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Normal Dendrite Growth in *Drosophila* Motor Neurons Requires the AP-1 Transcription Factor

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Abstract

During learning and memory formation, information flow through networks is regulated significantly through structural alterations in neurons. Dendrites, sites of signal integration, are key targets of activity-mediated modifications. Although local mechanisms of dendritic growth ensure synapse-specific changes, global mechanisms linking neural activity to nuclear gene expression may have profound influences on neural function. Fos, being an immediate-early gene, is ideally suited to be an initial transducer of neural activity, but a precise role for the AP-1 transcription factor in dendrite growth remains to be elucidated. Here we measure changes in the dendritic fields of identified *Drosophila* motor neurons *in vivo* and in primary culture to investigate the role of the immediate-early transcription factor AP-1 in regulating endogenous and activity-induced dendrite growth. Our data indicate that (a) increased neural excitability or depolarization stimulates dendrite growth, (b) AP-1 (a Fos, Jun heterodimer) is required for normal motor neuron dendritic growth during development and in response to activity induction, and (c) neuronal Fos protein levels are rapidly but transiently induced in motor neurons following neural activity. Taken together, these results show that AP-1 mediated transcription is important for dendrite growth, and that neural activity influences global dendritic growth through a gene-expression dependent mechanism gated by AP-1.

Keywords

dendrites; *Drosophila*; AP-1; plasticity; development

INTRODUCTION

Protein synthesis dependent structural alterations in neurons are widely believed to underlie long-term plasticity, the ability of neurons to change during learning and memory formation (Bailey et al., 2004). Persistent changes in postsynaptic specializations, or dendrites, are likely to result from synthesis of new proteins derived from translation of either stored, translationally

silent mRNAs or new mRNAs derived from nuclear gene expression. In this context, nuclear transcription provides a global control of dendritic growth (West et al., 2002).

Studies of activity dependent changes in vertebrate neuronal dendrites have highlighted the role of calcium responsive signaling mechanisms (Wong and Ghosh, 2002; Konur and Ghosh, 2005). For instance, high potassium induced depolarization of cultured hippocampal neurons results in globally increased dendritic growth through a mechanism that requires the activation of CaMKIV followed by induction of the transcription factor CREB (Redmond et al., 2002). Similarly, induction of the ERK cascade also promotes CREB mediated transcription (Wayman et al., 2006). Recently, Mef-2A, a transcription factor regulated by calcineurin and p38K signaling has been shown to negatively affect dendritic growth in hippocampal (Flavell et al., 2006) and cortical (Shalizi et al., 2006) neurons.

In this study, we tested the hypothesis that the transcription factor AP-1 (heterodimers of Fos and Jun) plays an important role in regulating dendritic growth and plasticity. We focused on AP-1 because (a) Fos has previously been strongly implicated in plasticity regulation (Cole et al., 1989; Hiroi et al., 1998; Nestler et al., 2001; Sanyal et al., 2002), (b) Fos is an immediate-early gene, well-positioned to mediate a rapid genome-wide transcriptional response (McClung and Nestler, 2003; Etter et al., 2005), (c) expression of Fos is strongly regulated by electroconvulsive seizures, and general neural stimulation (Daval et al., 1989; Hope et al., 1994; Hiroi et al., 1998), and finally, (d) addiction behavior in rodents correlates very strongly with changes in Fos isoform expression in the nucleus accumbens (Hope et al., 1992, 1994; Kelz et al., 1999).

Studies of sensory neuron dendrites in *Drosophila* have uncovered many regulators of normal dendrite development (Grueber et al., 2003; Emoto et al., 2004; Parrish et al., 2006). To specifically analyze activity-dependent mechanisms of dendritic growth, we evaluated structural and functional aspects of dendrites of *Drosophila* larval motor neurons, cells that have previously been shown to display activity-dependent forms of presynaptic plasticity, and cells that receive input from presynaptic neurons (Collins and DiAntonio, 2007). We genetically labeled and manipulated identified motor neurons, to ask whether motor neuron dendrites respond structurally to neural activity and are sensitive to AP-1 dependent transcription. Our results demonstrate that (a) our experimental system is capable of detecting changes in dendrite growth *in vivo*, (b) motor neuron dendrites in *Drosophila* are plastic and are regulated by neural activity, (c) normal dendritic growth during development is dependent on AP-1 function, (d) enhancement of dendritic growth by experimental alteration of motor neuron excitability requires AP-1, and (e) depolarizing stimuli rapidly but transiently upregulates Fos expression in motor neurons. In sum, these results indicate that AP-1 is required for dendrite growth both during development and dendrite expansion induced by elevated neural activity.

MATERIALS AND METHODS

Drosophila Stocks, Culturing, and Genetics

Flies were reared on standard corn meal medium at 25°C and were grown in 12 h light-dark cycles. The C380-GAL4 line has been described previously (Budnik et al., 1996) and expresses in most motor neurons, plus other unidentified neurons. The Cha-GAL80 (Choline-acetyl transferase promoter driven GAL4) line was obtained from Dr. Toshihiro Kitamoto, and has been shown to suppress GAL4 activity in cholinergic neurons (Kitamoto, 2002). The eve^{RRK}-GAL4 line and related eve^{RRA}-GAL4 line (RRA-GAL4) were obtained from Miki Fujioka and expresses in aCC and RP2 motor neurons (Fujioka et al., 2003). UAS-GFP, UAS-FLP, act-FRT-GAL4, and *kayak* alleles were obtained from the *Drosophila* stock collection in Bloomington. UAS-Fbz, UAS-Jbz, UAS-Fos, and UAS-Jun have been described previously

(Eresh et al., 1997) and were obtained from Marianne Bienz. UAS-eag(DN) flies were from Dr. Ralph Greenspan (Broughton et al., 2004), UAS-Sh(DN) (Mosca et al., 2005), and UAS-Sh[act] (EKO) (White et al., 2001) were from Dr. Haig Keshishian and UAS-Fos-RNAi was from Dr. Dirk Bohmann (Uhlirva and Bohmann, 2006). A recombinant including both UAS-Sh(DN) and UAS-eag(DN) was made and termed “Electrical Knock In (EKI)”. UAS-nod-lacZ flies were obtained from Dr. Y.N. Jan (Clark et al., 1997) and UAS-Kinesin-GFP flies were made by Patty Estes in the Ramaswami laboratory.

Immunohistochemistry, Imaging, Sholl Analysis, and 3D Reconstruction

Antibodies—To generate anti-dFos antibodies, we scanned the *Drosophila* Fos sequence to select peptides that are unique (no close matches returned with BLAST) and highly antigenic. A 21 amino acid peptide (ERTTKKPAIRKPEDPDPAEED) was synthesized by Fabgennix, Shreveport, LA and after conjugation with KLH, injected into rabbits for antibody production. Following standard immunization and boosting protocols, the antibody was affinity purified against immobilized peptide and used in our assays. Increased Fos immunoreactivity was detected in appropriate tissues as well as in Western blots when Fos was expressed transgenically using the GAL4-UAS system. Western blots were carried out using standard protocols. NIH Image J was used to quantify protein levels in these blots.

Immunocytochemistry—Immunofluorescent labeling techniques of *Drosophila* tissue have been previously described (Sanyal et al., 2002). Tissue was dissected in PBS (milliQ H₂O + 130 mM NaCl + 5 mM Na₂HPO₄ + 5 mM NaH₂PO₄) followed by three rinses in PBS. The tissue was fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. Fixative was removed with 3 × 10 min washes of PBS and preparation placed in block (PBS + 2% bovine serum albumin + 0.1% Triton X-100 + 5% Goat Serum) for 2 h at room temperature. Block was replaced with primary antibody at appropriate dilution in 200 µL block and kept overnight at 4°C. Phalloidin-rhodamine (Molecular Probes, R-415) was used to stain peripheral muscle at a concentration of 1:1000. Anti-GFP (Molecular Probes, A-11120) was used to label the axon of motor neurons with GAL4 expression at 1:200. Anti-VGlut (Daniels et al., 2004) was used to label a vesicular glutamate transporter of neurons in culture at 1:5000. β-Gal (Promega, Z3781) was used to label nod-LacZ localization in culture at 1:50. The preparations were rinsed three times quickly, followed by 3 × 10 min washes in PBS before being placed in 500 µL secondary antibody at appropriate dilution (1:1000 used for all secondaries) for 1 h at room temperature. The secondary was washed with large volumes of TBS (PBS + 0.1% Triton X-100), 3 quick + 3 × 10 min washes with the last wash in PBS. The preparations were mounted on Superfrost Plus slides (VWR Scientific, 48311-703) in 80% glycerol + PBS and covered with a glass coverslip. The preparations were stored at −20°C until imaging.

Quantification of Fos staining in cultured motor neurons was carried out using our anti-Fos peptide antibodies (see earlier). Cells were fixed for 30 min in 4% paraformaldehyde followed by incubation in Triton X-100 containing (0.15%) blocking solution. Cells were incubated with primary antibody (1:50 dilution in block) overnight followed by washes and incubation in A-568 conjugated anti-mouse IgG (Molecular Probes, Invitrogen). Cells were mounted in Vectashield (Vector Laboratories) and imaged using a Nikon laser scanning confocal microscope. At least 10 isolated neurons were imaged from each culture dish based on strong GFP fluorescence (indicating motor neuron identity) on both red and green channels. Postacquisition processing was performed using Metamorph (Molecular Devices) and included background subtraction followed by measurement of average intensity of Fos staining in neuronal nuclei. Means from each experimental set were evaluated for significant differences using SigmaPlot (Jandel Scientific).

Imaging and 3D Reconstruction—We used the eve-flipout line for all our experiments requiring *in vivo* dendritic analysis (see Results). “Isolated” RP2 neuron containing segments were selected for imaging. Only abdominal segments 3–6 were used since the size of the neuropile varies by segment. Images were gathered such that the *z* steps matched pixel dimensions in the *x*–*y* plane to make cubic voxels for 3D reconstruction. Fluorescence intensity was normalized between samples to the intensity of the axon and the cell body. The color palette function in the Zeiss LSM program was used to ensure that images were not saturated in the axonal and dendritic regions. At these settings, the cell body, however, was routinely saturated. Several sets of experiments were performed such that controls were done simultaneously to minimize variability arising from differences in culture conditions, GFP expression and antibody staining. Imaging was always carried out double-blind such that different genotypes were interleaved. Maximum projections of AP-1 images tend to appear brighter due to a higher density of dendrites; however, single sections show comparable fluorescence across genotypes. The voltex function in AMIRA was used to construct a 3D image, followed by volume-edit to select the neuron of interest. Isosurface labeling was used to create the “shell” around the entire dendritic field. The volume contained in this shell was used as a measure of the dendritic field. All data were plotted and analyzed using SigmaPlot (Jandel Scientific) and Microsoft Excel. Statistical significance was determined using unpaired *t*-tests; an asterisk represents $p < 0.05$. All data are represented as mean $\pm$ SEM.

Sholl Analysis—Sholl analysis was performed as described previously (Sholl, 1953). Briefly, maximum projections of RP2 motor neuron images were aligned on concentric circular grids with the cell body in the center. The number of intersections of dendritic branches with successive circles were counted and plotted against the circle number (from the center outwards). Greater complexity is indicated by a higher number of intersections.

Tissue Culture Techniques and High K⁺ Protocols

Cell cultures were prepared as described previously (Kraft et al., 1998) with a few modifications. All dissection equipment was soaked in 70% EtOH for 10 min before dissection. Larvae (C380-GAL4, UAS-CD8GFP; ChaGAL80 crossed with either Oregon-R to obtain labeled control motor neurons or with various transgenic strains) were selected and sterilized by placing the animal in 70% EtOH for 1 min. The larvae were rinsed three times in sterile water before the CNS was removed in sterile *Drosophila* medium (Schneider’s medium + 10% fetal bovine serum + 50 μ g/mL insulin). The cephalic lobes were discarded and only the thoracic plus abdominal ganglia were used in cultures. The ganglia were micro-dissected to remove ventral lying GFP positive neurons that have not been identified. The ganglia were enzymatically treated in liberase for 1 h at room temperature (10 μ L 2 $\times$ liberase stock/1 mL Rinaldini’s saline [800 mg of NaCl + 20 mg of KCl + 5 mg of NaH₂PO₄*H₂O + 100 mg of NaHCO₃ + 100 mg of glucose + 100 mL of H₂O]). The ganglia were rinsed twice in 1 mL *Drosophila* Medium, triturated with a fire polished glass Pasteur pipette, further triturated with an Eppendorf tip and plated on Con-A/Laminin coated dishes in a 100 μ L bubble. The cells were plated at a density of one micro-dissected ganglion/dish. The dishes were flooded 24 h later with 1 mL *Drosophila* Medium and imaged 24 h after flooding. The cells were maintained in a 25°C, humidified incubator.

The culture dishes were made by drilling an 8-mm hole in 35-mm Petri dishes (Corning 430165). A 12-mm round coverslip (Bellco 1943–10012) was then glued under the Petri dish with Sylgard 184 to create a well. The dishes were placed under UV light for 2 h to sterilize. The well was coated with 70 μ L of ConA (Sigma C-2010) + laminin (Becton-Dickinson 354232) + milliQ H₂O and incubated for 2 h at 37°C. Dishes were then rinsed with 6 mL sterile H₂O and used within 1 month.

The motor neurons in the high K⁺ experiments were fed medium with a 10 mM increase in [K⁺] from 22 mM in normal *Drosophila* culture medium to 32 mM by the addition of KCl. This is predicted to depolarize the neurons by about 10 mV. Control cells for the high K⁺ experiments were fed medium with the osmolarity adjusted to match the treated cells. As for all experimental manipulations, treated and control cultures were paired such that cells obtained from the same dissociation were divided into treated and control dishes. This minimized variability due to animals, dish coating, media and the dissociation procedure itself. Similarly, comparisons among neurons of different genotypes involved cultures that had been prepared together.

Cycloheximide was administered to cultures to inhibit protein synthesis (Mizel and Bamberg, 1976) by adding 1×10^{-2} mg/mL to the *Drosophila* medium. *Plectreuryx* Toxin (PLTX-II, 5×10^{-2} μM, Alomone Labs) were administered to the cultures as previously described (Jiang et al., 2005). The pharmacological agent was administered to neurons after 24 h *in vitro*, and the cells were imaged 24 h later. A live cell assay was performed on cycloheximide and high K⁺ exposed cells to test their viability using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, L-3224). The culture media was washed with 500 mL of PBS and then placed in 4 M EthD-1 solution in PBS and incubated at room temperature for 20–40 min.

To selectively visualize motor neurons in culture, the C380-GAL4 driver (Budnik et al., 1996; Sanyal et al., 2003) was combined with a choline-acetyl transferase (*cha*) GAL-80 construct (Kitamoto, 2002) to suppress GAL4 activity in cholinergic neurons. This restricted GAL4 expression to a small population of dorsal motor neurons [Fig. 1(B)] and a few unidentified ventral neurons that were micro-dissected out prior to cell culture preparation. To determine which specific dorsal motor neurons were labeled with GFP, rhodamine-phalloidin staining of body wall muscles was used to visualize the peripheral innervation pattern of these neurons [Fig. 1(A)]. Muscles 1–4, 6, 7, 9, 10, 13, 15, 16, 19, and 20 were innervated in each hemisegment. We conclude that MN1-Ib (aCC; ipsilateral axon with contralateral dendritic tuft), MNISN-1s (RP2; ipsilateral axon), and MNSNb/d-1s (RP5; contralateral axon) are labeled in this line based on their central morphology and prior identification of motor neuron muscle innervation patterns (Choi et al., 2004). The strongest GFP expression was consistently observed in MNISN-1s, which is also known as RP2. For analysis, fluorescent living cells, not in contact with other neurons, were imaged at 2 days *in vitro* and the total neurite length, total branch number, and higher order branching patterns measured through digital reconstruction of the neurons [Fig. S1(G,H)]. Because one thoracicoabdominal ganglion was dissociated and plated per culture dish, there was a maximum of about 60 labeled motor neurons per dish. In practice, about 20 labeled motor neurons were present in each culture dish.

Motor neurons maintained a glutamatergic identity in culture, as they stained with a *Drosophila* vesicular glutamate transporter antibody [Fig. 1(E,F)], α-VGlut (Daniels et al., 2004). They were also polarized since the microtubule minus-end directed reporter, nod-LacZ (Clark et al., 1997), and the plus-end directed kinesin-GFP (P. Estes and M. Ramaswami, unpublished), fusion proteins were transported selectively when expressed by UAS transgenes in cultured motor neurons [Fig. 1(C,D)]. In greater than 90% of the neurons, kinesin-GFP was localized within a single primary process (i.e., a neurite that emerged directly from the cell body), whereas the remaining primary processes were marked by nod-lacZ. Similar segregation of Kinesin and Nod fusion proteins, to axons and dendrites respectively, is observed *in vivo* within *Drosophila* sensory neurons (Clark et al., 1997), mushroom body Kenyon cells (Lee et al., 2000), motor neurons (Sanchez-Soriano et al., 2005), and also within cultured Kenyon cells (Kraft et al., 2006).

Analysis of Cultured Motor Neurons

The living cells were imaged with a Hamamatsu c4742-96 camera using Wasabi software version 1.4 on a Nikon inverted scope at 60 $\times$. Both phase/contrast and fluorescent images were obtained for each cell. The motor neurons had to meet the following criteria in order to be included in the analysis: alive and healthy with no signs of lifting off the dish, bright GFP signal representing a high level of UAS expression, and isolation from neurite interactions with other neurons. The Tiff image files were analyzed by tracing the fluorescent image by hand on a Wacom tablet PC. The parameters of total neurite length, total branch number, higher order branching patterns, and cell body circumference were recorded using Simple PCI software. All data were plotted and analyzed using SigmaPlot (Jandel Scientific) and Microsoft Excel. Statistical significance was determined using unpaired *t*-tests; an asterisk represents $p < 0.05$. All data are represented as mean $\pm$ SEM.

Electrophysiology

Cultures were prepared as described from third instar larvae. Whole cell current or voltage clamp recordings were obtained from GFP-labeled motor neurons with patch pipettes that were pulled from borosilicate glass (Friedrich and Dimmock, Millville, NJ). Similar results were obtained using different driver lines, although as indicated in the text, the baseline level of excitability varied consistently among lines. Therefore, for each of the transgenic manipulations described, comparisons are made with the appropriate driver line as control. An Axopatch 1D amplifier, Digidata 1320 interface, and PClamp software (Axon Instruments, Sunnyvale, CA) were used for data acquisition and analysis. The extracellular solution included (mM): NaCl, 140; KCl, 3; CaCl₂, 1; MgCl₂, 4; HEPES, 5. The intracellular pipette solution (modified from Jiang et al., 2005), included: potassium gluconate, 120; NaCl, 20; CaCl₂, 0.1; MgCl₂, 2; HEPES, 10; EGTA 1.1. Whole cell recordings from RP2 motor neurons *in vivo* were obtained using previously published methods (Choi et al., 2004). The intracellular solution also included a low concentration of rhodamine-dextran, which allowed positive identification of motor neuron identity based upon dendritic morphology.

RESULTS

Direct Measurement of Motor Neuron Dendritic Fields in the Central Nervous System

We adapted a strategy used previously in developmental studies to reproducibly label individual or small subsets of motor neurons optically isolated from their neighbors in the CNS.

The *eve*-GAL4^{RRK} line expresses in aCC and RP2 neurons in the dorsal medial motor neuron cluster (Fujioka et al., 2003). The expression of this GAL4 line is strongest in embryos, and by larval stages a single copy of this transgene does not drive GFP above levels of detection. We combined this GAL4 line with three other transgenes; UAS-Flp (a source of the recombinase, flippase), an actin promoter separated from a GAL4 by a spacer DNA fragment flanked by two FRT sites (target sites for the Flp recombinase), and a UAS-mCD8-GFP reporter [Fig. 2(A)]. Larval central nervous systems from our test line showed a mosaic pattern of GFP expression, owing to chance flip-out events in aCC and RP2 neurons during early embryonic stages [Fig. 2(B)]. RP2 and aCC neurons can be easily distinguished based on their dendritic field [Fig. 2(C,D)] (Choi et al., 2004) and peripheral innervation [Fig. 2(E–H)]. We selected segments where RP2 dendrites (owing to their simpler morphology) can be imaged cleanly without conflicting labeling from other neurons in adjoining segments [Fig. 2(B), I; see materials and methods]. After generating a series of images representing the entire thickness of the motor neuron using laser scanning confocal microscopy, we reconstructed the dendritic field using AMIRA (Vortex function). We then created an external bounding surface that tightly enclosed all the dendrites [Fig. 2(I)]. The internal volume contained in this shell was consistent across samples and sensitive enough to detect small changes in dendritic outgrowth (roughly

10%). These results demonstrate the utility of this simple technique which allows for the measurement of dendritic fields in 3D space.

A key advantage of this scheme is that it allows the expression of transgenes of interest in selectively labeled motor neurons. Thus, *Drosophila* Fos (dFos, *kayak*) expression via this “flipout” system causes robust overexpression of Fos in all GFP-labeled neurons [Fig. 2(J–L), arrowheads]. This allowed us to test the effect of altering protein function in targeted neurons without changing the surrounding milieu [compare Fos staining intensity in neurons overexpressing AP-1 (arrowhead) with neuron displaying endogenous lower levels of Fos (arrow) in Fig. 2(K)]. This is particularly relevant since loss-of-function mutations in both Fos and Jun often result in pleiotropic phenotypes and are typically lethal. Selectively altering protein function in these neurons largely overcomes these experimental limitations.

AP-1 Dependent Transcription Modulates Dendrite Growth

AP-1 is normally expressed by larval motor neurons [note detectable nuclear staining marked by an arrow in non-GFP expressing neurons in Fig. 2(K)]. To test if *Drosophila* motor neuron dendrites are regulated by AP-1 dependent transcription, we asked how dendritic growth was affected following inhibition or overexpression of AP-1 components (Fos and Jun) in RP2 neurons. Figure 3(A) summarizes this set of experiments and shows that AP-1 positively regulates dendrite growth *in vivo*. Although overexpression of AP-1, but not Fos or Jun alone, increased dendrite volume by 92%, inhibition of AP-1 by expressing a dominant-negative Fos or Jun (the bZip domain of either dFos or dJun; Fbz or Jbz) significantly reduced dendrite volume as compared to control animals. The nervous systems remained functional in these animals. Chronic pan-neuronal expression of Fbz or Jbz did not noticeably affect action potential production or synaptic transmission in the CNS (see supplementary data and Fig. S2).

To compare our method of volume measurement with well established techniques for assessing neuronal branching, we performed Sholl analysis (Sholl, 1953) on images gathered from three test genotypes, wild-type controls, animals expressing wild-type AP-1 protein in RP2 neurons, and those expressing dominant-negative Jun (Jbz) protein [Fig. 3(C)]. These measurements suggest that although maximum projections of these motor neurons do not allow easy reconstruction of dendrite branches, we are conservatively able to detect significant differences between test genotypes. Thus, expression of wild-type AP-1 proteins in these neurons led to an expansion of the dendritic field [Fig. 3(D)]. Conversely, AP-1 inhibition using the bZip only dominant-negative Jbz construct produced fewer dendrites.

As shown in Figure 3(A), established and newly isolated loss-of-function alleles of dFos show significantly reduced RP2 dendritic fields. It is noteworthy that both *kay*² and *kay*⁰⁸²³² are lethal alleles and affect motor neuron dendrites when heterozygous over a wild-type Fos allele [Fig. 3(A)]. *kay*¹²⁷¹⁰, though inserted close to the Fos gene, is not expected to affect Fos expression since the insertion is far upstream of the Fos transcription start site. Finally, expression of a validated Fos-RNAi construct (Uhlirova and Bohmann, 2006) in RP2 neurons also results in dendritic fields that are 41% of controls [Fig. 3(A)]. Taken together these results strongly suggest that endogenous AP-1 mediated transcription influences dendritic morphology in motor neurons.

The cell autonomy of AP-1 function for regulating dendritic plasticity, indicated by *in vivo* analyses of mosaic animals, is supported by parallel studies *in vitro*, which additionally allow direct measurements of branch number in AP-1 perturbed neurons. Results from overexpressing or inhibiting AP-1 in cultured motor neurons are consistent with *in vivo* findings Figure 3(B). Thus, AP-1 increased total neurite length by 80% while Fbz strongly reduced neurite length. Expression of Jbz did not reduce total neurite length significantly in this assay. We speculate that this is probably due to either insufficient inhibition achieved with the C380-

GAL4; Cha-GAL-80 system that was used to drive expression for cell culture experiments as compared to the strong actin-GAL4 *in vivo*, or due to a weaker dominant-negative effect of the Jbz protein. Both branch number and total length were increased by AP-1 perturbation, suggesting AP-1 dependent changes in growth and complexity of neurons *in vitro* [Fig. 3(B), gray bars]. These results suggest that effects of AP-1 on dendrite growth derive from autonomous action in motor neurons, and are not dependent for instance, on altered retrograde signaling from postsynaptic cells.

Experimental Strategies to Influence Neural Activity in Motor Neurons *In Vivo* and *In Vitro*

AP-1 expression is commonly induced by increased neuronal activity. To develop models of activity driven dendrite growth and test if AP-1 is required in this paradigm, we used composite GAL4 lines mentioned earlier (RN2-flipout, RRA-GAL4, and C380-Gal4; ChaGal80) to drive transgenic modifiers of neural excitability or activity in GFP labeled motor neurons *in vivo* and *in vitro*. We attempted to increase motor neuron activity by coexpressing both *Shaker* and *ether-a-go-go* dominant-negative transgenes (*UAS-Sh(DN)*, *UAS-eag(DN)*) which reduce potassium currents (Broughton et al., 2004; Mosca et al., 2005). The *Shaker* dominant-negative transgene causes increased branching of motor axon terminals when expressed in an *ether-a-go-go* mutant background (Mosca et al., 2005), as previously reported in *Shaker/ether-a-go-go* double mutants (Budnik et al., 1990). For our experiments we used a recombinant chromosome termed “EKI” (for electrical knock-in), which included both transgenes.

Identified motor neurons showed increased excitability in EKI larvae. Motor neurons recorded *in situ* from third instar larvae express sodium, potassium and calcium currents (Worrell and Levine unpublished observation; 2007, SFN Abstract). The resting membrane potentials of EKI and control motor neurons were not different (EKI: -61.8 mV; $n = 8$; Control: -61.5 mV; $n = 8$). Neither control nor EKI expressing motor neurons produced action potentials spontaneously during recordings. Upon injection of depolarizing current, however, TTX-sensitive action potentials were produced. Motor neurons in EKI larvae had similar action potential thresholds but significantly higher action potential frequency in response to the injection of positive current, as compared to the same identified (RP2 and aCC) motor neurons in control larvae [Fig. 4(C,D)]. The postspike hyperpolarization was smaller in EKI larvae, often resulting in spike doublets. In addition, the characteristic delay to first spike, which is a consistent feature of RP2 (Choi et al., 2004), was reduced in EKI larvae. RP2 and aCC motor neurons expressing two copies of the “electrical knock-out” transgene (EKO) (White et al., 2001), encoding a modified *Shaker* channel lacking the inactivation domain, had similar resting membrane potentials to control motor neurons (EKO: -59.7 mV; $n = 7$; Control: -59.6 mV; $n = 5$). The action potential thresholds of EKO motor neurons were also similar to control. In general, fewer action potentials were evoked with the injection of depolarizing current into EKO expressing motor neurons, but the differences from control were not significant [Fig. 4(A,B)]. Note that different driver lines were used for the EKI and EKO motor neuron physiology experiments to facilitate GFP labeling (see Figure legend).

To confirm that the effects of EKI and EKO transgene expression reflected intrinsic motor neuron excitability rather than altered synaptic drive, whole cell recordings were also obtained from isolated larval motor neurons in cell culture. The resting membrane potentials of the control motor neurons were somewhat depolarized and variable *in vitro*, relative to those measured *in vivo*, probably reflecting damage inflicted by recording from the fragile cells. The mean resting potential for control motor neurons was -27 mV (range, -20 to -37 mV; $n = 18$). The resting potentials cultured motoneurons of transgenic animals were similar to control (EKI: mean, -21 mV; range -20 to -35 mV, $n = 11$; EKO: mean, -23 mV; range -10 to -44 mV, $n = 15$). In voltage-clamp experiments cultured motor neurons from control and EKI larvae expressed potassium currents at 2.5 and 4 days *in vitro* [Fig. 5(A)]. In both control and EKI

motor neurons unclamped, TTX-sensitive events were observed after 4 days *in vitro*, with the frequency of these events being higher in EKI motor neurons [Fig. 5(A), arrows]. As with control motor neurons, no action potentials were produced spontaneously in EKI motor neurons at 4 days. Unlike control, however, in which none of the motor neurons produced action potentials when depolarized in current clamp mode at day 2.5, 33% of the EKI motor neurons recorded at 2.5 days ($n = 24$) already produced action potentials. By day 4 *in vitro*, 91% ($n = 11$) of the EKI motor neurons were capable of spiking as compared to 60% ($n = 18$) of the control cells. By contrast, EKO caused the expression of large voltage-dependent potassium currents in motor neurons [Fig. 5(A)]. None of the EKO-expressing motor neurons produced action potentials at 2.5 days *in vitro*, and only 27% ($n = 15$) were capable of spiking at 4 days.

Neural Activity Stimulates Motor Neuron Dendrite Growth and Requires AP-1 Function

Given that motor neuron excitability could be influenced as predicted by transgene expression, we next tested whether the effects of AP-1 on dendritic growth *in vivo* could be mimicked by EKI expression. The dendritic field volumes of RP2 motor neurons were significantly increased *in vivo* in EKI larvae [Fig. 6(A)]. By contrast, expression of EKO did not influence dendritic growth significantly [Fig. 6(A)]. To help confirm that the effects of EKI expression reflected a change in intrinsic cell excitability, parallel experiments were performed in cell culture. Motor neurons cultured from EKI larvae displayed increased growth and branching *in vitro* [Fig. 5(B)]. As with the cells measured *in vivo*, however, EKO expression did not alter growth or branching of cultured motor neurons [Fig. 5(B)].

An alternative strategy that is commonly used to depolarize cultured neurons is to increase the potassium concentration in the culture medium (Aizawa et al., 2004; Green et al., 2007). Larval motor neurons in cultures that were exposed to elevated extracellular K^+ for 24–48 h *in vitro* (10 mM increase in $[K^+]$), remained viable [supplementary Fig. S1(A–F)] and increased neurite length and branching significantly as compared to control cells maintained for 48 h in normal medium [Fig. 5(B–D)]. Administration of the translational inhibitor cycloheximide to motor neuron cultures reduced both basal and induced levels of neurite growth, indicating the necessity for protein synthesis in motor neuron growth and branching *in vitro* [Fig. 5(C)]. It is likely that the induced growth reflected the dendritic compartment of motor neurons *in vitro*. Whereas the number of primary processes arising from the cell body was increased, the number of neurons with more than one kinesin-labeled primary process was not. Moreover, there was no increase in the total length of kinesin labeled primary processes and their higher-order sub-branches, whereas the total length of the non-kinesin labeled processes increased with high K^+ exposure [Fig. 5(D)].

Results reported in the previous sections demonstrate separately that dendritic growth in *Drosophila* motor neurons is responsive to neural activity and requires AP-1 function. Is the activity-driven component of dendrite growth dependent on AP-1? A model in which AP-1 is a major mediator of activity-dependent growth makes three predictions. First, neural activity dependent growth should be suppressed by AP-1 inhibition; second, elevation of AP-1 activity might promote growth even under depressed neural activity and third, AP-1 or its constituents, Fos and Jun might be rapidly regulated following changes in neural activity. We describe experiments below that test and confirm these predictions.

To ascertain if AP-1 is required for activity-dependent growth of motor neuron dendrites, we inhibited AP-1 function by coexpressing UAS-Fbz in neurons expressing eag (DN) and Sh (DN) channel subunits (EKI). Under normal conditions, EKI results in dramatic growth of dendrites *in vivo*, but AP-1 inhibition completely abolished this activity-induced growth [Fig. 6(A)]. Indeed, dendritic volume was statistically indistinguishable in EKI-Fbz and Fbz animals (Fbz: $61\% \pm 6$ and EKI-Fbz: $68\% \pm 9$, as compared to wild-type controls). The observed genetic epistasis of Fbz indicates that AP-1 function is essential for neural activity induced dendritic

growth. *In vitro* experiments using high potassium stimulation also supported this conclusion [Fig. 6(B)]. Fbz blocked high-K⁺ driven dendritic growth. Jbz, a weaker inhibitor of AP-1, had a similar, though smaller effect on depolarization-induced growth. These results are consistent with the idea that neural activity stimulates AP-1 dependent transcription to promote dendrite growth.

Our working model also predicts that experimentally induced increases in neural activity may have no further effect on dendritic growth in AP-1 overexpressing neurons. To test this prediction, we asked how high potassium stimulation affected dendritic growth in AP-1 overexpressing motor neurons. Figure 6(B) shows that adding high potassium to AP-1 expressing motor neurons *in vitro* did not cause any additional growth. Thus, our data argue that AP-1 overexpression stimulates a physiologically relevant pathway normally recruited for activity-dependent dendritic expansion.

A potential mechanism by which neural activity might lead to increased AP-1 activity is through increased calcium entry. To test if increased calcium currents are responsible for dendritic growth seen in EKI expressing neurons, we inhibited voltage-gated calcium channels using *Plectreurys* toxin, PLTX. PLTX has been shown previously to inhibit some insect voltage-gated calcium channels (Jiang et al., 2005). We chose EKI over highK stimulated cultures for these experiments since we could dependably observe depolarization mediated changes in motor neurons both *in vitro* and *in vivo*. When added to normal cultures, PLTX inhibited neurite growth consistent with a role for calcium currents in mediating growth under basal conditions. Interestingly, PLTX completely prevented EKI mediated neurite outgrowth [Fig. 6(C)]. This suggests that elevation of calcium currents in EKI neurons is required for increased neurite outgrowth *in vitro*. We next tested if AP-1 expression can bypass the need for increased neural activity. Indeed, when AP-1 expressing motor neurons were treated with PLTX *in vitro*, they demonstrated robust outgrowth comparable to untreated AP-1 neurons (AP-1: 180%; AP-1+PLTX: 173% as compared to controls) [Fig. 6(C)]. Further, neurons in the same culture dish not expressing AP-1 (distinguishable due to lack of GFP expression) displayed reduced growth, similar to PLTX treated control cells. These results indicate that even under conditions of reduced calcium entry, AP-1 dependent transcription is capable of generating a genomic response that drives dendrite growth. Taken together, these results strongly suggest that EKI mediated depolarization of motor neurons increases calcium entry through PLTX-sensitive calcium channels, likely followed by the induction of signaling mechanisms that finally enhance AP-1 activity leading to dendrite growth. The essential elements of this model are depicted in Figure 6(F).

To explore potential mechanisms by which neural activity might influence AP-1 function, we tested levels of Fos protein in stimulated versus control neurons. We focused on Fos because it is a well-studied immediate early gene and our experiments consistently showed more severe effects of Fos inhibition both *in vivo* and *in vitro*. We raised and tested an antibody against a *Drosophila* Fos derived peptide. This antibody faithfully detects increased Fos protein achieved through GAL4 driven Fos overexpression. As shown in Figure 6(D), increased nuclear Fos is detected in dorso-medial motor neurons in the larval ventral ganglion (arrow) as expected from C380-GAL4 driven expression of UAS-Fos. Similarly, we were able to detect a band of the predicted molecular weight in western blots from adult head protein extracts. In these experiments, we also confirmed increased expression of Fos when AP-1 was expressed pan-neuronally using the C155-GAL4 driver. Next, using this antibody, we tested if acute induction of neural activity alters Fos levels in motor neuron nuclei. We stimulated cultured motor neurons with high potassium containing saline and measured Fos immunoreactivity following 0, 4, and 20 h of treatment. Figure 6(E) shows rapid induction of Fos within 4 h. Prolonged treatment with high potassium saline did not cause an increase in Fos staining,

presumably indicating that protein levels return to baseline with maintained stimulation. These results support a model where neural activity promotes dendrite growth by upregulating Fos.

DISCUSSION

Like CREB (Redmond et al., 2002), AP-1 (Fos and Jun dimer) is likely to be an important activity-dependent regulator of dendritic plasticity. Fos is upregulated rapidly in neuronal populations following activity (Cole et al., 1989; Kimpo and Doupe, 1997; Sgambato et al., 1997; Filipkowski et al., 2000; Strekalova et al., 2003; Svarnik et al., 2005) and can generate a rapid genomic response to incoming stimuli since it is an immediate-early gene not requiring protein synthesis for its own induction (Greenberg et al., 1986). At the *Drosophila* NMJ AP-1 controls both structural and functional aspects of long-term plasticity (Sanyal et al., 2002, 2003).

To test if AP-1 plays a role in activity-dependent dendritic plasticity, we first established experimental systems to study dendrites of the RP2 motor neuron *in vivo* in the larval CNS and complemented this with an *in vitro* culture system using a strategy to enrich for larval motor neurons. This dual analysis utilizes technical strengths of both systems and permits cross-validation of results. Results reported in this study support three main conclusions: (a) motor neuron dendrites in *Drosophila* show activity, or depolarization, dependent plasticity, (b) normal dendrite growth requires AP-1, and (c) activity or depolarization driven dendritic growth is gated by AP-1.

Motor Neuron Dendritic Growth Is Activity-Dependent

In the absence of prior demonstration, it was important to initially establish that motor neuron dendrites in *Drosophila* display robust changes in response to neural activity. We used both pharmacological and genetic manipulations to alter neural activity *in vivo* and *in vitro*. As shown in Figure 5(C,D), maintaining cultured motor neurons in high K⁺ medium enhanced the growth of neurites that have the characteristics of dendrites. Expression of dominant-negative subunits of the potassium channels *eag* and *Sh*, increased excitability and caused increased dendrite growth both *in vivo* and *in vitro* (Fig. 4–Fig 6). These experiments suggest that a conserved mechanism of plasticity operates in these motor neurons to regulate dendritic growth. Additionally, by developing the first assay for activity-stimulated dendritic growth in *Drosophila*, we now enable a wide range of experiments to further dissect underlying signaling and cell biological pathways.

It is worth noting that the cell culture approach allowed the comparison of acute depolarization (high potassium) with the more chronic alteration of excitability caused by the expression of *eag* and *shaker* dominant-negative transgenes. Despite the interesting possibility of compensatory changes following long-term transgenic manipulations, the functional properties of larval motor neurons *in vivo* were stably altered by reducing potassium channel function. By contrast, the effects of elevated potassium reflect an acute manipulation after cells are placed in culture. In spite of these differences the effects of EKI expression on cell growth *in vitro* were similar to those induced by elevated potassium. The lack of an effect on outgrowth by EKO expression may reflect a cellular compensatory mechanism. Although effects on cell physiology were detected, the difference in excitability measured *in vivo* was not significant. Two alternatives are that there is a basal level of normal growth that is not activity-sensitive, or that reduced activity had an influence on growth or branching that our analysis did not detect.

We suggest that the depolarization by high potassium or the increased excitability caused by EKI expression would lead to an increase in calcium influx through voltage-dependent channels (Berke and Wu, 2002; Jiang et al., 2005; Peng and Wu, 2007). The voltage-dependent properties of *Eag* and *Shaker* potassium channels (Wu et al., 1983), suggests that EKI

expression would not necessarily be expected to alter the resting membrane potential. Even without altering the resting membrane potential, however, the reduction in voltage-dependent potassium currents might allow increased calcium influx in response to spontaneous depolarization or calcium waves (Jiang et al., 2005). This would be augmented, *in vivo*, by the higher action potential frequency evoked by depolarization of motor neurons in EKI larvae as compared to wild type. It is also possible that the expression of eag-DN alters a modulatory function of the potassium channel subunit (Zhong and Wu, 1993; Sun et al., 2004). Consistent with this hypothesis, we find that inhibiting PLTX-sensitive calcium channels prevents EKI mediated neurite outgrowth. A similar dependence of neuronal growth on calcium channels has been demonstrated convincingly in the *Drosophila* giant neuron culture system (Peng et al., 2007). Ultimately, it will be important to establish the specific parameters of activity and calcium flux that are essential for modulating the intracellular signals that mediate growth plasticity (Fields et al., 1997; Zhao et al., 2007).

Dendrite Growth Is AP-1 Dependent

We have shown previously that AP-1 regulates synaptic plasticity at *Drosophila* motor terminals (Sanyal et al., 2002, 2003). Our present results uncover a role for AP-1 in regulating dendritic growth. Reduction of AP-1 activity decreases, while enhancement of AP-1 increases, dendritic outgrowth *in vivo* and *in vitro* (see Fig. 3). These conclusions are further strengthened by the fact that known and predicted loss-of-function alleles of *kayak* (DFos) also reduce dendrite volume significantly, when present in heterozygous combinations with wild-type alleles. It is to be noted that these mutations in Fos are homozygous lethal at early developmental stages, and transgenic strategies for tissue specific genetic manipulations are especially useful in these contexts. Additionally, multiple Fos isoforms have been reported, and using a dominant negative construct offers a good way to inhibit these multiple Fos proteins (Giesen et al., 2003). In conclusion, the observations that (a) Fos is normally detectable in motor neuron nuclei, (b) Fbz, Jbz and Fos-RNAi inhibit and AP-1 increases dendrite growth, (c) loss-of-function alleles of *kayak* decrease dendrite volume, and (d) a variety of controls validate the genetic perturbations used in our study, strongly suggest that AP-1 functions physiologically to control dendrite growth.

Activity-Dependent Dendritic Plasticity Requires AP-1

To test if neural activity driven growth requires AP-1 dependent transcription, we inhibited AP-1 function in a background of elevated neural activity. Figure 6(C) indicates that neural activity induced dendrite growth is completely abolished *in vivo* by the coexpression of Fbz. Further, *in vitro* experiments with High K⁺ induced dendrite growth show that expression of either Fbz or Jbz substantially reduces the extent of depolarization induced growth. Our results also indicate that the extent of overgrowth seen through AP-1 induction cannot be increased any further by High K⁺ induced depolarization [Fig. 6(B)]. Furthermore, calcium channel inhibition by PLTX toxin does not preclude AP-1 driven neurite growth, suggesting that AP-1 functions downstream of neural activity and calcium entry to enhance neurite growth [Fig. 6 (D)]. These results are consistent with the idea that AP-1 is a major contributor to activity-induced plasticity of dendrites.

Finally, to ascertain the mechanism by which neural activity might influence AP-1 function, we tested expression of Fos protein following acute induction of activity in cultured motor neurons. As depicted in Figure 6(E), Fos protein levels are increased by ~35% as compared to uninduced controls. This induction is rapid, occurring within 4 h, but transient, as Fos levels return to baseline during 20 h of stimulation. Although modest, this change in cellular Fos levels is consistent with previous observations (Morgan et al., 1987) and suggests, though it does not prove, a model where neural activity recruits AP-1 possibly through synthesis of new Fos protein, to promote dendrite growth. Hence, our results confirm that AP-1, positively

regulates plasticity in postsynaptic compartments, as demonstrated earlier for presynaptic terminals (Sanyal et al., 2002), and establishes it as a key component in activity-dependent neuronal plasticity.

A key goal for future studies must be to determine how neural activity is translated into transcription factor activity (Fields et al., 1997; Zhao et al., 2007). Calcium dependent signaling is arguably the most important pathway for the activation of both CREB and Mef2A, two transcription factors that influence plasticity in opposite ways (Redmond et al., 2002; Flavell et al., 2006; Shalizi et al., 2006), as well as AP-1 (Zhao et al., 2007). A second question is that of interaction between transcription factors. Transcriptional regulation of Fos by CREB (and *vice versa*) has been described in some detail, but the temporal sequence in which these proteins function or their relative importance in plasticity have not been assayed rigorously in the same preparation (Liu and Graybiel, 1996; Kang et al., 2001; Sanyal et al., 2002; McClung and Nestler, 2003). Finally, further experiments to identify the downstream targets of these transcription factors, will enable the description of a composite transcription factor network that mediates protein synthesis during long-term neural plasticity.

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Figure 1.

Motor neuron cultures from thoracic-abdominal ganglia of third instar *Drosophila* larvae. (A) Immunocytochemistry of axons (GFP) and staining of body wall muscles (rhodamine-phalloidin) reveals the peripheral innervation pattern (see arrows at sites of some of the synapses) in the C380-GAL4,UAS-CD8-GFP;chaGAL80 line. Muscles 1–4, 6, 7, 9, 10, 13, 15, 16, 19, and 20 are innervated in each segment. We conclude that MN1-Ib (aCC innervating muscle 1), MNISN-1s (RP2 innervating muscles 1–4, 9, 10, 19, and 20) and MNSNb/d-1s (RP5 innervating 6, 7, and 13) are labeled in this line. (B) C380-GAL4,UAS-CD8-GFP;chaGAL80 drives expression in a subset of identified late third instar *Drosophila* motor neurons as seen in this dorsal confocal projection. (C, D) The polarity of motor neurons can be detected *in vitro* with the use of microtubule associated motor reporter proteins. (C) UAS-nod-LacZ causes expression of a minus end directed fusion protein that localizes in all processes except one dominant process (see blue arrows; left = phase; right = fluorescence). (D) UAS-kinesin-GFP encodes a plus end directed motor protein that congregates in one major process of most motor neurons *in vitro* (see red arrows). (E, F) GFP positive neurons in culture (E) are glutamatergic, in that they stain positive with the VGlut antibody (F).

Figure 2.

Scheme to measure dendrite volume of RP2 motor neurons *in vivo*. (A) A schematic describing the “flipout” strategy used to generate somatic mosaics enabling the visualization of aCC and RP2 motor neurons in the larval ventral ganglion. Production of flippase driven by expression of the *eve*-GAL4 line in embryonic stages excises the spacer DNA and brings the actin promoter in proximity to a GAL4 gene. This postmitotic event randomly converts aCC and RP2 neurons into act-GAL4 cells. (B) A representative mosaic ventral ganglion showing selective labeling of neurons (marked with anti-elav) and cd8-GFP based visualization of dendrites. aCC neurons can be unambiguously differentiated from RP2 neurons based on dendritic branching and peripheral muscle innervation (C, E, and F for RP2 and D, G, and H for aCC). (I) Dendrites of optically isolated neurons can be reconstructed using AMIRA and the volume occupied in space can be measured as the internal volume of a “shell” that encloses the entire dendritic field (silver in cut-away shows internal surface). (J, K, L) These images show that transgenic Fos expression using the flipout system overlaps with cd8-GFP mediated labeling of cells. Anti-Fos antibodies also demonstrate that high degree of Fos expression is achieved by the actin-GAL4 that becomes active in these neurons (arrowhead) as compared to baseline expression of Fos in other surrounding neurons (arrow). Scale bar in L is 50 μm.

Figure 3.

AP-1 regulates dendrite growth in *Drosophila* motor neurons. (A) *In vivo* experiments that either inhibit or activate AP-1 function in selected motor neurons. Inhibition of AP-1 using UAS-Jbz, UAS-Fbz, or UAS-Fos-RNAi inhibits dendrite growth. The same effect is seen using loss-of-function mutations in *kayak* (dFos) that are heterozygous over a wild-type allele. Overexpression of AP-1 protein in these neurons (but not of either Fos or Jun alone) has the opposite effect of enhancing dendrite growth as compared to control animals. (*kay*²: 76% ± 5, *p* < 0.05; *kay*⁰⁸²³², a P-element insertion allele in the middle of the Fos gene: 74% ± 8, *p* < 0.05; *kay*¹²⁷¹⁰, a P-element insertion 6 kb upstream of the Fos gene: 101% ± 6, *p* > 0.1; AP-1: 192% ± 21; *p* < 0.01; Fbz: 61% ± 6; *p* < 0.01; Jbz: 54% ± 4; *p* < 0.01). Number of samples are control = 12; Jbz = 7; Fbz = 6; Fos-RNAi = 6; *kay*[2] = 11; *kay*[08232] = 8; *kay*[12710] = 8; Fos = 5; Jun = 5; AP-1 = 7. (B) *In vitro* experiments that reduce or increase AP-1 in motor neurons also show effects on growth. Fbz expressing neurons show reduced neurite length while the opposite is achieved with AP-1 overexpression. The effect of Jbz expression was not significant. (AP-1: 180% ± 18, *p* < 0.01; Fbz: 35% ± 3, *p* < 0.01; Jbz: 85% ± 7, *p* > 0.1). Similar results are shown for total number of branches. Number of cells counted are control = 16; AP-1 = 28; Jbz = 23; Fbz = 25. (C, D). Sholl analysis indicates that expression of AP-1 increases,

whereas AP-1 inhibition reduces dendritic complexity of RP2 motor neurons *in vivo*. (C) Sholl analysis technique used to measure dendritic complexity of RP2 neurons. The cell body is placed at the center and the number of times dendrites intersect with the circular lines is plotted against the circle number. (D) Sholl analysis plot shows that AP-1 expression in RP2 neurons results in greater complexity, while AP-1 inhibition through expression of Jbz reduces complexity as compared to wild-type controls. ($n = 6$ for each genotype). Sholl analysis is performed on the same data set used in (A) Sample single section confocal images demonstrate that AP-1 expression leads to greater dendrite growth whereas AP-1 inhibition results in fewer dendrites. All images were acquired using constant settings and normalized for fluorescence intensity in the soma and axon. Samples were scored blind and interleaved during acquisition.

Figure 4.

Genetic manipulation of motor neuron activity *in vivo*. (A) Whole cell current clamp records obtained *in situ* (*in vivo*) from identified aCC motor neurons in control and EKO expressing third instar larvae. Traces show the responses to positive current pulses 400 ms in duration; 20, 50, and 100 pA in amplitude. Note that more action potentials were evoked in the control neuron. Similar results were obtained for RP2 motor neurons. (B) Number of action potentials evoked in aCC motor neurons during 400 ms depolarizing current pulses of different amplitudes. Standard error bars are shown ($n = 5$ for both control and EKO). Similar results were obtained for RP2 motor neurons. The differences between EKO and control were not significant. For these experiments, RRA-GAL4 was used to drive EKO and GFP expression in RP2 and aCC. Cells were identified by GFP expression and confirmed by injection of rhodamine-dextran through the recording patch pipette. The RRA-GAL4; UAS-GFP line was used as control. Similar results were obtained by driving EKO with RN2-flp or C380; chaGAL80 lines, although the number of action potentials generated for a given current level was less for the C380;chaGAL80 control line (see C,D). (C) Whole cell current clamp records obtained *in situ* (*in vivo*) from identified aCC motor neurons in control and EKI expressing third instar larvae. Traces show the responses to positive current pulses 400 ms in duration; 20, 50, and 100 pA in amplitude. Note that fewer action potentials were evoked in the control neuron. Arrows denote clear differences in spike number. Similar results were obtained for RP2 motor neurons. (D) Number of action potentials evoked in aCC motor neurons during 400 ms depolarizing current pulses of different amplitudes. Standard error bars are shown ($n = 3$ for EKI and 6 for control). Asterisks indicate current injection levels for which the differences were significant. Similar results were obtained for RP2 motor neurons. For these experiments,

C380; chaGAL80 was used to drive EKI expression in RP2 and aCC. Cells were identified by a GFP tag carried by the shaker dominant-negative transgene and confirmed by injection of rhodamine-dextran through the recording patch pipette. The C380; chaGAL80 line was used as control. Similar results were obtained by driving EKI with RN2-flp or RRA-GAL4 lines, although, as noted above, the number of action potentials evoked for a given level of current injection was greater for the RRA-GAL4 control line.

Figure 5.

(A) Whole-cell voltage-clamp records of identified larval motor neurons after 4 days in culture. The membrane potential was stepped from the holding potential (-70 mV) to command potentials of -30 to $+90$ mV in seven 20 mV steps. No channel blockers were used. Note that there are unclamped action potentials (arrows indicate examples) in the control and EKI records. There is a large voltage-dependent outward current in the EKO motor neuron. (B) Identified larval motor neurons responded to transgenic (EKI) or high-potassium manipulation with enhanced neurite growth and branching *in vitro*. Expression of EKO did not influence growth significantly compared to controls. Number of cells are control = 20; HighK = 20; EKI = 16; EKO = 17. (C) Depolarization with elevated potassium ion in the culture medium induces growth and branching of motor neuron neurites that is dependent on protein synthesis. High K⁺ increases the number of primary branches in cultured neurons significantly as compared to controls, as well as the total number of branches and the total length of all branches. Cycloheximide inhibits both basal and depolarization induced neurite growth and branching. Asterisks indicate significant changes from the control population with $p < 0.05$. Number of cell measured are control = 16; HighK = 14; cyclo = 17; cyclo+HighK = 21. (D) High extracellular potassium did not increase total axonal length (kinesin positive neurites) but increased total dendrite length (kinesin negative neurites) significantly (20 cells measured in each case).

Figure 6.

Growth of dendrites *in vitro* and *in vivo* is regulated by neural activity and requires AP-1 function. (A) Larval RP2 motor neuron developed a significantly larger dendritic field *in vivo* in EKI preparations. Coexpression of Fbz and EKI completely abolishes the enhanced growth seen in EKI-alone. Number of samples: control = 12; EKO = 9; EKI = 11; EKI+Fbz = 8. (B) High K⁺ induced neurite outgrowth *in vitro* is reduced by both Fbz and Jbz, indicating the physiological requirement of AP-1 dependent transcription during activity induced growth. Neurite outgrowth produced through AP-1 expression is not enhanced any further by High K⁺ induced depolarization. (These two results are depicted in separate graphs since they derive from separate experiments with corresponding internal controls). (HighK: 177% ± 14; Fbz + HighK: 41% ± 4, $p < 0.01$; Jbz+HighK: 136% ± 11, $p < 0.05$; AP-1: 244% ± 17; AP-1+HighK: 251% ± 17, $p > 0.1$ between AP-1 and AP-1+HighK). Number of cells are control = 16; HighK = 14; HighK + Fbz = 14; HighK + Jbz = 23; AP-1 = 20; HighK + AP-1 = 20; control for AP-1 and HighK = 20. (C) EKI induced growth is dependent on I_{Ca}⁺². Blocking I_{Ca}⁺² with the insect voltage gated I_{Ca}⁺² blocker PLTX, significantly reduced EKI induced growth and branching.

Further, expression of AP-1 in neurons treated with PLTX completely rescues the growth inhibition observed in PLTX-treated cells. AP-1 and AP-1 + PLTX cells show mutually indistinguishable growth as compared to controls. This suggests that AP-1 affects dendrite growth downstream of neural activity and calcium entry. Note that AP-1 expressing neurons in this experiment show approximately 180% increase over controls as compared to ~250% in 6B. This apparent discrepancy is due to variability arising from these two experiments being performed at different times. As explained in the materials and methods section control cells were plated at the same time as each individual experiment to account for such variability. Asterisks indicate significant changes as compared to wild-type controls. (D) The anti-Fos antibody recognizes nuclear Fos protein in motor neurons (arrow) in the ventral ganglion of animals overexpressing Fos using a C380-GAL4 driver. This antibody also recognizes the predicted Fos band on western blots of proteins from adult heads. Increased Fos expression is detected in animals overexpressing Fos using a pan-neuronal C155-GAL4 line. When normalized to tubulin loading controls, GAL4 driven expression of Fos was 147% of control, while the UAS-AP-1 transgene alone showed 107% Fos expression as compared to C155-GAL4. (E) High K⁺ induced depolarization rapidly increases Fos protein levels in neuronal nuclei *in vitro*. This increase is transient; it occurs within 4 h of treatment and returns to baseline during prolonged (20 h) treatment. (134% ± 6; $p < 0.01$ as compared to uninduced controls). (F) Schematic model proposing that neural activity and calcium signaling regulates dendrite growth in *Drosophila* motor neurons by activating AP-1 dependent global gene expression and protein synthesis.