synapses performed as well as control ones (Figure 4C). The rate and extent of decline, reserve pool recruitment and recovery post-stimulation are not significantly different between knockdown and control animals suggesting normal vesicle dynamics at these synapses. Taken together, results in this section imply that behavioral deficits following neuronal knockdown might not result from obvious changes in baseline synaptic transmission at the neuro-muscular junction. This is in apparent conflict with earlier reports in *Drosophila* (Chen et al., 2003) and several studies in rodents (Kong, 2009; Ruiz, 2010; Martinez, 2012). However, these studies did not selectively remove *Smn* in neurons, and synaptic transmission phenotypes in these situations could have arisen from developmental changes triggered by reduced SMN in both neurons and muscles and indeed in other tissues. In a situation where *Smn* was selectively reduced in rodent motor neurons, synaptic and behavioral defects were also mild and improved dramatically over time (Park, 2010).

Smn perturbation in muscle affects quantal size

Increasingly, a role for normal SMN function in muscle is being appreciated (Dachs, 2011; Lee, 2011; Martinez, 2012). These studies suggest that SMN in muscle might contribute to the regulation of normal muscle development and synaptic physiology. In *Drosophila*, previous experiments have clearly documented post-synaptic enrichment of SMN protein, synaptic growth phenotypes at the larval neuro-muscular junction that have resulted from *Smn* knockdown (Chang, 2008), strong muscle atrophy in *Smn* mutants (Rajendra et al., 2007) and genetic interactions between *Smn* and *FGF*

signaling pathways in muscle (Sen et al., 2011). Based on this information, we asked whether *Smn* perturbations in muscle might result in synaptic phenotypes at the larval NMJ.

how^{24B}-GAL4 driven expression of full-length *Smn* or *Smn*-RNAi resulted in reduced mean EJP and mEJP amplitude (Figure 5A and B) resulting in indistinguishably similar quantal content of release across genotypes. Although it seems surprising that both over-expression and knockdown of *Smn* lead to equivalent synaptic phenotypes, this is in line with the observation that both over-expression and knockdown of *Smn* in muscle leads to pupal/adult lethality (Chang, 2008) and abnormally long pupal cases (our unpublished observations). While the reasons behind the similarity of these phenotypes are unclear, it seems likely that these are indeed due to perturbations in muscle SMN function. Quantal size phenotypes prompted us to examine the staining intensity and distribution of post-synaptic glutamate receptors. A severe disorganization of post-synaptic Acetylcholine receptors at the NMJ has been widely observed in rodent models of SMA (Kariya, 2008; Murray, 2008; Kong, 2009; Ling, 2010; Ruiz, 2010; Dachs, 2011; Lee, 2011, Biondi, 2008) and a loss of glutamate receptor staining has also been reported in *Drosophila Smn* mutants (Chen et al., 2003). However, we did not observe any changes in overall glutamate receptor staining intensity or distribution when *Smn* was knocked down in muscles using the stronger N4 RNAi transgene (Figure 5C). Similarly, no change in glutamate receptor distribution is observed following neuronal knockdown of *Smn* either (data not shown). These results suggest that a reduction in SMN exclusively in the muscle (or in neurons) is not sufficient to cause strong synaptic transmission defects or receptor mis-aggregation, although it is sufficient to cause adult

lethality (Chang, 2008). It is possible that neuro-muscular symptoms in SMA arise from much stronger reduction in SMN and/or distorted NMJ development or maintenance precipitated by a loss of SMN in both compartments (Martinez, 2012).

Pre-synaptic knockdown of *Smn* abolishes long-term homeostatic compensation at the NMJ

Rodent models of SMA show early synaptic transmission deficits at the NMJ that typically worsen with age (Kariya, 2008; Murray, 2008; Kong, 2009; Ling, 2010; Ruiz, 2010; Dachs, 2011; Lee, 2011). Additionally, recent studies suggest that homeostatic feedback mechanisms that maintain parity of synaptic transmission might be compromised when SMN levels are reduced in motor neurons. In one study, selective reduction of SMN in motor neurons resulted in reduced quantal content of transmission at post-natal day 8 (PND8) in mice (Park, 2010). Strikingly, these defects were sharply reduced by PND10-12, and by 3 months, NMJs showed overcompensation as they displayed higher mini-end plate potentials (mEPPs), EPPs and quantal content than control animals (Park, 2010). These data reveal aberrations in homeostatic mechanisms at the synapse following neuronal knockdown of *Smn*. Conversely, two other studies have addressed the tissue-specificity of SMN by rescuing SMN expression in either neurons or muscle in an *Smn* mutant background (Gogliatti, 2012; Martinez, 2012). While neuronal, but not muscle, add-back of SMN was able to rescue synaptic transmission defects at the NMJ in *Smn* mutants, surprisingly, neuronal supplementation of SMN also prevented loss of central motor neuron synaptic input. This observation suggests that under normal

conditions neuronal SMN might regulate retrograde signaling mechanisms that maintain homeostatic drive at synapses.

To directly evaluate whether SMN plays a role in homeostatic signaling mechanisms in neurons, we used two assays that test either a rapid, gene expression-independent or a long-term, gene expression requiring form of homeostasis at the larval NMJ. An activity-dependent block of post-synaptic glutamate receptors at the larval NMJ using the wasp venom Philanthotoxin (PhTox) triggers a fast homeostatic response from the pre-synapse that results in a significant increase in quantal content, while quantal size remains predictably reduced (Frank, 2006; Frank, 2009). We tested whether *Smn* knockdown in neurons or muscles affected this homeostatic response. Figure 6A shows that PhTox-dependent homeostatic responses remained robust in either case. Thus, reduction of SMN using the N4 RNAi transgene did not alter the synapse's ability to engage a fast homeostatic increase in quantal content following glutamate receptor attenuation with PhTox (reflected in the reduced mEJP amplitudes in Figure 6A). These data suggest that local signaling mechanisms that are involved in this form of homeostasis do not require SMN activity.

Since aberrations in homeostasis in rodent models of SMA occur over a developmental time period, we next tested a form of homeostatic compensation at the larval NMJ that is longer term and likely requires changes in gene expression. A strong genetic reduction in one glutamate receptor subunit, *GluRIIA*, leads to severely attenuated post-synaptic currents and smaller mEJP amplitudes (Davis et al., 1998; Petersen et al., 1997). However, over developmental time, the pre-synapse compensates through an increase in quantal content (Davis et al., 1998 1999; Haghighi et al., 2003; Penney et al.,

2012; Petersen et al., 1997). When we reduced SMN in neurons in a *GluRIIA* mutant background, we saw a virtual absence of homeostatic compensation (Figure 6B and C). By contrast, SMN reduction in muscles did not affect this form of homeostasis. These observations point to very specific neuronal functions for SMN in the control of long-term homeostatic responses. Such a function would also be consistent with a general role for SMN in the regulation of RNA metabolism and gene expression (Figure 6D).

DISCUSSION

Although there are obvious differences between *Drosophila* and mammalian neuro-muscular organization, modeling SMA in flies is a valid and powerful approach given the high degree of conservation in SMN function and the ease and rapidity of genetic analysis in flies (Dimitriadi et al., 2010). Indeed, previous work in *Drosophila* has suggested that *Smn* regulates U snRNP biogenesis (Kroiss, 2008; Shpargel, 2009; Cauchi, 2010; Lee, 2009) and loss of SMN results in both synaptic and motor defects that are comparable to those seen in mouse models of SMA (Chan, 2003; Chang, 2008; Rajendra, 2007; Shpargel, 2009). Recent work has also made use of RNA interference to knockdown *Smn* in target tissues (Chang, 2008; Sen, 2011) to better mimic the situation in SMA patients. Experiments with these validated RNAi reagents have uncovered morphological phenotypes at the NMJ when SMN was knocked down in either neurons or muscle (Chang, 2008; Sen, 2011). In the current study we have used the same RNAi reagents in conjunction with the GAL4-UAS system to reduce SMN in neurons or muscle tissue followed by an assessment of such perturbation on motor performance and synaptic transmission at the NMJ (Figure 1). Muscle knockdown of SMN did not result in strong behavioral or synaptic transmission defects, though similar manipulations have been shown to affect synaptic morphology in larvae and lead to adult lethality (Chang, 2008) (Sen et al., 2011). This suggests a situation in *Drosophila* that is very similar to that in mice, where SMN plays a role in muscles to ultimately impact lifespan, but it is not required for normal synaptic function (Gogliotti, 2012; Martinez, 2012). On the other hand, we find distinct deficits in visuo-motor performance when SMN is reduced

neuronally, though baseline synaptic transmission under these conditions is largely unaffected. This contrasts with earlier findings in *Drosophila* (Chen et al., 2003) and is likely due to the relatively mild and compartment-specific knockdown of SMN in our experiments. However, in the absence of a severe decrement in synaptic function, we were able to identify a very specific loss of homeostatic compensation between the pre- and post-synapse when SMN was reduced neuronally (Figure 6D). We propose that this might reflect an early event in SMA etiology that predates more dramatic SMA sequelae comprising loss of normal NMJs and neuro-muscular degeneration.

Severe mouse models of SMA (e.g. $Smn^{-/-}$; $SMN2^{+/+}$) show clearly discernible loss of neuro-muscular morphology, strong synaptic transmission defects, loss of pre-synaptic inputs to spinal motor neurons, muscle degeneration and eventual death of motor neurons (Monani, 2000; Jablonka, 2000; Balabanian, 2007). Several studies have also used Cre-loxP derived conditional knockout models of SMA in mice to address the question of tissue-specificity of SMN vis-à-vis the incidence of SMA-like symptoms. Complete removal of exon 7 in specific tissues led to cell lethality, a situation, though consistent with a vital role for SMN in all cells, significantly different from that found in SMA patients (Frugier, 2000; Cifuentes-Diaz, 2001; Cifuentes-Diaz, 2002; Nicole, 2003; Vitte, 2004). Recent studies have more successfully approximated the patient condition, while trying to distinguish tissue-specific functions for SMN *in vivo* (Park, 2010; Gogliotti, 2012; Martinez, 2012). In one study, *Smn* exon 7 was removed in neurons in a background where *SMN2* was added back (Park, 2010). Surprisingly, these mice showed mild SMA symptoms that improved with age. However, this manipulation did uncover

subtle homeostatic regulatory phenotypes at the neuro-muscular junction (NMJ) that may not have been noticed in the presence of the typically severe SMA phenotypes (Murray, 2010; Murray, 2008; Ruiz, 2010; Michaud, 2010; Kariya, 2008, Kong, 2009, Biondi, 2008). In another study, full-length SMN protein was added back to either neurons or muscle in a mutant *SMN* background that otherwise results in viable "SMA" mice (Lutz, 2011; Martinez, 2012). This study showed that while both neuronal and muscle add-back of SMN could significantly rescue survival and motor behavior, only neuronal expression rescued synaptic function at the NMJ and also motor neuron somal synapses, through putative homeostatic mechanisms (Martinez, 2012). A third study showed, by adding back SMN selectively to motor neurons using an *Hb9-Cre*, that SMN supplementation in motor neurons could rescue the vast majority of motor defects and restored normal sensory-motor synapses (Gogliotti et al., 2012). Together, these observations hint at neuron-specific roles for SMN in the homeostatic regulation of normal synaptic connectivity and transmission.

Phenotypic end-points in severe SMA models closely mirror SMA pathology in patients, particularly Type1 SMA. However, the progressive nature of these phenotypes, increased fetal expression of *SMN* and the general observation that SMA phenotypes can be most effectively rescued by adding back full length SMN perinatally, suggest that defining events in SMA pathology might occur at very early stages of development (Gabanella, 2005; Burlet, 1998; Baumer, 2009; Murray, 2010; Foust, 2010; Narver, 2008; Butchbach, 2010; Hammond, 2010). Thus, obvious target tissues in SMA such as motor neurons and muscle might be more susceptible to a reduction in SMN function such that

subtle defects in neuro-muscular development or physiology are the first to appear at these loci – consistent with the threshold hypothesis for SMA (Grice et al., 2011). Milder models of SMA in mice such as those mentioned in the previous section, seem to support this idea in some ways since they reveal aberrations in the homeostatic regulation of synaptic connectivity that may not be discernible in the presence of more severe phenotypes (Park, 2010; Gogliotti, 2012; Martinez, 2012). These observations point to a fundamental impairment in communication between motor neurons and their pre-synaptic terminals following loss of SMN. Over time, this might lead to an asynchrony in an otherwise tightly coordinated program of synaptic development.

Our experiments in *Drosophila* probably represent one of the mildest manipulations of *Smn* and reveal deficits in synaptic homeostasis. Consistent with this idea, animals are largely viable and do not show the kind of striking locomotor deficits that are seen in *Smn* loss-of-function mutants (Chan, 2003; Shpargel, 2009). However, neuronal knockdown of *Smn* does result in locomotor dysfunction that can only be discerned with sophisticated visuo-motor assays. At the NMJ, though most parameters of synaptic transmission are near normal, we detect a specific defect in homeostatic compensation when *Smn* is knocked down in neurons. A severe impairment of synaptic transmission as described previously (Chen et al., 2003), might not have allowed the detection of this phenotype. In general, this phenotype finds resonance with defects in homeostatic regulatory mechanisms that have been highlighted previously in mild SMA models in mice (Park, 2010; Gogliotti, 2012; Martinez, 2012) and might represent a "weak link" that is most susceptible to a loss of SMN in neurons. It is also conceivable

that an SMN-dependent loss of calibration in homeostatic drive is one of the earliest events that disrupts developmental coupling between the pre- and post-synapse during a critical period in synaptogenesis. Further experiments are required to test whether this prediction holds in mouse models of SMA.

Our results might also have a bearing on the molecular function of SMN in neurons. While SMN is clearly involved in U snRNP biogenesis, it is not clear whether this function of SMN is the one most compromised in neurons (Buhler, 1999; Pellizzoni, 1999; Gabanella, 2007; Wan, 2005; Shpargel, 2005). For example, in *Drosophila*, *Smn* null mutant show normal mRNA splicing (Rajendra, 2007; Praveen, 2012). In addition, low transgene-mediated expression of wild type SMN rescues SMA-like phenotypes in flies without any improvement in snRNA levels (Praveen et al., 2012). While snRNA levels have not been tested in our manipulations, phenotypic analysis shows clearly that a gene expression-requiring homeostatic compensation is completely abolished through a neuron-specific reduction in SMN (Figure 6D). By contrast, a rapid gene expression-independent form of homeostasis at the NMJ is normal, suggesting perhaps a role for SMN in the regulation of nuclear gene expression. Whether this occurs through control of mRNA metabolism, remains unclear. However, it is interesting to note that this form of long-term homeostasis also requires BMP/TGF-*b* signaling in the pre-synapse, a signaling pathway recently shown to interact with SMN in flies (Haghighi, 2003; Chang, 2008). In this context, one might envisage SMN playing a role either in the relay of a homeostatic “signal” in the pre-synaptic compartment, or in the execution of a suitably graded response (Figure 6D). Although our studies have previously shown a role for

SMN in the regulation of FGF signaling in the muscle, this function does not seem to be necessary for homeostatic regulation of synaptic transmission (Sen et al., 2011). Finally, it is of note that motor neurons in *Drosophila* are glutamatergic. Whether GluR-dependent homeostatic signaling in flies has a bearing on cholinergic neuro-muscular synapses or glutamatergic sensory-motor synapses in mammals remains to be seen.

MATERIALS AND METHODS

Drosophila strains, genetics and husbandry

Drosophila strains were reared in standard corn meal–dextrose–yeast containing food at 25 °C in controlled humidity incubators under a constant 12 h light:dark cycle. *elav*^{C155}-GAL4 and *how*^{24B}-GAL4 have been described previously (Brand and Perrimon, 1993; Luo et al., 1994). UAS-SMN, UAS-SMN-RNAi(C24) and UAS-SMN-RNAi(N4) were from Spyros Artavanis-Tsakonas and have been validated and described earlier (Chang et al., 2008). *GluRIIA* mutants (*GluRIIA*^{Sp16}) and deficiency (*Df(2l)cl-h4*) were from Pejmun Haghighi (Penney et al., 2012).

Antibodies and immuno-histochemistry

Larval dissection, staining and confocal microscopy were performed according to standard protocols (Franciscovich et al., 2008). Briefly, larvae were dissected in Ca^{2+} free HL3 ringer's solution, fixed in 4% paraformaldehyde, stained with primary antibody overnight, and followed by incubation in Alexa Fluor conjugated secondary antibody. Rabbit anti-dGluRIII (Marrus et al., 2004) was used at 1:1000 and Alexa-568 conjugated anti-HRP (Molecular Probes) was used at 1:500. Anti-rabbit Alexa 488 conjugated secondary antibody (Molecular Probes) was used at 1:400. An inverted 510 Zeiss LSM microscope was used for imaging. For quantitative fluorescence care was taken to prepare samples identically. Samples were imaged and analyzed double blind, and all imaging was interleaved such that control and experimental samples were imaged

alternately on the same day. Confocal settings including black level (offset/contrast), gain, pixel dwell time and the number of iterative samplings for noise reduction were kept constant.

Electrophysiology

Baseline evoked and spontaneous junction potentials were measured as described previously (Sen et al., 2011). Briefly, wandering third instar larvae were dissected in normal HL3 ringer's solution (Stewart et al., 1994) with 1 mM Ca^{2+}. Intracellular recording electrodes with tip resistances between 25-50 MW were filled with 3 M KCl. Only those recordings were used where the resting membrane potential was more polarized than -60 mV and the muscle input resistance was greater than 10 MW. Muscle 6 (VL2) in abdominal segment A2 was used for all recordings. A train of 25 supra-threshold stimuli at 0.5 Hz was delivered in each experiment from which mean peak EJP values were obtained by averaging the last 20 traces. EJP amplitudes were corrected for non-linear summation using Martin's correction factor, assuming a reversal potential of 0 mV and a membrane capacitance factor of 0.55 (Kim, 2009; McLachlan, 1981). 8–10 separate animals were used for each genotype. A 2 minute continuous recording was used to measure mEJP amplitude. Traces were low-pass filtered at 1 kHz and analyzed using Clampfit or Mini-Analysis programs (Synaptosoft). 8–10 separate animals were analyzed for each genotype. Quantal content was determined by dividing the mean corrected EJP amplitude for a given synapse by the mean mEJP amplitude. Statistical significance was determined using one-way ANOVA. Representative traces were plotted using MS-Excel from episodic recordings exported from Clampfit as axon text files. High-frequency

stimulation was carried out in a modified HL3 Ringer's solution containing 10 mM Ca^{2+} as described previously (Seabrooke and Stewart, 2011). Rapid PhTox (Sigma Aldrich Inc.) mediated homeostasis was measured as described earlier in 0.3 mM Ca^{2+} containing HL3.1 Ringer's solution (Feng, 2004; Frank, 2006). Recordings in a loss of *GluRIIA* background were carried out in HL3.1 Ringer's with 0.3 mM Ca^{2+}.

Behavioral analysis

2-3 day old flies were collected and their wings cut close to the thorax. These flies were then allowed to recover for an additional 3 days. Flies were individually placed on a circular platform 10 cm in diameter and surrounded by a moat of water 2 cm in width. Flies normally walked back and forth between two diametrically opposite vertical black bars within a brightly illuminated cylinder. A 5 mpi webcam mounted centrally above the platform recorded their movement for a period of 5 minutes from within a Buridan tracking program from Bjorn Brembs. These recordings were then analyzed using custom designed Buridan analysis software (from Bjorn Brembs) written in the statistical package *R*. Results from this analysis were plotted in MS-Excel. Statistical significance was determined using one-way ANOVA. Single representative fly tracks and occupancy plots were also generated using the Buridan analysis software.

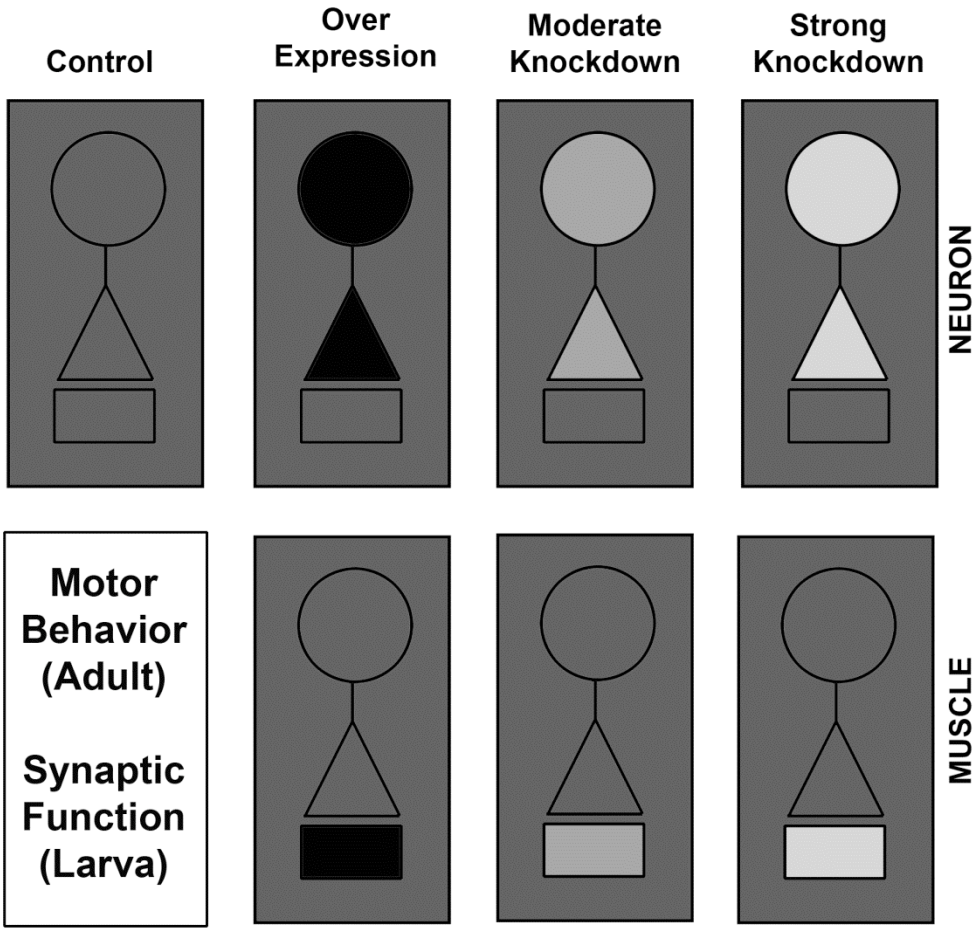
Control
Over Expression
Moderate Knockdown
Strong Knockdown
NEURON
Motor Behavior (Adult)
Synaptic Function (Larva)
MUSCLE

Figure 1

Figure 1. Experimental paradigm to test tissue-specific requirement for SMN in the maintenance of normal motor physiology. The experimental strategy to test compartment-specific functions for SMN is outlined schematically. SMN is expressed widely in many tissues (grey outer box). In this study, SMN has either been over-expressed (black) in neurons or muscles, or has been reduced through RNAi mediated knockdown (lighter shades of grey reflected moderate to strong knockdown). SMN levels in other tissues are presumed to be intact and comparable to wild type control animals. Experimental animals are then subjected to behavioral assays to measure motor function in adults. Electrophysiological measurements are used to evaluate various aspects of synaptic function at the third instar larval NMJ. In all figures, circles represent the neuronal soma, triangles represent the pre-synaptic compartment and small rectangles represent post-synaptic muscle.

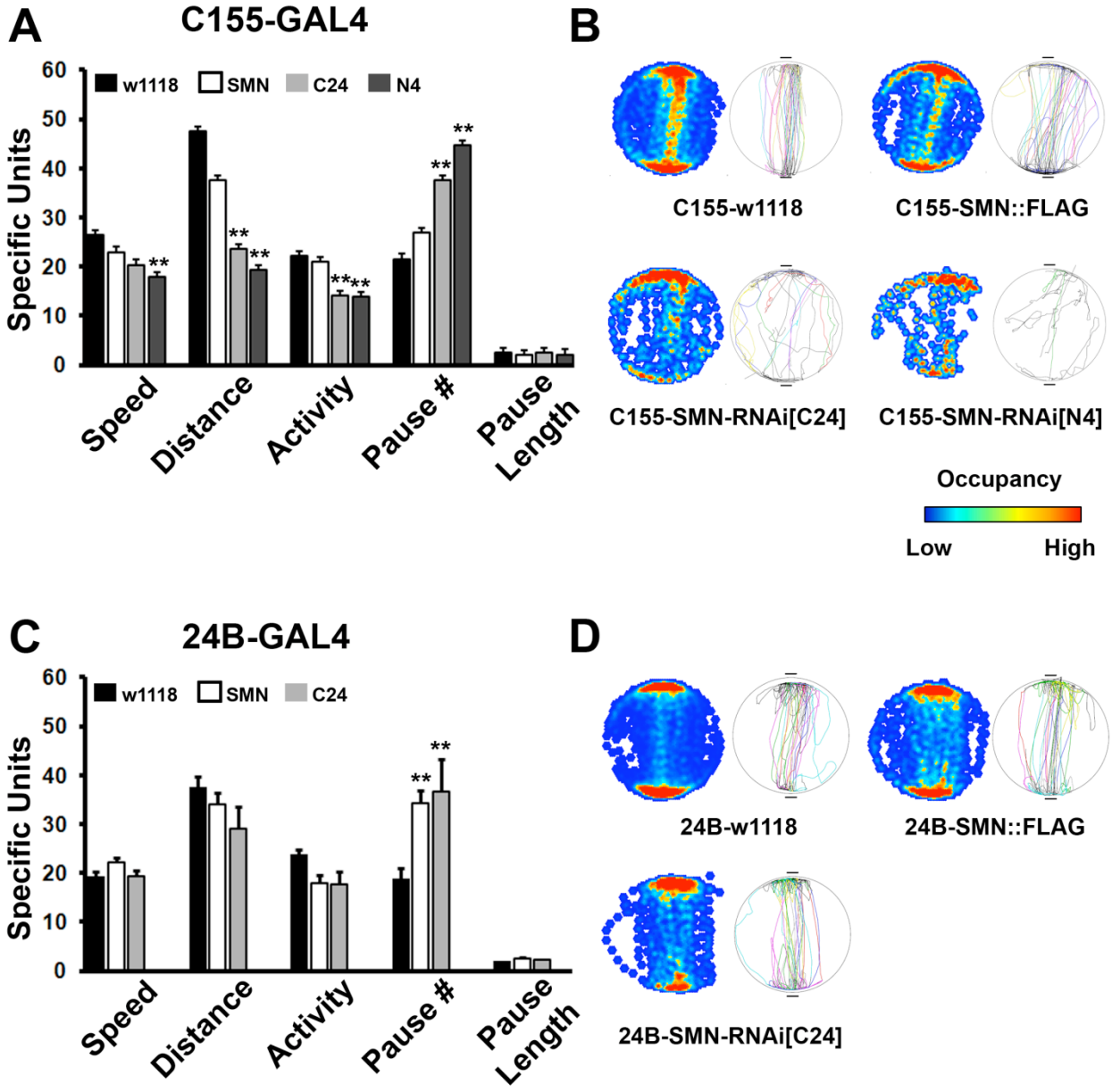
A
C155-GAL4
w1118
SMN
C24
N4
Specific Units
60
50
40
30
20
10
0
Speed
Distance
Activity
Pause #
Pause Length
B
C155-w1118
C155-SMN::FLAG
C155-SMN-RNAi[C24]
C155-SMN-RNAi[N4]
Occupancy
Low
High
C
24B-GAL4
w1118
SMN
C24
Specific Units
Speed
Distance
Activity
Pause #
Pause Length
D
24B-w1118
24B-SMN::FLAG
24B-SMN-RNAi[C24]

Figure 2

Figure 2. Perturbation of SMN in either the nervous system or musculature disrupts normal motor behavior. (A) Various parameters of locomotion as measured by the Buridan's assay are shown for neuronal perturbations of SMN using the *elav*^{C155}-GAL4 driver line. SMN knockdown using two RNAi lines results in decreased speed, distance covered and overall activity, while the number of pauses is increased significantly without affecting mean pause length. **(B)** Single representative fly traces (left circles) and cumulative occupancy plots (right circles with heat maps) show disrupted locomotor behavior following pan-neuronal knockdown of SMN. **(C)** Muscle perturbation of SMN using the *how*^{24B}-GAL4 driver line. The number of pauses is significantly increased following either over-expression or knockdown of SMN in the mesoderm. **(D)** Single fly tracks and cumulative occupancy plots show mild impairment of locomotion. Note that muscle expression of SMN-RNAi-N4 is pupal lethal as reported previously. 10 individual animals that are 5-6 days old are tested for each genotype. Error bars are SEM, double asterisks represent $p<0.01$ as tested by ANOVA.

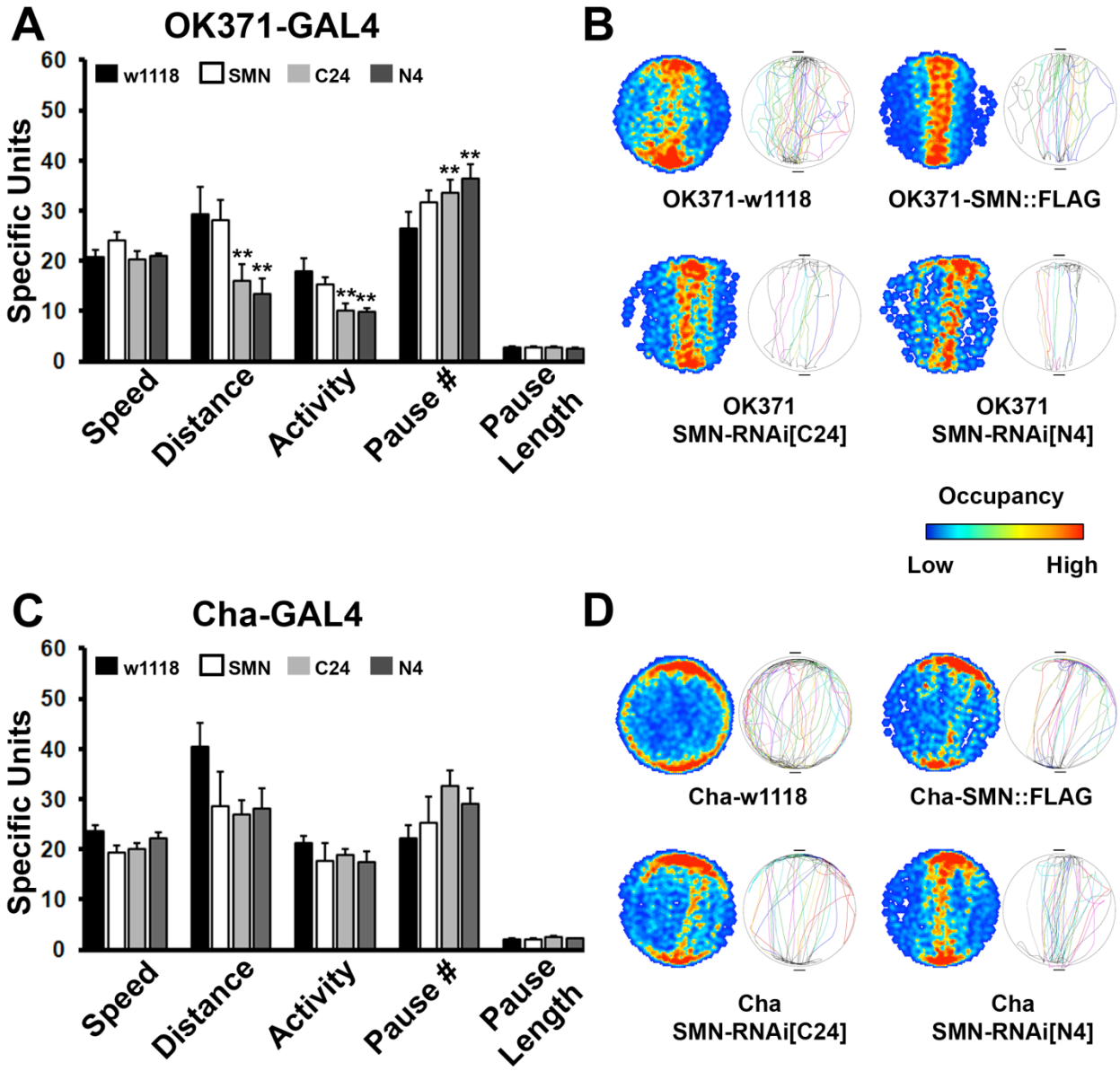
A
OK371-GAL4
w1118
SMN
C24
N4
Specific Units
60
50
40
30
20
10
0
Speed
Distance
Activity
Pause #
Pause Length
**
B
OK371-w1118
OK371-SMN::FLAG
OK371 SMN-RNAi[C24]
OK371 SMN-RNAi[N4]
Occupancy
Low
High
C
Cha-GAL4
D
Cha-w1118
Cha-SMN::FLAG
Cha SMN-RNAi[C24]
Cha SMN-RNAi[N4]

Figure 3

Figure 3. SMN reduction in motor neurons is sufficient to impair motor function. **(A, B)** Parameters of locomotion, single fly tracks and occupancy plots in animals with glutamatergic neuron specific perturbations of SMN using the VGlut-GAL4 driver (OK371). RNAi mediated knockdown of SMN in glutamatergic neurons recapitulates in large part the effect of pan-neuronal knockdown. Distance and activity are reduced, while the total number of pauses is increased. SMN over-expression does not have any discernible influence on locomotion. **(C, D)** Results from the Buridan's assay for cholinergic neuron specific perturbations in SMN. None of the parameters for locomotion are significantly affected by altering SMN function in cholinergic neurons. 10 animals are tested for each genotype, error bars are SEM and double asterisks represent $p<0.01$ as determined by ANOVA.

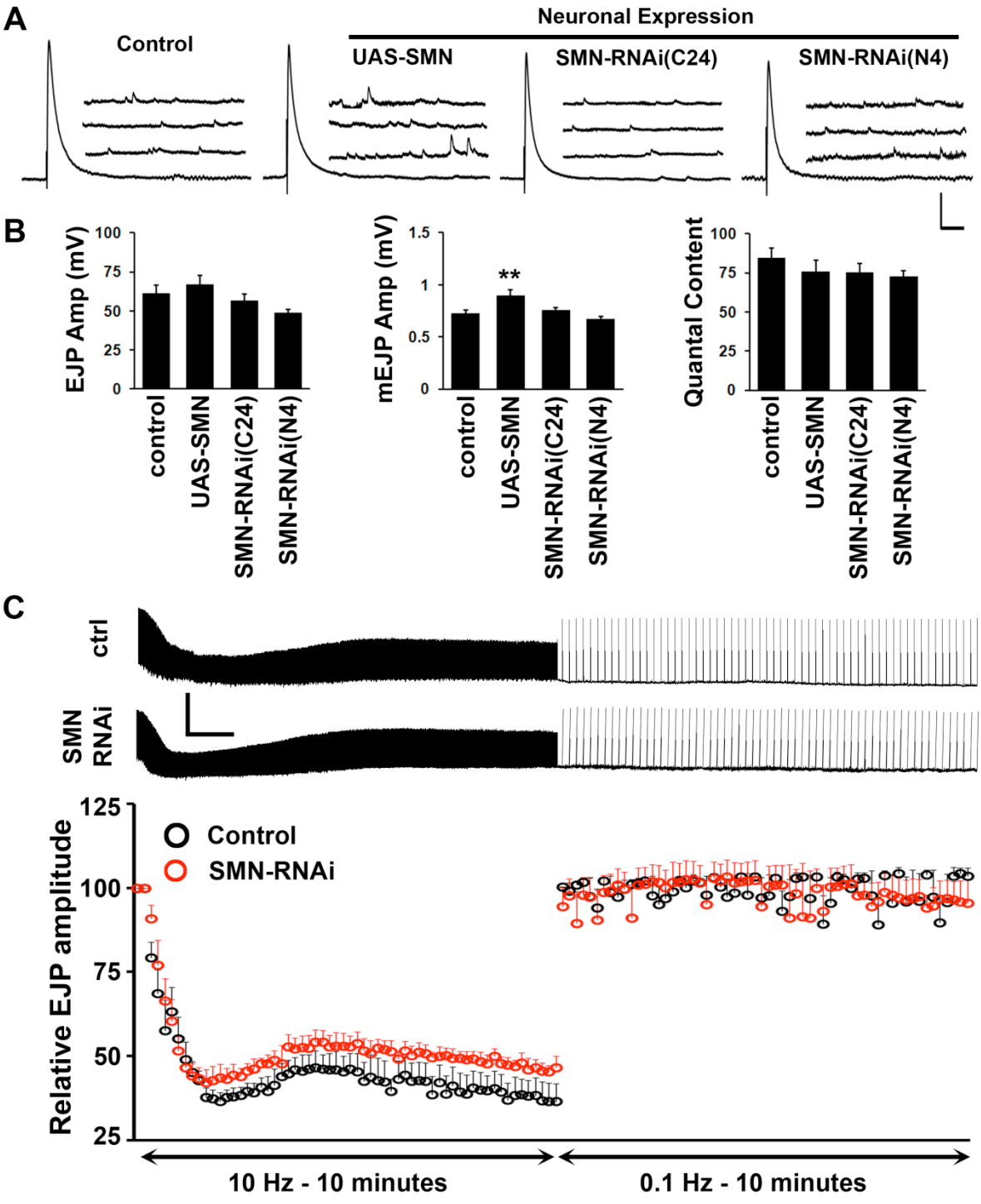
A
Control
Neuronal Expression
UAS-SMN
SMN-RNAi(C24)
SMN-RNAi(N4)
B
EJP Amp (mV)
mEJP Amp (mV)
Quantal Content
**
control
UAS-SMN
SMN-RNAi(C24)
SMN-RNAi(N4)
C
ctrl
SMN RNAi
Relative EJP amplitude
Control
SMN-RNAi
10 Hz - 10 minutes
0.1 Hz - 10 minutes

Figure 4

Figure 4. Synaptic transmission is largely normal under conditions of neuronal SMN modulation. (A, B) Representative traces (A) and quantification of baseline synaptic transmission at the larval NMJ (muscle 6/7 synapse at abdominal segment A2) following pan-neuronal SMN perturbation (B). mEJP amplitude (quantal size) is significantly different in SMN over-expressing synapses. However, EJP amplitudes or quantal content remain unchanged. 9-10 animals are tested for each genotype. Vertical scale bar = 10 mV for EJP and 5 mV for mEJP; Horizontal scale bar = 10 ms for EJP and 200 ms for mEJP. **(C)** High-frequency (10 Hz) stimulation in high-calcium (10 mM) containing Ringer's solution leads to gradual synaptic depression to approximately 50% of the initial EJP amplitude, followed by almost instantaneous recovery under low-frequency (0.1 Hz) stimulation. This profile is unchanged when SMN is knocked down pan-neuronally (red spheres) as compared to control animals (black spheres). Errors bars are SEM. 5 animals were tested for each genotype.

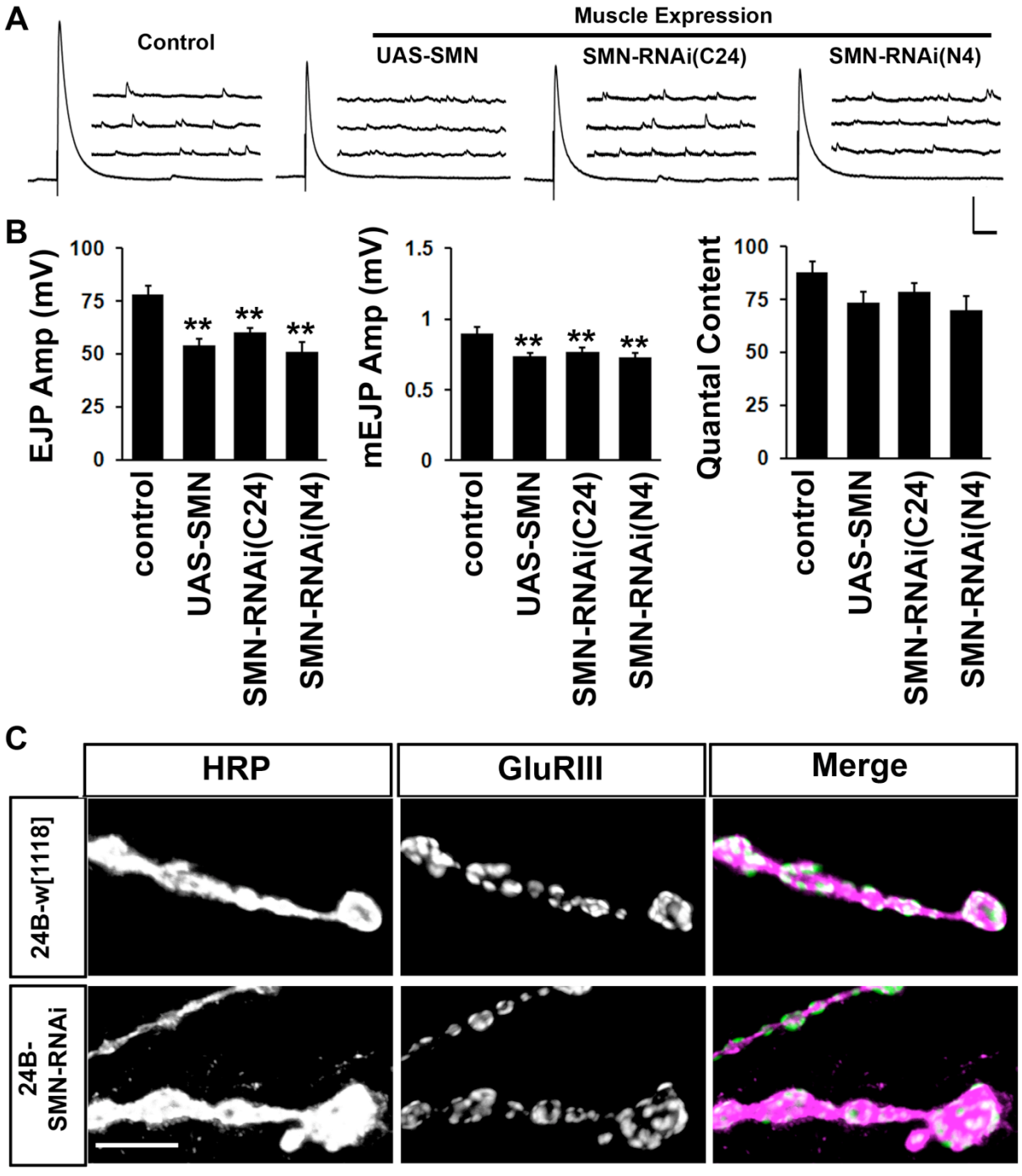
A
Control
Muscle Expression
UAS-SMN
SMN-RNAi(C24)
SMN-RNAi(N4)
B
EJP Amp (mV)
mEJP Amp (mV)
Quantal Content
control
UAS-SMN
SMN-RNAi(C24)
SMN-RNAi(N4)
**
C
HRP
GluRIII
Merge
24B-w[1118]
24B-SMN-RNAi

Figure 5

Figure 5. SMN knockdown in muscle affects the quantal size of pre-synaptic transmitter release. (A, B) Representative traces and quantification for baseline synaptic transmission at the third instar larval NMJ following muscle-specific perturbation in SMN. Both over-expression and knockdown of SMN result in smaller EJP and mEJP amplitudes. As a result quantal content is not significantly altered in experimental animals. 9-10 animals are tested for each genotype. Vertical scale bar = 10 mV for EJP and 5 mV for mEJP; Horizontal scale bar = 10 ms for EJP and 200 ms for mEJP. **(C)** Staining of third instar synapses with anti-HRP and anti-GluRIII shows indistinguishable staining intensities and pattern of glutamate receptor distribution following SMN knockdown in muscles. Scale bar = 1 mm.

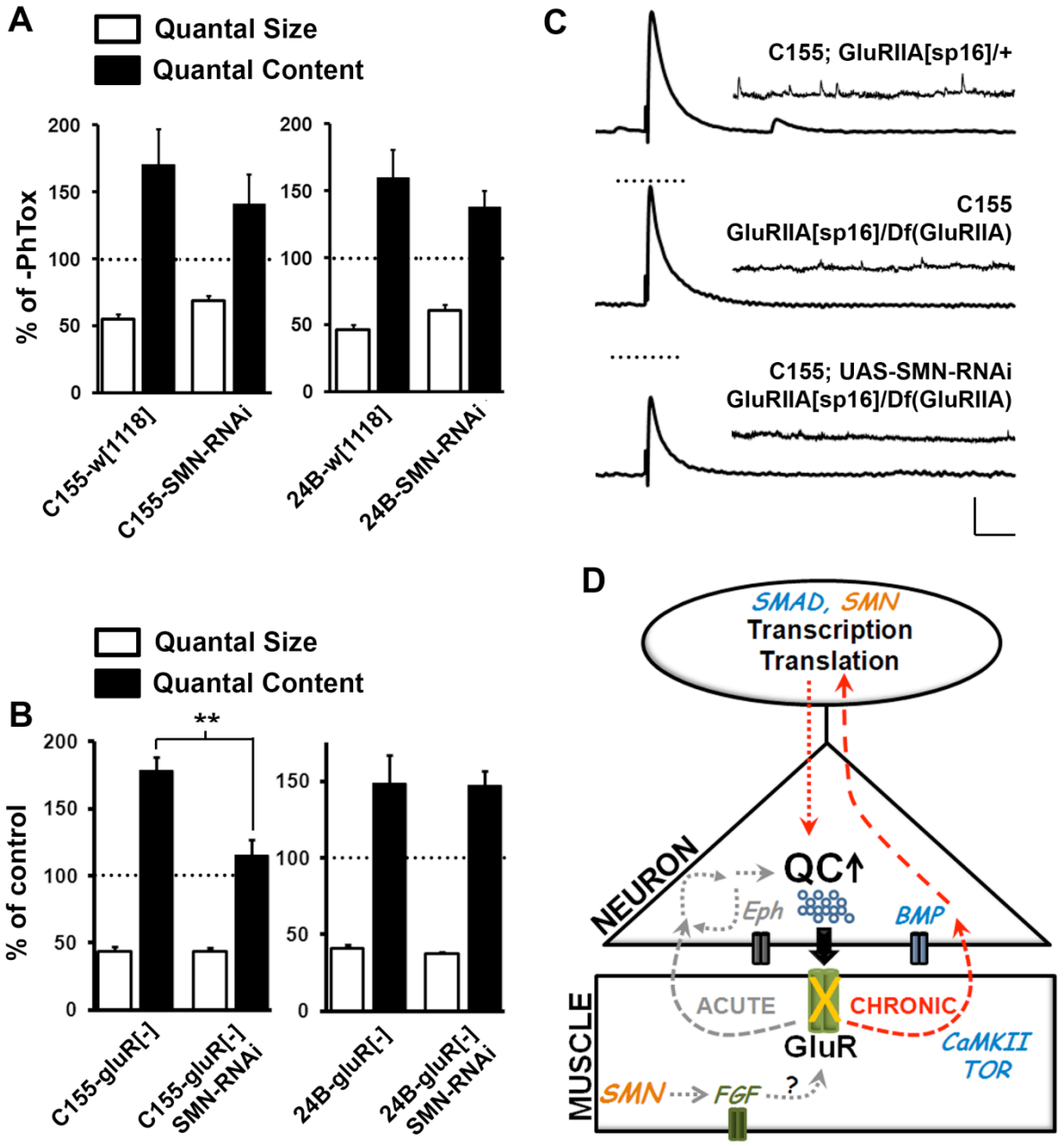
A
Quantal Size
Quantal Content
% of -PhTox
200
150
100
50
0
C155-w[1118]
C155-SMN-RNAi
24B-w[1118]
24B-SMN-RNAi
B
Quantal Size
Quantal Content
**
% of control
C155-gluR[-]
C155-gluR[-] SMN-RNAi
24B-gluR[-]
24B-gluR[-] SMN-RNAi
C
C155; GluRIIA[sp16]/+
C155 GluRIIA[sp16]/Df(GluRIIA)
C155; UAS-SMN-RNAi GluRIIA[sp16]/Df(GluRIIA)
D
SMAD, SMN
Transcription
Translation
NEURON
QC↑
Eph
BMP
ACUTE
CHRONIC
GluR
CaMKII
TOR
MUSCLE
SMN
FGF
?

Figure 6

Figure 6. Loss of SMN in neurons impairs sustained long-term homeostasis at the neuro-muscular junction. (A) PhTox induced rapid homeostatic compensation at the NMJ is normal following SMN knockdown in either neurons or muscle. Shown are the percentage reduction in mEJP amplitude (quantal size) and percentage increase in quantal content induced by a 10 minute bath application of PhTox. 10 animals were tested for each genotype. **(B)** Persistent developmental homeostasis (increased quantal content) induced by a genetic reduction in the GluRIIA subunit of glutamate receptors at the NMJ is lost when SMN is knocked down pre-synaptically in neurons. Reduction of SMN in muscles, however, does not impair this form of homeostatic compensation. **(C)** Representative traces showing the absence of homeostasis in neuronal SMN knockdown animals. 8-10 animals were tested for each genotype. Vertical scale bar = 10 mV for EJP and 2 mV for mEJP; Horizontal scale bar = 10 ms for EJP and 1 s for mEJP. **(D)** Model schematic showing a role for SMN in chronic homeostatic plasticity at the NMJ. Interestingly, this form of plasticity also requires normal BMP signaling, a pathway known to genetically interact with SMN. Chronic homeostasis is likely to rely on long-term changes in gene expression that might require normal SMN function in neurons. SMN also functions in muscles to modulate outputs of FGF signaling that control post-synaptic properties. Overall, SMN is required both pre- and post-synaptically for normal NMJ physiology.

Chapter 5

References

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