

AP-1 functions upstream of CREB to control synaptic plasticity in *Drosophila*

Subhabrata Sanyal*†, David J. Sandstrom*†‡, Charles A. Hoeffler* & Mani Ramaswami*

* Department of Molecular and Cellular Biology, and Arizona Research Laboratories, Division of Neurobiology, University of Arizona, Tucson, Arizona 85721, USA

† These authors contributed equally to this work

‡ Present address: Laboratory of Molecular Biology, NIMH Building 36, Room 1B10, National Institutes of Health, Bethesda, Maryland 20892, USA

Activity-regulated gene expression mediates many aspects of neural plasticity, including long-term memory. In the prevailing view, patterned synaptic activity causes kinase-mediated activation of the transcription factor cyclic AMP response-element-binding protein, CREB. Together with appropriate cofactors,

CREB then transcriptionally induces a group of ‘immediate-early’ transcription factors and, eventually, effector proteins that establish or consolidate synaptic change¹. Here, using a *Drosophila* model synapse, we analyse cellular functions and regulation of the best known immediate-early transcription factor, AP-1; a heterodimer of the basic leucine zipper proteins Fos and Jun². We observe that AP-1 positively regulates both synaptic strength and synapse number, thus showing a greater range of influence than CREB³. Observations from genetic epistasis and RNA quantification experiments indicate that AP-1 acts upstream of CREB, regulates levels of CREB messenger RNA, and functions at the top of the hierarchy of transcription factors known to regulate long-term plasticity. A Jun-kinase signalling module provides a CREB-independent route for neuronal AP-1 activation; thus, CREB regulation of AP-1 expression⁴ may, in some neurons, constitute a positive feedback loop rather than the primary step in AP-1 activation.

Synaptic signalling modulates a transcriptional cascade gated by the cyclic AMP-responsive element-binding protein (CREB) and immediate-early transcription factors such as AP-1, c/EBP and Zif-268 to control gene expression that underlies persistent forms of synaptic plasticity¹. A role for CREB at the top of the transcriptional

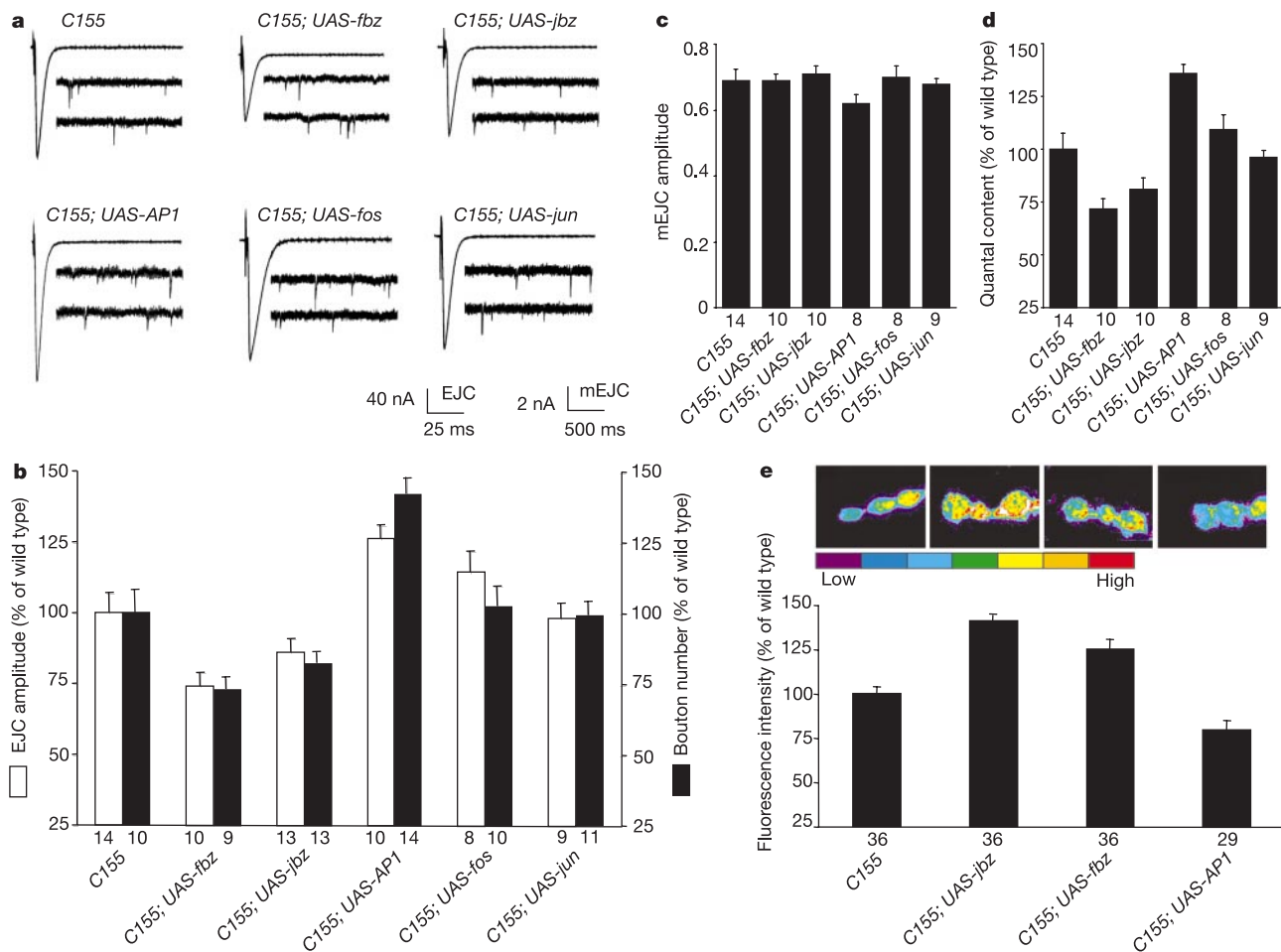


Figure 1 Neuronal AP-1 controls bouton number and synaptic strength. **a, b**, EJCs and mEJC traces (**a**), and histogram representation of EJC amplitude and bouton number (**b**) from larvae in which either wild-type or dominant inhibitory forms of Fos (Fbz) or Jun (Jbz) are expressed. Fbz causes a 30% reduction and Jbz a 25% reduction in synapse size ($P < 0.005$ and $P < 0.02$, respectively) and strength ($P < 0.001$ and $P < 0.03$, respectively). Although Fos or Jun alone have minimal or no effects on bouton number and synaptic strength, together they cause an increase of 30% ($P < 0.001$ for bouton number and synaptic strength). **c, d**, Miniature (m)EJC sizes in all genetic combinations tested are

not significantly altered (**c**); thus, alterations in synapse strength arise from reduced quantal content (**d**). **e**, The pseudocolour scale indicates levels of synaptic fasciclin II (top panel). The histogram shows quantitative fluorescence measurements indicating increased fasciclin II in Fbz- ($P < 0.001$) and Jbz- ($P < 10^{-5}$) expressing preparations, and decreased fasciclin II in AP-1-induced preparations ($P < 0.004$). The number of larvae examined is indicated below the bars. For **e**, the number of type 1b termini (4–6 per larva) is indicated.

cascade is strongly indicated by demonstrations that induction of an activator isoform of CREB substantially enhances long-term memory in *Drosophila* and rodents, and long-term facilitation in the sea slug *Aplysia*⁵⁻⁷. These findings, together with substantial data to indicate the CREB responsiveness of several immediate-early genes⁸, has led to a 'switch' model for CREB, in which CREB acts upstream of all transcriptional processes required to effect enduring synaptic change¹. Motivated by observations potentially inconsistent with this model^{3,9}, we investigated neural functions of AP-1—a heterodimeric transcription factor composed of Fos and Jun—and contrasted these to known functions of CREB. Detailed analysis of a Fos-, Jun- and Jun N-terminal kinase (JNK) signalling module's function in *Drosophila* development provides a genetic framework¹⁰ for a dissection of AP-1 function during synaptic plasticity at the *Drosophila* larval neuromuscular junction (NMJ). The maturation of this synapse as it grows in size (number of boutons) and strength (stimulus evoked response) during the three instars of larval growth

involves activity-dependent mechanisms that also underlie long-term plasticity and memory¹¹. Thus, genetic manipulation of neuronal activity or cAMP levels results in predictable alterations in synaptic size and strength¹². Hyperactivity- or cAMP-induced increases in synaptic strength are CREB dependent; this requires increases in bouton number that occur through a reduction in levels of fasciclin II, a synapse cell adhesion molecule whose orthologue in *Aplysia* is rapidly downregulated after induction of long-term facilitation^{3,13,14}.

Our results show that neuronal AP-1 positively regulates both bouton number and synaptic strength; such extensive control of plasticity processes is unique among known transcription factors. Increased neural expression of both Fos and Jun, but not of either alone, results in a 30% potentiation in evoked junctional currents (EJCs) and a parallel increase in the number of synaptic boutons (Fig. 1a, b). Inhibition of either protein by means of targeted neural expression of Fbz and Jbz, dominant negative forms of Fos and Jun,

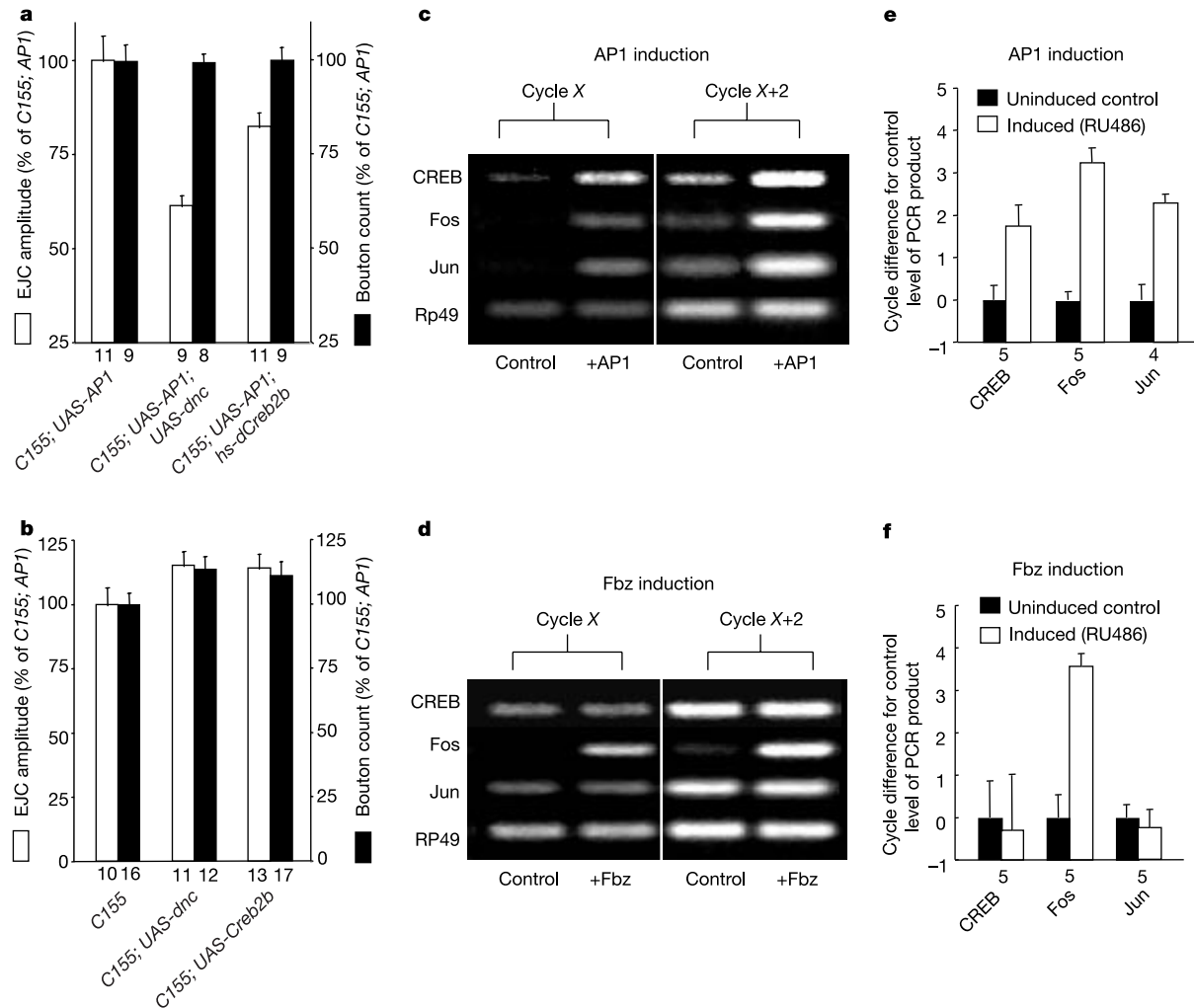


Figure 2 Both CREB and a cAMP-sensitive activity are required downstream of AP-1 to regulate synapse strength but not bouton number. **a**, In a genetic background where AP-1 is induced in neurons, induction of either an inhibitory isoform of CREB (through *hs-Creb2b*) or cAMP phosphodiesterase (through *UAS-dnc*) does not perturb the effect of AP-1 on bouton number. In contrast, both *hs-Creb2b* ($P < 0.02$) and *UAS-dnc* ($P < 0.001$) significantly inhibit the effect of AP-1 on synaptic strength. (*C155; AP-1; hs-dCreb2b* animals have 108%, and *C155; AP-1; UAS-dnc* have 83% average EJC amplitude as compared with wild type.) **b**, In a wild-type background, identical induction of *hs-Creb2b* or neuronal expression of *UAS-dnc* has no effect on either synapse size or

strength. **c-f**, AP-1 induction in neurons leads to rapid threefold upregulation of CREB2 mRNA. **c**, CREB2 transcript levels are elevated in AP-1-induced neurons when compared with uninduced control animals. **d**, Ru486 induction of Fbz does not significantly alter levels of Jun and CREB2 mRNA. **e, f**, Quantitative comparison of mRNA levels by Q-PCR. After induction of AP-1, *Cerb2* mRNA levels increase threefold relative to uninduced controls ($P < 0.02$). **f**, After Fbz induction, Fbz transcript levels are increased 12-fold whereas the average levels of Jun and CREB2 remain unchanged when compared to mRNA from uninduced controls.

respectively¹⁵, causes opposite phenotypes at the larval NMJ (a roughly 30% reduction in bouton number and synaptic strength, Fig. 1a, b). The similarity and specificity of these effects indicate that Fbz and Jbz act by specifically inhibiting endogenous AP-1 activity, a premise supported by demonstrations that developmental phenotypes caused by induction of Fbz or Jbz are identical to those caused by loss-of-function mutations in D-Fos or D-Jun¹⁶. The observation that Fos and Jun positively regulate synapse plasticity in a manner that requires the presence of both molecules, but is sensitive to inhibition of either, indicates that Fos and Jun function as a heterodimer to control synapse plasticity. This is important, given various data to suggest that AP-1 is a heterogeneous collection of molecularly distinct transcription factors¹⁷. Expression of AP-1, Fbz or Jbz through a promoter relatively restricted to motor neurons¹⁸ was sufficient for the observed effects; perturbation of AP-1 activity in postsynaptic muscle had little or no effect on the synaptic parameters analysed (data not shown). Together these data are consistent with a cell-autonomous role for AP-1 in regulating neural plasticity.

AP-1 regulates synapse strength by controlling the quantal content (number of vesicles fusing per stimulus) of presynaptic transmitter release (Fig. 1c). Thus, whereas AP-1 manipulations affect EJCs, responses to individual synaptic vesicles (miniature (m)EJC amplitudes) remain unchanged (Fig. 1a, c). Synapse expansion mediated by AP-1 is accompanied by reduced (by approximately 25%) levels of synaptic fasciclin II; Fbz- or Jbz-mediated reductions in synapse size are accompanied by increased (by about 35%) levels of synaptic fasciclin II (Fig. 1e). This is consistent with a model in which AP-1-mediated changes in synapses occur through the classical activity-regulated pathway for synapse plasticity¹⁴.

Previous work has demonstrated a requirement for CREB in determining synaptic strength but not bouton number at the *Drosophila* larval NMJ³. In this study, the observed consequences of AP-1 manipulations were not consistent with a simple model of CREB exerting its effects through Fos and Jun expression. Rather, they suggested a model in which CREB acts downstream of AP-1 in the pathway, leading to altered synaptic strength. We tested this hypothesis by asking whether induction of a CREB-blocker¹⁹ transgene (*hs-Creb2b*) would inhibit AP-1-induced synaptic changes. Induction of *hs-Creb2b* (ref. 3) had no effect on synaptic size and strength in a control genetic background; however, it specifically blocked the AP-1-induced increase of synaptic strength, without affecting the AP-1-induced increase of bouton number (Fig. 2). This specific effect of CREB blocker strongly suggests that the effect of AP-1 on synaptic strength occurs through a pathway that involves the activation of CREB; however, its effect on bouton number occurs by means of the CREB-independent pathway, suggested by previous studies.

One formal explanation for these observations is that AP-1 induction in neurons results in general hyperexcitability; that is, it acts to turn on the activity- and cAMP-dependent signalling pathway in neurons. In this case, reduction of neuronal cAMP through targeted overexpression of the *dunce* (*dnc*) cAMP phosphodiesterase¹⁸ would be expected to block all effects of AP-1 overexpression. This was not the case. Neural induction of cAMP phosphodiesterase did not alter the influence of AP-1 on bouton number (Fig. 2b); this is consistent with a function of AP-1 downstream of the cAMP-regulated stage in structural synaptic plasticity. In contrast, reduced neuronal cAMP completely blocked the effect of AP-1 on synaptic strength. As with the CREB-blocker transgene, identical induction of cAMP phosphodiesterase had no effect on synaptic size or strength in a control genetic background; thus, low cAMP specifically affects the AP-1-induced potentiation of synaptic strength. The simplest interpretation of these observations is that the activity of AP-1 is not substantially reduced at low levels of cAMP achieved in our experiment; however, activity of a downstream molecule required for the effect of AP-1 on synaptic strength is inhibited

under these conditions. These observed consequences of cAMP and CREB inhibition on the effects of AP-1 confirm and extend previous studies demonstrating independent control of synapse size and strength³. The similarity of low cAMP and CREB-inhibition effects on AP-1-induced plasticity (Fig. 2) is consistent with a model in which the downstream target of cAMP signalling is CREB itself.

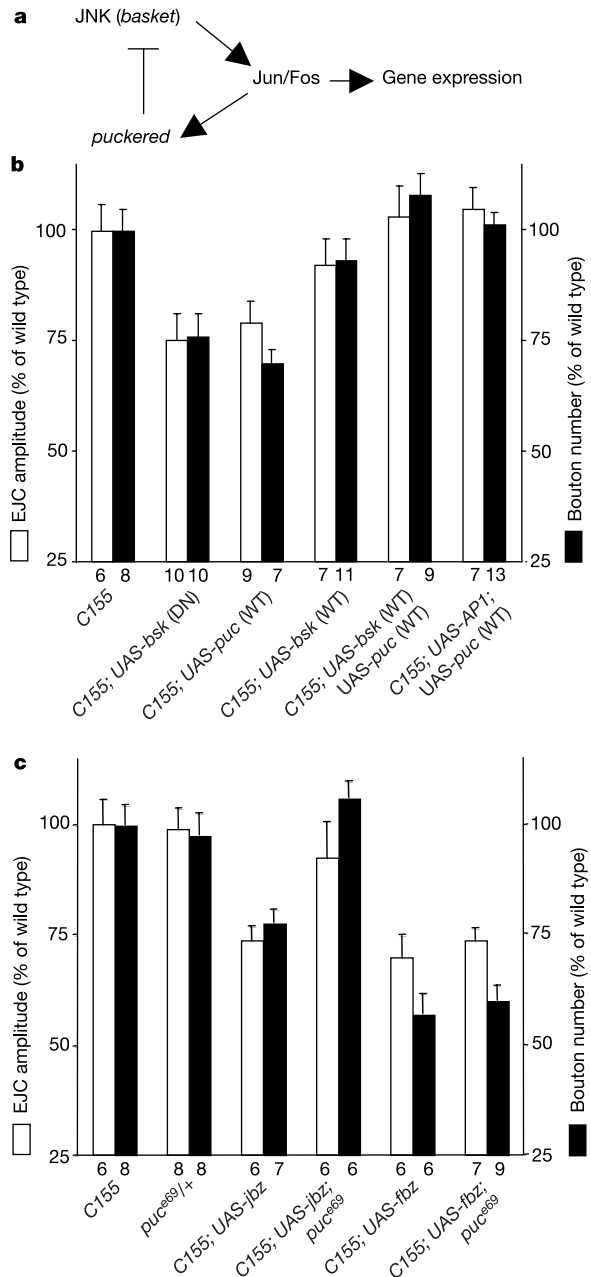


Figure 3 JNK signalling regulates AP-1-dependent synaptic plasticity. **a**, Outline of proximal components of the JNK signalling module in *Drosophila*¹⁰. **b**, The JNK signalling pathway positively regulates both structural and functional synaptic plasticity. JNK inhibition through neural expression of either a dominant negative JNK (*C155; UAS-bsk* (DN)) or wild-type JNK-phosphatase (*C155; UAS-puc* (WT)) reduces synapse size ($P < 0.0001$ and $P < 0.0001$) and strength ($P < 0.04$ and $P < 0.03$) by about 25–30%. These effects of Puc are suppressed by simultaneous overexpression of wild-type JNK. Neural overexpression of Puc neutralizes all effects of AP-1 induction ($P < 0.01$). **c**, Genetic reduction of JNK phosphatase in flies heterozygous for a *puc* hypomorphic allele (*puc^{eb9}/+*) has no observable effect in a wild-type background, but restores normal bouton number and EJC amplitude to Jun-inhibited motor neurons.

One potential mechanism for AP-1 regulation of CREB is suggested by the presence of consensus AP-1-binding sites in the *Drosophila* CREB2 promoter. Consistent with a direct transcriptional control mechanism, we observed a robust increase in CREB mRNA levels after a brief pulse of increased neural AP-1 expression. Brief induction of AP-1 in the *Drosophila* nervous system was achieved using the *elavGS-GAL4* transgene, which causes pan-neural expression of a steroid-dependent, inactive variant of the Gal4 transcription factor. In *elavGS-GAL4; UAS-AP-1* animals, a roughly ninefold increase in levels of neural AP-1 is induced by a 6-h incubation on food doped with the steroid analogue Ru486 (Fig. 2c)²⁰. Under these conditions, CREB mRNA levels are increased about threefold. That this increase is caused by AP-1, rather than unidentified consequences of doping with Ru486 for instance, is evidenced by the unchanged levels of CREB mRNA in Ru486-fed animals expressing Fbz under *elavGS-GAL4* control (Fig. 2d).

Our proposal that AP-1 functions upstream of CREB to regulate synaptic plasticity, predicts the existence of a CREB-independent signalling pathway that can act as a primary activator of neuronal AP-1. One candidate is the JNK pathway, known to regulate AP-1 function in several contexts in mammals and *Drosophila*^{21,22}. Several reports^{23–25} point to the existence of a Ca²⁺-dependent route to JNK activation in the nervous system. Two critical elements of the JNK pathway expressed in the *Drosophila* nervous system are *basket* (*bsk*), which codes for JNK²², and *puckered* (*puc*), which codes for a phosphatase that, by dephosphorylating JNK, negatively regulates JNK signalling²⁶ (Fig. 3a). We tested how altered JNK signalling affected synapse plasticity. Reduced JNK signalling by neural overexpression of either Puckered or dominant negative Basket (*Bsk*^{DN}), mimicked the synaptic effects of AP-1 inhibition. Thus both treatments result in a roughly 30% decrease in synapse strength and bouton number (Fig. 3b). Although overexpression of Basket JNK causes no phenotype on its own, it completely neutralizes the effect of Puckered overexpression; this indicates that effects of Puckered on synapse plasticity occur through its documented inhibition of JNK signalling (Fig. 3b). In contrast to reduced cAMP (Fig. 2), reduced JNK signalling (achieved by neural expression of Puckered) blocked effects of AP-1 on both structural and functional synaptic plasticity (Fig. 3b). This is consistent with a model in which JNK signals through AP-1 to regulate synaptic change.

To ascertain further that the effects of JNK on synaptic change are mediated by AP-1, we studied the effects of increased JNK signalling on synapses inhibited for AP-1 (Fig. 3c). If JNK acts by increasing Jun function, then increased JNK signalling should alleviate the consequences of AP-1 inhibition that derive from reduced Jun activity. We increased JNK signalling by using a genetic background

heterozygous for *puc*^{E69}, a loss-of-function allele of *puc*. Although the resulting enhancement of JNK signalling was not sufficient to drive synaptic change in a wild-type background, it completely suppressed effects of neural Jbz expression (Fig. 3c). This observation, that reduced endogenous *puc* function can compensate when neuronal AP-1 is inhibited, demonstrates that the neural AP-1 activity *in vivo* is positively regulated by JNK signalling.

We demonstrate that AP-1, under JNK regulation, functions upstream of CREB to control a wider range of plasticity processes than anticipated; indeed, our data are consistent with a model in which AP-1 activation is sufficient for transcriptional control of long-term plasticity. Our finding that AP-1 activates CREB, probably through the regulation of CREB mRNA levels, contrasts with previous demonstrations that CREB positively regulates AP-1 transcription⁴. These apparently conflicting observations may be rationalized if CREB induction of AP-1 is considered as part of a positive feedback loop rather than the primary mechanism for AP-1 activation (Fig. 4). Observations presented here demonstrate unanticipated functions of AP-1 and expose limitations of current models for transcriptional control of long-term plasticity. Further studies in other neuronal subtypes are required to establish the generality of our observations. □

Methods

Drosophila strains and genetic controls

We used the following strains: wild type (Oregon R; D. Brower); Gal4-responsive *UAS-fos*, *UAS-jun*, *UAS-jbz* and *UAS-jbz* transgenes (M. Bienz); *hs-Creb2b* (J. Yin and T. Tully); neural Gal4 line, *C155* and muscle line *MHC-GAL4* (C. Goodman); muscle and neural Gal4 lines, respectively *24B* and *elav-GAL4* (L. Luo); *D42* (G. Boulianne); *UAS-bsk* and *UAS-bsk*^{DN} (M. Mlodzik); *UAS-puc* and *puc*^{E69} (A. Martinez Arias). *ElavGS-GAL4* lines were from T. Osterwalder and H. Keshishian. In general, experimental animals were generated by crossing males homozygous for UAS transgenes with virgin females homozygous for the Gal4 driver; wild-type controls consisted of the Gal4 driver crossed to the wild type (Oregon R). In cases where this was not feasible, larvae of the same sex as the experimental animals, from closely matched genetic backgrounds, served as controls. We induced the CREB-blocker transgene, *hs-Creb2b*, as described previously². For inducing Gal4 activity in adult *Drosophila* expressing the *elavGS-GAL4* transgene, 1–3-day-old flies were starved for 6 h and transferred to glass bottles with standard food containing 0.02 mg ml⁻¹ Ru486 (Sigma) for 6 h before decapitation and RNA extraction. We reared flies at 25 °C.

Immunocytochemistry and antibodies

Bouton number in segment A2 was assessed by counting DSYT2 (from H. Bellen) immunoreactive varicosities on muscles 6 and 7. Muscle surface areas were not significantly different in all genotypes analysed. To quantify levels of fasciclin II, synapses were fluorescently labelled with 1D4 antibodies (from C. Goodman), and three terminal type 1b boutons of segment A2 or A3 were imaged at maximum resolution (68 nm pixel diameter) using a cooled charge-coupled device camera (Princeton Instruments) and Metamorph imaging software (Universal Imaging). After background subtraction, the average pixel intensity in these boutons and surrounding subsynaptic reticulum was measured and analysed.

Quantitative PCR (Q-PCR)

For Q-PCR, approximately 50 fly heads were collected for each sample; total RNA was extracted using the RNAeasy kit (Qiagen) and purified from genomic DNA using the DNA-free DNase kit (Ambion). Two micrograms of total RNA were used to synthesize oligodT-primed complementary DNA with the Omniscript cDNA synthesis kit (Qiagen). The cDNA was diluted 1:5 with nuclease-free H2O (Invitrogen) for Q-PCR reactions performed on the Cepheid SMARTCycler using reaction ingredients and the standard protocol from the Quantitech kit (Qiagen). Messenger RNAs in all samples were diluted to ensure identical levels of Rp49. Transcript levels were determined using primer sets (details available on request) specific to Rp49, D-fos, D-jun and *Drosophila* (d)CREB2. Each Q-PCR reaction was repeated three times for five independent RNA preparations. For gel visualization, experimental and control PCR with reverse transcription (RT-PCR) reactions were stopped at the same cycle during the log-linear phase of growth, determined by monitoring the reactions in real time (Fig. 2c, d). Products were taken at cycle X and cycle (X+2), where X is the cycle at which the RT-PCR product derived from experimental animals enters the log-linear growth phase. PCR products were visualized after 2% agarose gel electrophoresis. For statistical comparisons of Q-PCR data, cycles at which RT-PCR products from Ru486-induced and uninduced control samples entered the exponential phase were compared. The difference in PCR cycles required for identical levels of RT-PCR product (at early log-linear growth phase) from experimental and control animals is plotted in Fig. 2e, f. A one-cycle difference represents a twofold difference in starting template concentration. Each sample set was compared using the Student's *t*-test, and only results with a *P*-value <0.05 were considered statistically significant.

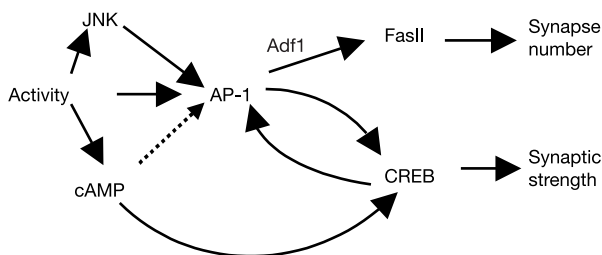


Figure 4 A model for plasticity regulation at the *Drosophila* larval neuromuscular synapse. AP-1 functions upstream of CREB to regulate plasticity. Adf1 influences bouton number but not synapse strength²⁷. AP-1 regulation by CREB is modelled as a positive feedback loop. An important cAMP-sensitive target (potentially CREB itself) functions downstream of AP-1 in regulation of synaptic strength. Upstream signals derive from neural activity-induced Ca²⁺ and cAMP signalling. The mechanism of a postulated cAMP-mediated activation of AP-1 is unknown; however, this study indicates the involvement of JNK signalling upstream of AP-1-dependent transcription.

Electrophysiology and data analysis

Synaptic currents were recorded in HL-3 saline from muscle 6 in segment A2 using a two-electrode voltage clamp²⁸. For EJC recordings, stimuli consisted of 0.2 ms pulses delivered at 1 Hz from an isolated pulse stimulator (AM systems 2100), gated by pClamp software (Axon Instruments). Intracellular glass microelectrodes were filled with 3 M KCl (voltage monitor electrode, resistance 7–10 MΩ) or saturated potassium citrate and 3 M KCl (current injection electrode, resistance 18–25 MΩ). Data were acquired from muscles clamped at –70 mV, using an Axoclamp 2B amplifier (Axon Instruments), and recorded with pClamp software. Data were filtered at 1 kHz before analysis. EJC amplitude was determined from 22–25 contiguous stimuli, and mEJC parameters were measured from one to two 41-s records, using the minianalysis software (Jaevin Software). Differences between means were compared using either *t*-tests (paired comparisons) or analysis of variance (ANOVA; multiple comparisons) in Microsoft Excel or SigmaPlot.

Received 26 November 2001; accepted 21 February 2002.

- Bailey, C. H., Bartsch, D. & Kandel, E. R. Toward a molecular definition of long-term memory storage. *Proc. Natl Acad. Sci. USA* **93**, 13445–13452 (1996).
- Curran, T. & Morgan, J. I. Fos: an immediate-early transcription factor in neurons. *J. Neurobiol.* **26**, 403–412 (1995).
- Davis, G. W., Schuster, C. M. & Goodman, C. S. Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. *Neuron* **17**, 669–679 (1996).
- Sheng, M., McFadden, G. & Greenberg, M. E. Membrane depolarization and calcium induce *c-fos* transcription via phosphorylation of transcription factor CREB. *Neuron* **4**, 571–582 (1990).
- Yin, J. C., Del Vecchio, M., Zhou, H. & Tully, T. CREB as a memory modulator: induced expression of a dCREB2 activator isoform enhances long-term memory in *Drosophila*. *Cell* **81**, 107–115 (1995).
- Bartsch, D. et al. Aplysia CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change. *Cell* **83**, 979–992 (1995).
- Josselyn, S. A. et al. Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. *J. Neurosci.* **21**, 2404–2412 (2001).
- Sheng, M. & Greenberg, M. E. The regulation and function of *c-fos* and other immediate-early genes in the nervous system. *Neuron* **4**, 477–485 (1990).
- Kelz, M. B. et al. Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature* **401**, 272–276 (1999).
- Kockel, L., Homys, J. G. & Bohmann, D. *Drosophila* AP-1: lessons from an invertebrate. *Oncogene* **20**, 2347–2364 (2001).
- Davis, G. W. & Goodman, C. S. Genetic analysis of synaptic development and plasticity: homeostatic regulation of synaptic efficacy. *Curr. Opin. Neurobiol.* **8**, 149–156 (1998).
- Zhong, Y., Budnik, V. & Wu, C. F. Synaptic plasticity in *Drosophila* memory and hyperexcitable mutants: role of cAMP cascade. *J. Neurosci.* **12**, 644–651 (1992).
- Schuster, C. M., Davis, G. W., Fetter, R. D. & Goodman, C. S. Genetic dissection of structural and functional components of synaptic plasticity. Fasciclin II controls presynaptic structural plasticity. *Neuron* **17**, 641–654 (1996).
- Bailey, C. H., Chen, M., Keller, F. & Kandel, E. R. Serotonin-mediated endocytosis of apCAM: an early step of learning-related synaptic growth in Aplysia. *Science* **256**, 645–649 (1992).
- Eresh, S., Riese, J., Jackson, D. B., Bohmann, D. & Bienz, M. A CREB-binding site as a target for decapentaplegic signalling during *Drosophila* endoderm induction. *EMBO J.* **16**, 2014–2022 (1997).
- Zeitlinger, J. et al. Defective dorsal closure and loss of epidermal decapentaplegic expression in *Drosophila* fos mutants. *EMBO J.* **16**, 7393–7401 (1997).
- Karin, M., Liu, Z. & Zandi, E. AP-1 function and regulation. *Curr. Opin. Cell Biol.* **9**, 240–246 (1997).
- Cheung, U. S., Shayan, A. J., Boulianne, G. L. & Atwood, H. L. *Drosophila* larval neuromuscular junction's responses to reduction of cAMP in the nervous system. *J. Neurobiol.* **40**, 1–13 (1999).
- Yin, J. C. et al. Induction of a dominant-negative CREB transgene specifically blocks long-term memory in *Drosophila*. *Cell* **79**, 49–58 (1994).
- Osterwalder, T., Yoon, K., White, B. & Keshishian, H. A conditional tissue-specific transgene system using inducible Gal4. *Proc. Natl Acad. Sci. USA* **98**, 12596–12601 (2001).
- Chang, L. & Karin, M. Mammalian MAP kinase signalling cascades. *Nature* **410**, 37–40 (2001).
- Riesgo-Escovar, J. R., Jenni, M., Fritz, A. & Hafen, E. The *Drosophila* Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. *Genes Dev.* **10**, 2759–2768 (1996).
- Yang, D. D. et al. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the JNK3 gene. *Nature* **389**, 865–870 (1997).
- Schwarzschild, M. A., Cole, R. L. & Hyman, S. E. Glutamate, but not dopamine, stimulates stress-activated protein kinase and AP-1-mediated transcription in striatal neurons. *J. Neurosci.* **17**, 3455–3466 (1997).
- Sagasti, A. et al. The CaMKII unc-43 activates the MAPKKK nsy-1 to execute a lateral signalling decision required for asymmetric olfactory neuron fates. *Cell* **105**, 221–232 (2001).
- Martin-Blanco, E. et al. *puckered* encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*. *Genes Dev.* **12**, 557–570 (1998).
- DeZazzo, J. et al. *nalyot*, a mutation of the *Drosophila* myb-related Adf1 transcription factor, disrupts formation and olfactory memory. *Neuron* **27**, 145–158 (2000).
- Stewart, B. A., Atwood, H. L., Renger, J. J., Wang, J. & Wu, C. F. Improved stability of *Drosophila* larval neuromuscular preparations in haemolymph-like physiological solutions. *J. Comp. Physiol. A* **175**, 179–191 (1994).

Acknowledgements

We thank D. Bohmann and E. Hafen for strains, and T. Littleton and H. Bellen for anti-Syt antibodies. We also thank H. Keshishian and T. Osterwalder for sharing *elav*^{GS-GLA4} strains before publication; P. Etter for early assistance; D. Stimson for advice on electrophysiology; and P. Jansma for help with confocal microscopy. We thank P. Etter, R. Narayanan and members of the Ramaswami laboratory for useful discussions and/or comments on the manuscript. The work was funded by grants from the National Institute on Drug Abuse (primarily), the National Institute for Neurological Disorders and Stroke,

the Human Frontiers Science Program Organization, McKnight and Alfred P. Sloan Foundations to M.R., and NSF and NIH predoctoral fellowships to C.A.H.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to M.R. (e-mail: mani@u.arizona.edu).

Deafness and renal tubular acidosis in mice lacking the K-Cl co-transporter *Kcc4*

Thomas Boettger^{†‡}, Christian A. Hübner^{†‡}, Hannes Maier[‡], Marco B. Rust^{*}, Franz X. Beck[§] & Thomas J. Jentsch^{*}

^{*} Zentrum für Molekulare Neurobiologie, ZMNH, Universität Hamburg, Falkenried 94, 20246 Hamburg, Germany

[‡] HNO Klinik, Universitätsklinikum Eppendorf, Universität Hamburg, Martinistrasse 52, 20246 Hamburg, Germany

[§] Physiologisches Institut, Ludwig-Maximilians-Universität München, Pettenkoferstrasse 12, 80336 München, Germany

[†] These authors contributed equally to this work

Hearing depends on a high K⁺ concentration bathing the apical membranes of sensory hair cells. K⁺ that has entered hair cells through apical mechanosensitive channels is transported to the stria vascularis for re-secretion into the scala media¹. K⁺ probably exits outer hair cells by KCNQ4 K⁺ channels^{2,3}, and is then transported—by means of a gap junction system connecting supporting Deiters' cells and fibrocytes⁴—back to the stria vascularis. We show here that mice lacking the K⁺/Cl[−] (K-Cl) co-transporter *Kcc4* (coded for by *Slc12a7*) are deaf because their hair cells degenerate rapidly after the beginning of hearing. In the mature organ of Corti, *Kcc4* is restricted to supporting cells of outer and inner hair cells. Our data suggest that *Kcc4* is important for K⁺ recycling^{1,5} by siphoning K⁺ ions after their exit from outer hair cells into supporting Deiters' cells, where K⁺ enters the gap junction pathway. Similar to some human genetic syndromes⁶, deafness in *Kcc4*-deficient mice is associated with renal tubular acidosis. It probably results from an impairment of Cl[−] recycling across the basolateral membrane of acid-secreting α -intercalated cells of the distal nephron.

Electroneutral K-Cl co-transporters have roles in cell volume regulation, transepithelial transport, and in the regulation of intracellular chloride concentration ([Cl]_i). Of the four mammalian K-Cl co-transporters (*Kcc1–4*), *Kcc1* and *Kcc3* are broadly expressed, whereas *Kcc2* is neuron-specific⁷. In mice lacking *Kcc2*, the ensuing rise of neuronal [Cl]_i leads to excitatory responses to the normally inhibitory neurotransmitters GABA (γ -aminobutyric acid) and glycine, resulting in spasticity and early postnatal death⁸. Although the coupling of K⁺ to Cl[−] favours efflux and therefore lowers [Cl]_i under many circumstances, K-Cl co-transporters often operate near electrochemical equilibrium and may also mediate net ion uptake⁹.

We have now generated mice constitutively lacking *Kcc4* (Supplementary Information Fig. 1a), an isoform with hitherto unknown physiological function. It is predominantly expressed in kidney, heart, lung and liver¹⁰. Western blots indicated that *Kcc4* protein was absent in knockout mice (Supplementary Information Fig. 1c). *Kcc4*^{−/−} mice were born at the expected mendelian ratio