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Overexpression Screen in Drosophila Identifies Neuronal Roles of GSK-3β/*shaggy* as a Regulator of AP-1-Dependent Developmental Plasticity

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ABSTRACT

AP-1, an immediate-early transcription factor comprising heterodimers of the Fos and Jun proteins, has been shown in several animal models, including Drosophila, to control neuronal development and plasticity. In spite of this important role, very little is known about additional proteins that regulate, cooperate with, or are downstream targets of AP-1 in neurons. Here, we outline results from an overexpression/misexpression screen in Drosophila to identify potential regulators of AP-1 function at third instar larval neuromuscular junction (NMJ) synapses. First, we utilize >4000 enhancer and promoter (EP) and EPgy2 lines to screen a large subset of Drosophila genes for their ability to modify an AP-1-dependent eye-growth phenotype. Of 303 initially identified genes, we use a set of selection criteria to arrive at 25 prioritized genes from the resulting collection of putative interactors. Of these, perturbations in 13 genes result in synaptic phenotypes. Finally, we show that one candidate, the GSK-3β-kinase homolog, *shaggy*, negatively influences AP-1-dependent synaptic growth, by modulating the Jun-N-terminal kinase pathway, and also regulates presynaptic neurotransmitter release at the larval neuromuscular junction. Other candidates identified in this screen provide a useful starting point to investigate genes that interact with AP-1 *in vivo* to regulate neuronal development and plasticity.

THE ability of an animal to adjust to its environment relies significantly on the capacity of neuronal circuits to change in response to stimuli. This intrinsic plasticity of neurons results in persistent modifications that are orchestrated by the synthesis of new proteins, either through translation of preexisting mRNA or activation of nuclear transcription (Nestler *et al.* 2001; West *et al.* 2001; Lonze and Ginty 2002; Klann *et al.* 2004). Several transcription factors have thus been implicated in the regulation of neuronal plasticity both during development and during acute changes such as those required for memory formation. Of these, a fundamentally important candidate is the immediate-early transcription factor AP-1 comprising heterodimers of Fos and Jun (Kaminska *et al.* 1994; Hiroi *et al.* 1998; Morris *et al.* 2000; Kaczmarek *et al.* 2002; Sanyal *et al.* 2002; Etter *et al.* 2005). Experimental demonstrations in diverse systems have verified the central role of AP-1 in controlling the development, growth, survival, and plasticity of neurons. These include learning behavior in rodents, developmental plasticity in insect models, and cocaine addiction in mammals, to name a few (Sanyal *et al.* 2002; McClung and Nestler 2003; Yasoshima *et al.* 2006). Despite this obvious centrality of AP-1 function in neurons, additional proteins that interact with and modulate AP-1 function in neurons remain largely unknown.

Neural roles for Drosophila Fos and Jun, encoded by the genes *kayak* and *jra*, respectively, have been identified on the basis of experiments that explore AP-1 function in the developing compound eye and at the third instar larval neuromuscular junction (NMJ). For instance, Fos regulates G2/M transition in dividing ommatidia and also controls cell survival and growth (Ciapponi and Bohmann 2002; Hyun *et al.* 2006). More pertinently, studies using the motor synapse demonstrate that AP-1 positively regulates growth and presynaptic neurotransmitter release (Sanyal *et al.* 2002; Collins *et al.* 2006). Inhibiting AP-1 activity in differentiated postmitotic motor neurons results in smaller synapses with reduced evoked release of neurotransmitter. Conversely, coexpression of wild-type Fos and Jun proteins, but not either protein alone, leads to expanded synapses and enhanced transmitter release. These phenotypes suggest significant roles for the AP-1 protein in mediating changes in the structure and function of neuronal synapses that are likely to be relevant in the context of neuronal plasticity. Recent experiments also indicate a requirement for AP-1 in long-term olfactory conditioning in flies (A. Pimentel and M. Ramaswami, personal communication). Thus, taken together with cell biological and behavioral

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experiments in other vertebrate model systems, AP-1 fulfills a strongly conserved role in mediating cellular changes that underlie long-term neural plasticity.

Recently attempts have been made to identify AP-1 targets. The two AP-1 components, Fos and Jun, are canonical immediate-early genes because induction of their messenger RNA does not depend on protein synthesis. Consequently, AP-1 is well positioned to mediate an early response to incoming stimuli. Indeed several experimental paradigms that induce neural activity produce robust, rapid, and consistent changes in Fos mRNA and protein (Swank and Bernstein 1994; Robertson *et al.* 1995; Zhang *et al.* 2002). Additionally, AP-1 is also likely to regulate the transcription of a number of downstream targets in the cell. These considerations have stimulated the search for AP-1 targets in at least two experimental systems. First, microarray experiments have recently been performed to identify genes that have altered expression in the rodent brain reward pathway (nucleus accumbens) following transgenic expression of δ-FosB (an inhibitory isoform of mammalian Fos) (McClung and Nestler 2003). Persistent Fos isoform switching followed by prolonged presence of δ-FosB has long been recognized as a major regulatory event during chronic cocaine addiction. This study has identified a collection of genes that are potentially regulated by δ-FosB and contribute to cocaine addiction, but the functional relevance of these candidates remains to be confirmed. Second, experiments in Drosophila have used two separate techniques, microarrays and serial analysis of gene expression (SAGE), to discover genes that are under AP-1 regulation in neurons (Etter *et al.* 2005). Following the initial identification of a set of candidates, very few were confirmed when tested individually. In a way, this study demonstrates the limitations of molecular techniques when applied to complex tissues. Neurons from whole brains or even brain regions are likely to be heterogenous in their ability to respond to signals increasing the likelihood of experimental noise. In the absence of synchronized populations of cells, these techniques, though powerful, will identify putative candidates that need careful validation.

To fully appreciate how AP-1 controls neuronal plasticity and complex behavior, it is essential to study other proteins that are active in AP-1-regulated processes. These would include not only downstream target genes, but also other proteins that interact with and regulate AP-1 function. Owing to the constraints of molecular strategies described above, we decided to perform a functional screen in Drosophila for genetic interactors of AP-1. The compound eye was used as a convenient prescreening tool to scan a large collection of previously described enhancer and promoter (EP) lines and the newly generated EPgy2 collection to search for genes that when overexpressed or misexpressed modify an AP-1-dependent eye phenotype (Bellen *et al.* 2004). Inhibition of AP-1 in the developing eye through a dominant-negative Fos transgene (bZip domain of dFos) using the GAL4-UAS system results in a malformed small eye phenotype (Cerrato *et al.* 2006). We hypothesized that although AP-1 is likely to perform somewhat different functions in the eye as compared to motor neurons, we would be able to rapidly screen through a large number of genes to generate a short list that could then be tested more rigorously at the motor synapse. Here, we describe results from our screen that confirm these predictions. We report a set of genes that have significant synaptic phenotypes at the neuromuscular junction. Using experiments that test genetic interactions we also show that several of these candidates predictably alter AP-1-derived phenotypes. Finally, we demonstrate a novel role for the GSK-3β-homolog *shaggy* in regulating the function of the AP-1 transcription factor through the Jun-N-terminal kinase (JNK) pathway control growth and neurotransmitter release at this model synapse. In sum, this study identifies and functionally validates a collection of genes that are likely to perform important roles in AP-1-dependent regulation of neural development and long-term synaptic plasticity.

MATERIALS AND METHODS

Drosophila stocks and culture: The Oregon-R (OR) strain was used as the wild type in this study. The EP lines were a part of the Pernille Rorth collection (Rorth 1996); EPgy2 lines were obtained from the Drosophila Stock Center (Bloomington, IN) for screening (Bellen *et al.* 2004). EPgy2 constructs carry multiple upstream activating sequence (UAS) elements in the 3′ end of the transposon and are oriented to transcribe genes downstream of their insertion site. A total of 4307 lines were included in the screen. Ey3.5-GAL4 was used to drive expression in an *eyeless* domain in the developing eye disc. This GAL4 line is a transgenic construct made by Exelexis that contains one copy of the eyeless gene enhancer driving GAL4 expression. UAS-Fbz animals (a dominant-negative transgenic construct that expresses the Bzip domain of Drosophila Fos) were obtained from Marianne Bienz and have been used to inhibit AP-1 in a number of tissues (Eresh *et al.* 1997; Sanyal *et al.* 2002; Cerrato *et al.* 2006). UAS-Fos[WT] and UAS-Fos[WT] are described in the same references. UAS-sgg[DN] lines have been described previously and were obtained from the stock center (Bourouis 2002; Franco *et al.* 2004). The sgg::GFP protein-trap line was obtained from the FlyTrap collection (http://flytrap.med.yale.edu; Franco *et al.* 2004) and the sgg^{e6} allele from Ulrike Heberlein (Wolf *et al.* 2007). UAS-Bsk[DN] flies were obtained from the Drosophila Stock Center (Sanyal *et al.* 2002; Collins *et al.* 2006). *Wallenda* mutant flies were obtained from Aaron diAntonio (Collins *et al.* 2006). Flies were reared on standard cornmeal–dextrose–yeast medium at 22°–25°.

Screening method and genetics: A stock of ey3.5-GAL4 and UAS-Fbz was established and used as the tester line for the screen. Individual EP lines were crossed to this line, and the F_1 progeny were tested for suppression or enhancement of the Fbz-derived small-eye phenotype. To prevent accidental and progressive accumulation of extragenic modifiers of the Fbz phenotype, the ey3.5-GAL4; UAS-Fbz stock was maintained over a second chromosome Curly-O balancer line that con-

tains a GAL80 gene expressed ubiquitously from a tubulin promoter (tub-GAL80) obtained from Sean Sweeney (University of York, York, UK). GAL80 suppresses GAL4 activity and this was seen readily from the absence of an Fbz phenotype in the eye when the balancer was present in the stock. Putative candidates isolated from the screen were then maintained for the next round of analysis at the NMJ. To test the effect of overexpressing candidate genes from these EP or EPgy2 lines at the motor synapse, individual homozygous lines were crossed to the elav^{C155}-GAL4 driver line. This ensured expression in all postmitotic neurons. For genetic experiments with Fbz (AP-1 inhibition) or AP-1 (expression of both wild types, Fos and Jun), appropriate elav^{C155}-GAL4 lines containing balancers on the second or third chromosomes were first crossed to the EP line. Male progeny carrying the elav^{C155}-GAL4 on the X chromosome and the EP line balanced over a "green balancer" (GFP expression from the balancer chromosome is used to select larvae carrying the balancer chromosome) were mated with homozygous UAS-Fbz or UAS-Fos; UAS-Jun animals. F2 female progeny that lacked the balancer chromosome and hence carried the elav^{C155}-GAL4, EP transposon and either UAS-Fbz or UAS-Fos; UAS-Jun were selected for dissections. EP lines on the X chromosome were first combined with the UAS-Fbz or UAS-Fos; UAS-Jun containing chromosomes to generate animals that were homozygous for these transgenic combinations. These were then crossed to elav^{C155}-GAL4 animals.

Immunohistochemistry and confocal imaging: For immunohistochemistry, larvae were dissected in calcium-free HL3 saline (Stewart *et al.* 1994), fixed in 4% paraformaldehyde, and stained overnight in blocking solution containing 0.1% Triton X-100. Mouse anti-synaptotagmin was obtained from the Developmental Studies Hybrodima Bank (DSHB) and used at 1:1000. Alexa-568 dye-conjugated anti-mouse antibodies from Invitrogen/Molecular Probes (Eugene, OR) were used as secondary antibodies. Laser scanning confocal microscopy using a Zeiss 510 inverted miscroscope was used to image synaptic terminals at muscles 6/7 from larval abdominal segment A2. The total number of boutons per synapse was used as a measure of synapse size. Boutons were counted double-blind and at least 10 samples were used for each genotype. Statistical analysis was performed using SigmaPlot (Jandel Scientific) and Microsoft Excel. Standard unpaired *t*-tests were used to determine statistical significance.

Electrophysiology: Excitatory junction current (EJC) recordings were made in HL3 saline (Stewart *et al.* 1994) with 1 mM Ca^{2+} as described previously (Sanyal *et al.* 2003). All recordings were made from muscle 6 in segment A2 of wandering third instar larvae. Muscles were voltage clamped at −70 mV, and excitatory junctional currents were evoked by stimulating the segmental nerves such that both neurons innervating muscle 6 were recruited. The nerves were stimulated at 0.5 Hz and a train of 25 stimuli was averaged for each recording. For spontaneous events, a 2-min continuous recording was used to determine mEJC amplitude and frequency. Traces were analyzed using Clampfit (Molecular Devices) and the Mini Analysis software (Synaptosoft).

RESULTS

Screening for modifiers of an AP-1-dependent eye phenotype: Inhibition of AP-1 in the developing eye disc results in smaller aberrant adult eyes (Cerrato *et al.* 2006; Hyun *et al.* 2006). Recent studies have shown this to be due to perturbations in the G2/M transition during mitotic divisions in the eye disc (Hyun *et al.* 2006). Thus, AP-1 is involved in the regulation of cell cycle in this tissue. When we expressed a Fos-bZip-containing transgene in the eye disc using an ey3.5-GAL4, we could recapitulate the eye phenotypes that had been observed previously (Figure 1A) (Cerrato *et al.* 2006). We further confirmed that this phenotype results from AP-1 perturbation by coexpressing wild-type copies of dFos and dJun. AP-1 coexpression completely rescued the small-eye phenotypes, with all adults displaying normal wild-type eyes (Figure 1A).

To screen for genes that influence AP-1 function in neurons, we used a misexpression-based system used effectively in several screens. This strategy relies on the use of the GAL4-UAS system (Brand and Perrimon 1993) and a scheme to overexpress/misexpress different genes in the pattern of the GAL4 line of choice (Rorth 1996). Modifications of these original EP transposon lines have recently become available through a large-scale effort to tag all Drosophila genes (Bellen *et al.* 2004). We chose to use this system for two reasons. First, these individual lines are molecularly characterized. Their orientation and site of insertion have been mapped, leading to easy identification of the gene of interest. Second, expression of Fbz through the eyeless-GAL4 (ey3.5-GAL4) produces an incompletely penetrant phenotype. These adults have eyes that range from completely normal to severely reduced (Figure 1A). Hence, it becomes essential to test a substantial number of flies for each potential modifier, precluding a simple EMS-based F_1 screen. Since the EPgy2 lines were available from the stock center, we decided to cross individual lines to a background in which the ey3.5-GAL4 drives expression of Fbz (from a UAS-Fbz transgene; Figure 1C). Progeny from each of these crosses were then tested for suppression or enhancement of the Fbz-dependent phenotype. We screened lines from the original EP collection at Bloomington and also the new EPgy2 collection, for a total of >4000 lines.

Our strategy for selecting putative candidates was based on several criteria. As shown in Figure 1B, expression of Fbz resulted in a range of phenotypes. We classified these into four categories: completely normal, intermediate, bilaterally small, and missing eyes. We analyzed >200 animals of this genotype and determined the relative distribution of these categories. Finally, to simplify screening, we designated phenotypes as "big" or "small" eyes. From our test crosses (Figure 1C), a line was considered to be a suppressor if it completely eliminated the occurrence of missing eyes. By contrast, a line was an enhancer if it produced >50% animals with small or missing eyes. Since we were interested in genes that primarily modified an AP-1-dependent phenotype, we also crossed selected EPgy2 lines directly to the ey3.5-GAL4 line to test if this manipulation produced an eye phenotype by itself. Only lines that did not affect eye development when expressed alone but strongly modified an Fbz phenotype were retained for further analysis.

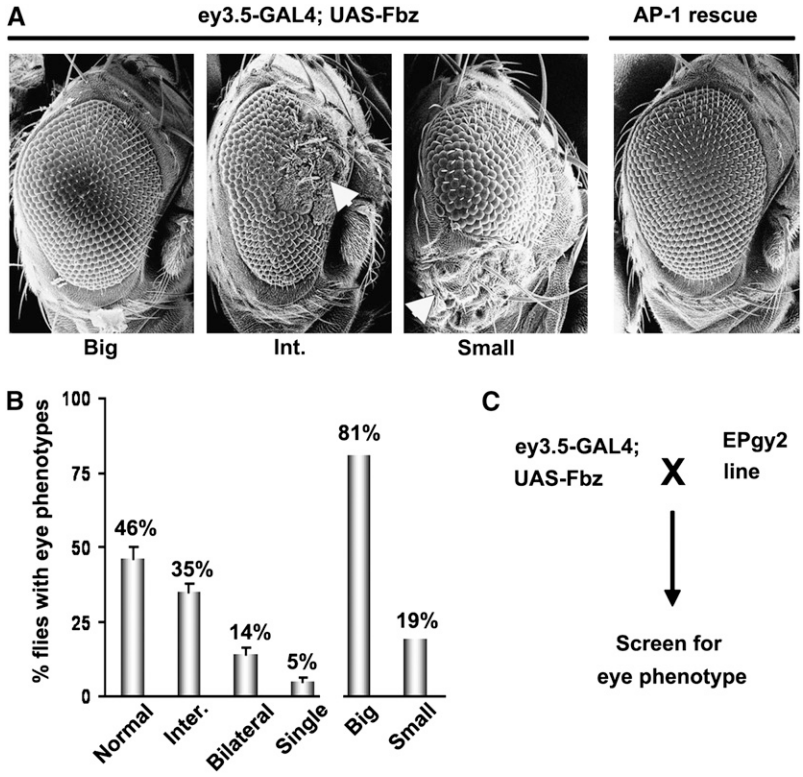

FIGURE 1.—An eye-based screen for genetic modifiers of AP-1 function. (A) AP-1 inhibition through the expression of a dominant-negative bZip-only Fos (Fbz) using an *ey3.5*-GAL4 driver line, results in small eyes with varying severity. This incompletely penetrant phenotype is rescued by the coexpression of wild-type dFos and dJun consistent with the idea that Fbz expression inhibits endogenous AP-1. (B) Distribution of eye phenotypes resulting from Fbz expression in the eye. Almost half the animals (from >200 animals tested) have near-normal eyes, with many others showing only mild deformations. These are pooled into the "big eye" category, which comprises 81% of all adults tested. Nineteen percent have significantly small or missing eyes. Enhancers are defined as those candidates that result in a larger number of small or missing eyes. Similarly suppressors are those that reduce the number of animals with small and missing eyes. (C) Genetic scheme for an F_1 screen to identify EP and EPgy2 lines that modify an Fbz-dependent phenotype in the adult eye. Between 30 and 50 animals were scored for each genotype.

Analysis of candidates and selection for synaptic screen: Upon conclusion of our screen, we recovered a total of 303 candidates. Of these, 54 are predicted genes (CG nos. that are predicted through the use of computer algorithms) while 249 have been functionally verified. At the outset, we had predicted that a main source of "noise" for our purposes would arise from genes that function solely in eye development. Since we could immediately ascertain the identity of our candidates, we selected "eye-specific" genes on the basis of previous studies and decided not to pursue these candidates further. However, in some cases we selected genes that play a role in eye development but also have demonstrated neural functions for further analysis (*e.g.*, *sty*).

A reasonable test of success would be the isolation of genes that we would predict to interact with AP-1 on the basis of prior experiments. Not all Drosophila genes have been tagged with the EPgy2 element and so even a few such candidates would suggest that the screen had been successful. Indeed, we isolated several such genes. Most notable among them were Ras85D, Bsk (dJNK), CycB, and Men (supplemental Table S1). All four genes have been tested separately for their interaction with AP-1 (SANYAL *et al.* 2002; CERRATO *et al.* 2006; HYUN *et al.* 2006). Since our main goal with this prescreen was to generate a short list of candidates that we could test at the synapse, we then used one or more of the following three criteria to narrow down the list of potential AP-1 interactors:

a. Previously described role in the nervous system: In addition to rejecting candidates with established roles in eye development, we prioritized our selection on the basis of existing information on the role of the candidate in the nervous system, either from expression or functional studies.
b. Known interaction with AP-1 in other tissues: We picked candidates that have been shown to influence AP-1 function in other cell types and physiological processes but not in neurons. For instance, we chose genes that interact with AP-1 to regulate embryonic dorsal closure (RIESGO-ESCOVAR and HAFEN 1997; CIAPPONI and BOHMANN 2002; JACINTO *et al.* 2002).
c. Strength of phenotype and isolation of multiple alleles: Strong eye phenotypes and candidates for which we had identified multiple insertion lines were preferred.

Following the application of one or more of these criteria, we generated a list of 25 genes of which 10 were isolated as suppressors and 15 as enhancers of Fbz-derived phenotypes in the adult eye (Table 1). Our next goal was to test if any of these genes affected the development of the NMJ, and more specifically, if they interacted with AP-1 at the NMJ. We further narrowed down our choice to 13 genes that fulfilled both of the following conditions (genes currently not included for further analysis, but incorporated in Table 1 for potential future studies are *chic*, *dimm*, *gry*, *slmo*, *ab*, *bon*, *sqh*, *thr*, *px*, *dad*, *Alh*, and *Mkp3*):

a. Orientation of *P*-element: We selected lines in which the UAS-containing *P*-element is inserted such that it is likely to overexpress the full-length downstream

TABLE 1

Prioritized list of candidates tested at the larval neuromuscular junction for their ability to regulate synaptic growth in the context of AP-1 function

Gene	Protein/function	Remarks
		Enhancers
chic	Actin binding	Central brain metamorphosis (Boquet *et al.* 2000)
cnk	Signal transduction	Regulates Ras activation of Raf (Douziech *et al.* 2006)
Cnx99A	Calcium binding, protein folding	Expressed in midline CNS neurons (Kearney *et al.* 2004)
dimm	Transcription factor	Activates neuropeptide expression (Hewes *et al.* 2006)
Fkbp13	Peptidyl-prolyl *cis–trans* isomerase	FK506 binding/calcium-ion binding (Spradling *et al.* 1999)
gry	Unknown	Mushroom body expression, learning defects (Dubnau *et al.* 2003)
Mkp3	MAPK phosphatase	ERK-specific phosphatase, neuroprotective (Levinthal and Defranco 2005)
Pde8	c-AMP phosphodiesterase	Regulates cAMP/cGMP (Day *et al.* 2005)
Sema-2a	Semaphorin, axon guidance	Synaptic phenotype (Matthes *et al.* 1995); neuronal expression (Kolodkin *et al.* 1993)
sgg	Glycogen synthase kinase-3b	Synaptic phenotype, olfactory habituation (Franco *et al.* 2004; Wolf *et al.* 2007)
slmo	Unknown	CNS expression, locomotory defects (Carhan *et al.* 2004)
sqh	Nonmuscle myosin II regulatory light chain	Development of eye and leg imaginal discs (Edwards and Kiehart 1996)
sty	Signal transduction	Inhibitor of Egfr/Ras signaling (Casci *et al.* 1999); neurodegeneration (Botella *et al.* 2003)
thr	Unknown	Peripheral nervous system development (Prokopenko *et al.* 2000)
Sdc	Transmembrane receptor, syndecans	Regulates slit signaling at CNS midline (Johnson *et al.* 2004)
		Suppressors
ab	Transcription factor	Sensory neuron dendritic branching (Sugimura *et al.* 2004)
bon	Transcription factor	Sensory neuron dendrite morphogenesis (Parrish *et al.* 2006)
Dad	BMP signaling	Motor neuron synaptic phenotypes (Sweeney and Davis 2002)
Ibm	Tetraspanin, signaling	Neural tetraspanin, synaptic development (Kopczynski *et al.* 1996)
Mbs	Myosin phosphatase	Synaptic phenotype with NSF2 (Laviolette *et al.* 2005); photoreceptor defects (Lee and Treisman 2004)
pigeon	Unknown	Learning mutants (Dura *et al.* 1993; Bolwig *et al.* 1995; Moreau-Fauvarque *et al.* 2002)
Pvf1	VEGF/PDGF receptor binding	Signals through the Drosophila PDGF/VEGF receptor (Harris *et al.* 2007)
px	Unknown	Regulates transcription (Matakatsu *et al.* 1999)
spag	Ser/Thr phosphatase	Cell survival and differentiation in imaginal discs (Roch *et al.* 1998)
Alh	Transcription factor	Maintains eve expression in CNS (Bahri *et al.* 2001)

The second column denotes protein function while the third column presents evidence in favor of selecting these candidates for further analysis at the neuromuscular junction. The first 14 genes were isolated as enhancers and the last 11 as suppressors of the Fbz eye phenotype.

gene. Although expression of partial gene segments or expression of antisense message from an inverted EP element could potentially lead to loss-of-function phenotypes, we initially restricted our analysis to those insertions that are predicted to overexpress downstream genes.

b. Existence of additional reagents: Two independent collections of flies became available during the course of the screen. One is the FlyTrap collection of GFP exon trap lines. These lines potentially produce GFP fusion proteins for identified genes *in vivo* providing the ability to follow expression patterns and subcellular localization of proteins of interest (Morin *et al.* 2001; Quinones-Coello *et al.* 2007). The other useful collection comprises the transgenic RNAi collections in Japan (NIG) and Austria (VDRC). These reagents greatly facilitate rapid loss-of-function analysis of selected genes and will be used extensively in our future studies.

Neuronal phenotypes and expression of selected screen candidates: To ascertain whether our candidates had neuronal roles, we first overexpressed these proteins in differentiated postmitotic neurons using the elav^{C155}-GAL4 driver line. Since Fbz expression leads to smaller motor synapses at the NMJ, we hypothesized that genes that had been isolated as enhancers of Fbz might also independently result in a smaller synapse. Similarly, suppressors of Fbz could result in expanded synapses. Our results summarized in Figure 2 indicate

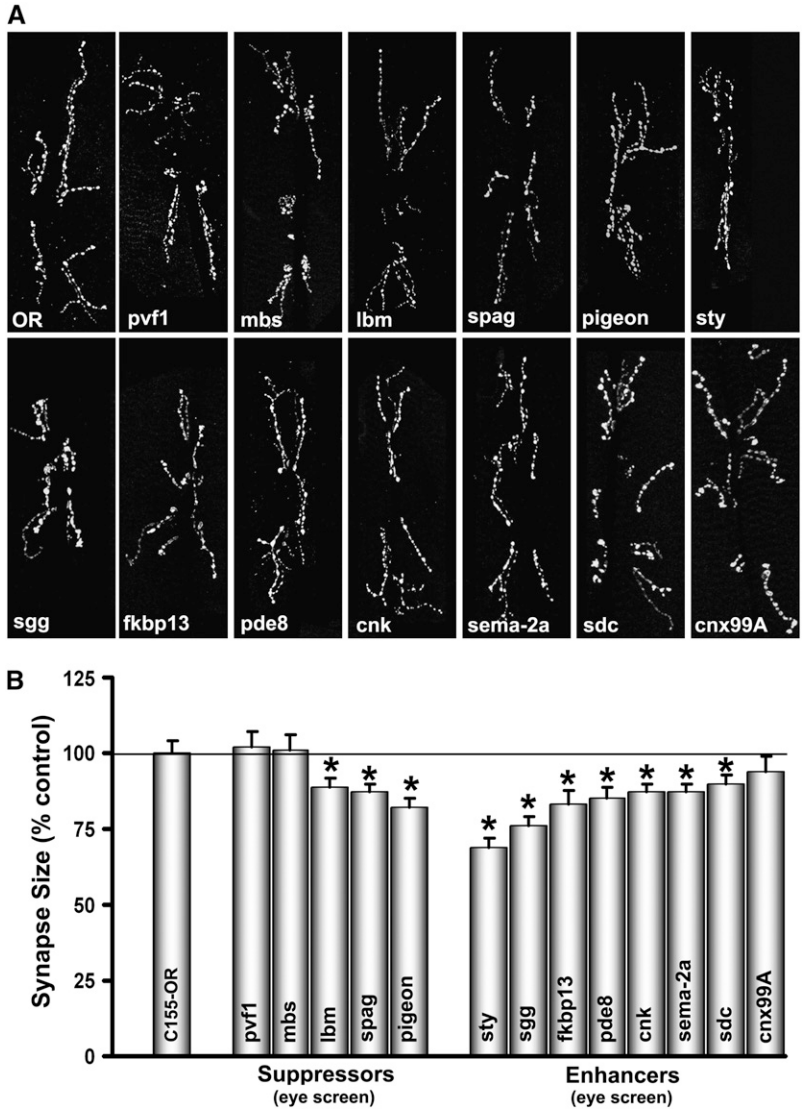

FIGURE 2.—Panneuronal expression of selected candidates results in synapse growth phenotypes at the larval neuromuscular junction. (A) Representative synapses imaged using antisynaptotagmin immunohistochemistry showing a range of phenotypes. A wild-type control synapse is shown for reference. (B) Mean percentage of synaptic boutons normalized with respect to control animals. Three candidates that suppress the eye phenotype and seven candidates that enhance the eye phenotype all produce significantly smaller synapses. Significant differences are denoted by asterisks and were determined using unpaired Student's *t*-test (*P*-values are Pvf1, <0.8; Mbs, <0.9; lbm, <0.04; spag, <0.008; pig, <0.0006; sty, <0.0000007; sgg, <0.0002; fkbp13, <0.01; pde8, <0.015; cnk, <0.02; Sema-2a, <0.01; Sdc, <0.04; and cnx99A, <0.3). More than 10 animals were included for each genotype and synaptic boutons were counted double-blind from coded images. C155-GAL4 denotes the panneuronal GAL4 driver *elav*^{C155}-GAL4.

that from the short list of 13 genes, seven of eight enhancers reduced synapse size, but none of the suppressors led to enlarged synapses. These genes were next tested for their ability to modify an AP-1-dependent synapse phenotype (next section).

Strongest phenotypes were observed through the overexpression of two genes, *sprouty* (69% of control synapses) and *shaggy* (76% of control). Interestingly, both of these genes are established negative regulators of specific intracellular signaling cascades. Sprouty is a ligand-activated inhibitor that antagonizes receptor tyrosine kinase signaling in several contexts, such as Ras/ERK signaling in the developing compound eye (KRAMER *et al.* 1999; HANAFUSA *et al.* 2002). More notably, sprouty2-dependent signaling negatively regulates survival, differentiation, and growth in cultured immature neurons by antagonizing brain-derived neurotrophic factor (BDNF)-dependent signaling (GROSS *et al.* 2007). Precise analysis of this negative regulator in neural development and plasticity, however, has been lacking so far. Considering mounting evidence for the role of Ras signaling in these processes, it is likely that *sty* plays an important modulatory role in Ras/ERK-dependent synaptic growth in Drosophila (KOH *et al.* 2002; HOEFFER *et al.* 2003). Shaggy (also known as *zeste white 3*), the fly homolog of the GSK-3β-kinase, has been investigated extensively and recent studies using Drosophila have uncovered unique functions of this gene in synaptic development (FRANCO *et al.* 2004). Briefly, *sgg* is a negative regulator of synaptic growth; *sgg* mutations result in expanded synapses with extra "satellite" boutons, while Sgg overexpression results in small synapses. This effect is mediated at least in part through the phosphorylation of a mictrotubule-binding MAP1B homolog in flies, *futsch*. In spite of the strong effects of *sgg* on synaptic growth regulation, underlying cellular mechanisms remain unexplored. Our results (see below) provide some of the first evidence that *sgg*-dependent regulation of the transcription factor AP-1 controls both structural and functional aspects of synaptic development and plasticity.

The genes *fkbp13* and *pde8* have not been explored systematically for their role in the nervous system. Both of these genes were isolated as enhancers and result in significantly smaller synapses when overexpressed neuronally. Fkbp13 is a relatively unstudied protein predicted to bind the pharmacological agent FK506. FK506 exerts its immunosuppressant activity by binding to Fkbp12 or other immunophilins thus activating a ligand-receptor cascade that finally targets the phosphatase calcineurin (Bram *et al.* 1993). Recent experiments have also highlighted the fact that FK506 binding proteins are present in the nervous system and control neuronal survival and growth (Edlich *et al.* 2006; Labrande *et al.* 2006; Nakajima *et al.* 2006). Our identification of *fkbp13* in the current screen opens up exciting new avenues for exploring the role of specific FK506 binding proteins in the nervous system. The gene *pde8* is predicted to encode a 3′-5′ c-AMP phosphodiesterase that has not been investigated hitherto. Owing to its expression in the nervous system, *pde8* might have neural roles similar to that of the better-studied phosphodiesterase *dunce* (Zhong and Wu 1991; Davis 1996). Further experiments are required to determine how *pde8* might regulate c-AMP signaling in neurons to affect aspects of growth and plasticity.

Functional analysis of AP-1 interactors at the NMJ: Since our screen relied on an Fbz expression-derived phenotype, we first tested our selected 13 candidates at the motor synapse for their ability to modify an Fbz-dependent small synapse phenotype. As expected, when expressed alone with elav^{C155}-GAL4, Fbz resulted in synapses that were 67% of control synapses in size (number of synaptic boutons as measured through synaptotagmin staining). From the lines tested, overexpression of four genes suppressed or partially reverted a small Fbz synapse toward a wild-type synapse (Figure 3A). These genes were *pigeon*, *lbm*, *Cnx99A*, and *sty*. While *pigeon* and *lbm* were isolated as suppressors, *Cnx*99A and *sty* were isolated as enhancers and resulted in significantly smaller synapses when overexpressed in neurons. This apparent contradiction suggests perhaps a more complex interaction between AP-1 function and negative regulation of receptor tyrosine kinase (RTK) signaling mediated by *sty* on one hand and calnexins encoded by *Cnx99A* on the other (Michalak *et al.* 2002).

The *pigeon* locus encodes a currently uncharacterized protein. Strikingly, however, transposon insertions between this gene and the neighboring *derailed* locus lead to deficits in olfactory memory (Dura *et al.* 1993; Callahan *et al.* 1995). Conflicting results point to memory defects arising from changes in transcript levels in either the *pigeon* or the *derailed* locus (Bolwig *et al.* 1995; Moreau-Fauvarque *et al.* 2002). The EP line isolated in our study is upstream of the *pigeon* locus and is not likely to drive expression of *derailed/linotte*. Interestingly, therefore, our study suggests the testable possibility that cellular deficits in neurons might arise from changes in *pigeon* transcripts. Additionally, genetic interactions with AP-1 in plasticity regulation provide a good starting point for analysis of this long-known learning mutant. Isolation of a *pigeon* allele in our screen also supports the efficacy of our approach in detecting genes of interest that are likely to play important roles in AP-1-mediated neural development and plasticity.

The *lbm* (late bloomer) gene specifies a Drosophila neural tetraspanin (Kopczynski *et al.* 1996), and mutations in *lbm* produce axon guidance and synapse de-

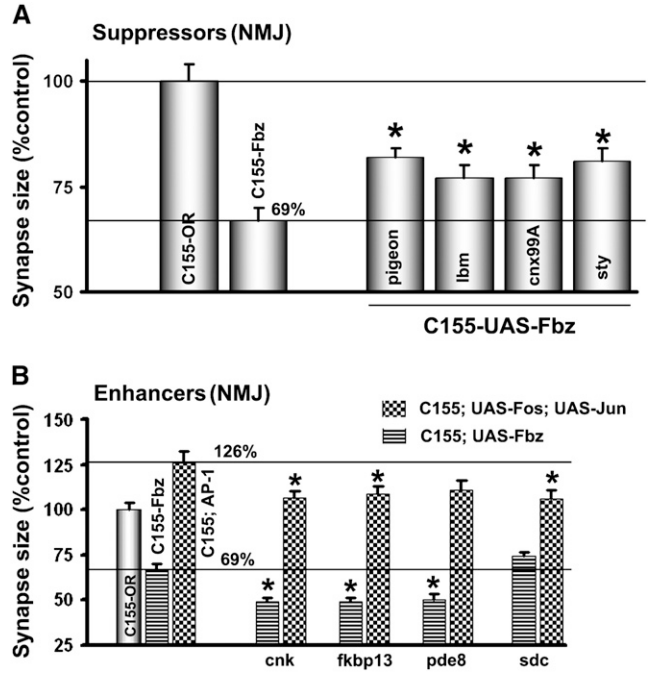

Figure 3.—Genes that modify AP-1-dependent synaptic phenotypes at the larval NMJ. (A) Four genes suppress an Fbz-dependent small synapse phenotype. Mean synapse size values for both wild-type controls and Fbz-expressing synapses are shown for comparison. Expression of Fbz panneuronally produces synapses that are 69% that of controls. Coexpression of *pigeon*, *lbm*, *cnx99A*, and *sty* suppresses this phenotype significantly. Asterisks denote differences from Fbz-expressing animals. More than 10 animals were analyzed for each genotype. (*P*-values: pig, $<6.3X10^{-5}$; lbm, <0.006; cnx99A, <0.019; and sty, <0.004). (B) Four of five genes tested enhance an Fbz-derived phenotype producing synapses that are smaller than Fbz-expressing animals (*cnk*, *fkbp13*, *pde8*, and *sgg*). Four of these genes (*cnk*, *fkbp13*, *sgg*, and *sdc*) also attenuate synaptic growth due to AP-1 overexpression. (*P*-values for comparison between Fbz and EP lines: cnk, $<1.8 \times 10^{-5}$; fkbp13, $<9.3 \times 10^{-6}$; pde8, $<7.3 \times 10^{-5}$; Sdc, <0.06); (*P*-values for AP-1 overexpression and EP lines: cnk, <0.008; fkbp13, <0.014; pde8, <0.033; and Sdc, <0.01). Lines that represent an Fbz synapse (synaptic size = 69% of controls) and an AP-1 synapse (synaptic size = 126% of controls) are shown for reference. Asterisks denote significant differences as compared to appropriate controls. Bars with horizontal lines denote experiments that test these candidates in an Fbz expression background. Checkered bars represent experiments to test them in an AP-1 overexpression background.

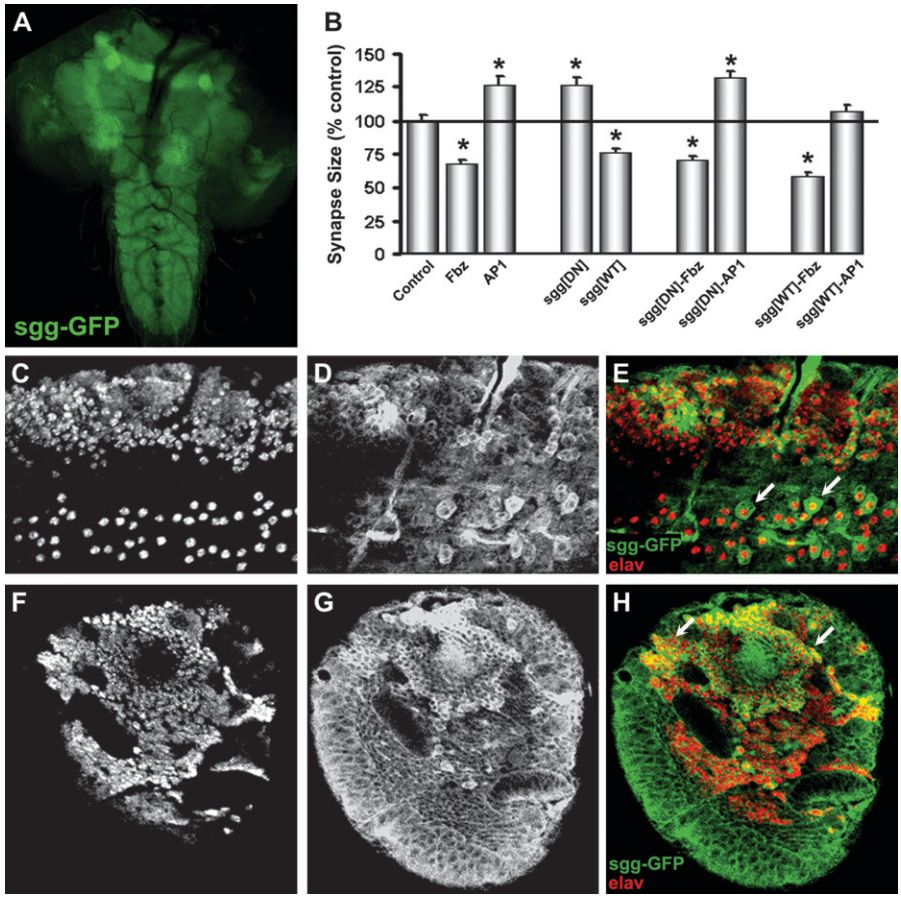

FIGURE 4.—The Drosophila GSK-3β-homolog *shaggy* is expressed in the central nervous system and functions upstream of AP-1 to regulate motor synapse growth. (A) *sgg* expression in larval brains is detectable as a fusion with GFP in a Fly-TRAP line that splices GFP in frame with the endogenous *sgg* gene. Expression is seen throughout the nervous system. (B) Analysis of synapse size at the ventral longitudinal muscle 6/7. Sgg inhibition increases while Sgg overexpression decreases the number of synaptic boutons at this synapse. Genetic analysis suggests that AP-1 functions downstream to *sgg* in the regulation of synapse size. Thus, AP-1 inhibition using a dominant-negative Fos transgene (UAS-Fbz) in a *sgg*-inhibited background results in synapses that are indistinguishable from Fbz synapses. Similarly, overexpression of AP-1 rescues smaller synapses produced by Sgg overexpression. (*P*-values as compared to controls: Fbz, $<6.9 \times 10^{-7}$; AP-1, <0.001; sgg[DN], $<7.9 \times 10^{-5}$; sgg[DN]-Fbz, $<2.3 \times 10^{-6}$; sgg[DN]-AP-1, $<4.8 \times 10^{-5}$). (C–E) Sgg::GFP is present at detectable levels in motor neurons in the larval ventral ganglion. Shown are consecutive hemisegments of the ventral ganglion (dorsal midline at the bottom). Sgg is present in the neuronal cytoplasm in dorsal medial motor neurons (arrows in E). Neurons are marked with anti-elav staining in red. (F–H) Sgg::GFP is also present in neurons of the brain lobes. Shown is a single confocal section through one hemisphere of the larval brain. Arrows mark neurons in the brain that are stained with anti-elav and show Sgg staining in the cytoplasm.

velopment defects in embryos. Tetraspanins are a class of membrane-bound proteins, also found on neuronal surfaces, that regulate a diverse array of cellular processes (HEMLER 2005). For instance, the tetraspanin peripherin 2 (also known as peripherin/rds) is enriched in mammalian photoreceptors and regulates membrane fusion and renewal. Analysis of the *lbm* tetraspanin at the NMJ can potentially illuminate novel aspects of this class of signaling molecules in the nervous system.

Overexpression of four genes of the ones tested enhanced the Fbz-dependent synaptic phenotype. Consistent with our hypothesis, all four were initially isolated as enhancers of the eye phenotype. In addition to *sgg*, which has been mentioned previously (and also see next section), the three other mutations that enhanced Fbz were *cnk*, *Fkbp13*, and *pde8* (Figure 3B). The *cnk* gene (connector enhancer of ksr) encodes a protein that regulates Ras-dependent Raf activation and has been studied extensively in the developing eye (THERRIEN *et al.* 1998; DOUZIECH *et al.* 2003). Significantly, isolation of *cnk* in our screen supports our initial hypothesis that several genes are likely to regulate eye development and also have important roles at the synapse in the context of AP-1 function. Given the emerging prominence of Ras signaling in neural plasticity, *cnk* is likely to exert a significant influence on the development of these motor synapses. Two other genes tested did not have any effect on synapses in Fbz animals. These two genes were *Sdc* (Drosophila syndecan homolog) and *Sema-2a* (Drosophila semaphorins).

Enhancers of Fbz, if they are indeed negative regulators of AP-1 function, are also likely to attenuate an AP-1 overexpression-dependent synapse expansion phenotype. To test this, we coexpressed dFos, dJun, and the gene of interest in all neurons and measured synaptic growth. Of the six lines tested, *cnk*, *Fkbp13*, *pde8*, *sgg*, and *Sdc* significantly reduced synapse growth in an AP-1 overexpression background (Figures 3B and Figure 4), while *Sema-2a* expression did not produce any change as compared to AP-1-expressing animals. It is relevant to point out that although *Sema-2a* and *Sdc* did not enhance an Fbz phenotype, these two genes were included for analysis since they show synaptic deficits and might restrain additional synaptic growth seen in AP-1-overexpressing animals. Thus, these five genes are *bona fide* genetic interactors of AP-1 and function antagonistically to AP-1 in motor neurons to regulate synapse growth.

***shaggy* acts upstream of AP-1 to control synaptic growth:** From the genes tested, we opted to further characterize in detail the Drosophila homolog of the GSK3-β-kinase, *shaggy*. Several considerations prompted this choice. First, GSK-3β-kinase is a component of several signaling cascades such as wingless and TGF-β (Harwood 2001; Doble and Woodgett 2003). Second, several independent lines of evidence support neural roles for GSK-3β: (i) GSK-3β is a confirmed target of lithium, a drug used extensively as an antidepressant (Jope 2003; Zhang *et al.* 2003); (ii) GSK-3β regulates neuronal polarity (Jiang *et al.* 2005; Yoshimura *et al.* 2005; Gartner *et al.* 2006); (iii) negative regulation of neuronal growth and survival is mediated by GSK-3β-dependent signaling (Davis 1996; Crowder and Freeman 2000; Li *et al.* 2000); (iv) this kinase is a target of wingless (*wnt*) signaling in neurons and provides compensatory negative feedback (Siegfried *et al.* 1992; Cook *et al.* 1996; Hedgepeth *et al.* 1997); (v) GSK-3β has been shown to prominently regulate tau phosphorylation and thereby potentially contribute to the development and progression of Alzheimer's disease (Lovestone *et al.* 1994; Phiel *et al.* 2003); (vi) in Drosophila, GSK-3β has been demonstrated to negatively regulate synapse growth through phosphorylation of the microtubule-binding protein *futsch* (this is similar to the regulation of axonal microtubules in cultured vertebrate neurons) (Franco *et al.* 2004); and (vii) GSK-3β has very recently been shown to regulate long-term plasticity in vertebrate brains (Hooper *et al.* 2007; Peineau *et al.* 2007; Zhu *et al.* 2007).

Drosophila Sgg is expressed robustly in the larval nervous system. As described previously, a GFP exon trap line expresses a Shaggy::GFP fusion protein from endogenous enhancer elements (Franco *et al.* 2004). We used this line to detect shaggy expression in the larval CNS. As shown in Figure 4A, live imaging of larval brains demonstrates robust Sgg expression throughout the nervous system. To further localize regions of Sgg expression, we co-immunostained these animals with an antibody to elav (a protein present in all differentiated neuronal nuclei). Figure 4, C, D, and E, shows a few consecutive hemisegments in the larval ventral ganglion (ventral midline at bottom). We detected strong Sgg::GFP expression in the cytosol of most motor neurons (dorsal–medial motor neurons are marked by arrows in Figure 4E). Similarly, single sections of the brain lobes (shown in Figure 4, F, G, and H) also confirm cytosolic localization of Sgg in brain neurons (arrows in Figure 4H). Together, these data reflect strong Sgg expression throughout the larval nervous system.

Since Sgg overexpression in motor neurons enhanced an Fbz-dependent synaptic phenotype, we decided to explore the role of *sgg* in the regulation of AP-1-mediated synaptic plasticity. Earlier studies have outlined negative regulation of synaptic growth at the NMJ through *sgg* (Franco *et al.* 2004). Thus, overexpression of Sgg results in a smaller synapse while *sgg* mutations and transgenic expression of a kinase-dead Sgg protein results in expanded synapses. We first verified these results to be true (Figure 4B). Next, to determine whether *sgg* controls AP-1 function, we expressed the dominant-negative *sgg* transgene together with Fbz using the elav^{C155}-GAL4 driver. Panneuronal expression of Sgg[DN] produces expanded synapses that are 127% of control synapses, while expression of Fbz results in synapses that are much smaller than control. Genetic experiments shown in Figure 4 indicate that blocking both Sgg and AP-1 in the same neurons, results in synapses that are similar to Fbz alone. These results suggest that *sgg* acts upstream of AP-1 to regulate synaptic growth. Consistent with this mechanism, upregulating Sgg signaling in the background of AP-1 overexpression results in synapses that are similar to wild-type and not expanded as expected for AP-1 animals. Taken together, these experiments are wholly consistent with a model in which *sgg* functions upstream of AP-1 to regulate its transcriptional activity in neurons.

***shaggy* regulates presynaptic neurotransmitter release at the NMJ:** Since *sgg* negatively controls synapse growth in an AP-1-dependent manner, we next tested if it affects transmitter release at this synapse. Electrophysiological recordings of evoked junction currents (EJCs) from muscle 6 in response to motor neuron stimulation provide an accurate measure of presynaptic transmitter release (Sanyal *et al.* 2002). Using this technique we found that unexpectedly, inhibition of Sgg in neurons reduces evoked transmitter release (Figure 5, A and B). The effects of *sgg* inhibition are strikingly pronounced, as neuronal expression of Sgg reduces transmitter release to 69% of controls. While paradoxical, there are precedents where genetic manipulations of signaling cascades have resulted in a larger synapse with lowered evoked transmitter release, seen, for instance, in *highwire* mutants (Wan *et al.* 2000). To rule out nonspecific background causes for this phenotype, we further confirmed these effects on synapse strength by either inhibiting or activating Sgg specifically in motor neurons using the OK6-GAL4 driver (Sanyal *et al.* 2003). This enhancer trap provides robust GAL4 expression in a motor neuron-enriched pattern. Using this system, we detected stronger effects on synaptic strength as compared to those seen with panneuronal expression, although in this case, both Sgg inhibition and overexpression resulted in reduced EJC amplitudes (Figure 5B). We also tested a potential *sgg* loss-of-function allele (*sgg*^{e6}) that had been isolated as a mutant in alcohol tolerance assays, but detected no significant changes in transmitter release (Wolf *et al.* 2007). We speculate that perhaps this result reflects a narrow range of Sgg signaling required to maintain normal transmitter release at these synapses. While clear interpretations are difficult, these results do serve to highlight inherent complexity in cellular mechanisms that regu-

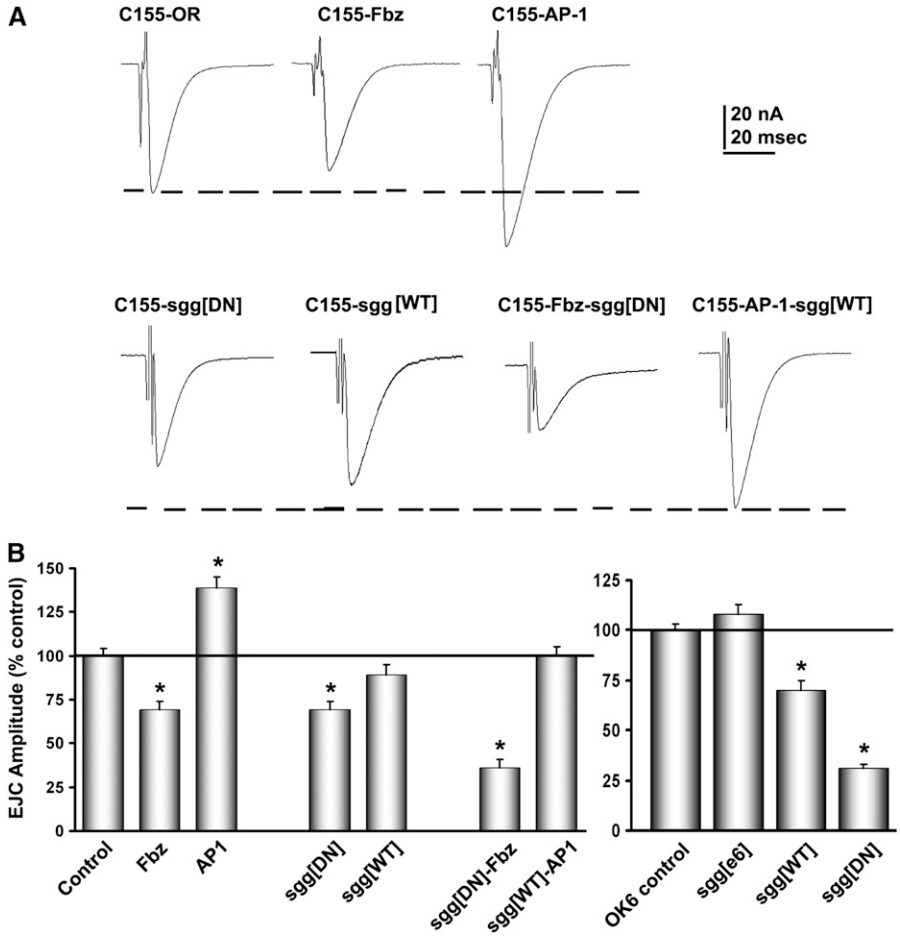

FIGURE 5.—*shaggy* activity regulates neurotransmitter release in larval motor neurons. (A) Representative evoked junction current (EJC) traces showing the effect of inhibition or activation of AP-1 (UAS-Fbz and UAS-AP-1). As shown previously, AP-1 activity positively regulates synaptic strength (peak EJC amplitude). Dotted lines mark the wild-type control amplitude. Panneuronal expression of Sgg[DN] strongly reduces transmitter release, while expression of wild-type Sgg does not result in a significant effect on EJC amplitude. Coexpression of Sgg[DN] and Fbz results in markedly reduced transmitter release, while expression of wild-type Sgg attenuates an AP-1-dependent increase in EJC amplitude. Parameters of spontaneous release are not affected by any of these experimental manipulations. (B) Histograms showing quantitative differences between the genotypes mentioned above. *sgg* function clearly affects neurotransmitter release, although its relation to AP-1 activity is unclear. Expression of *sgg* transgenes in a motor neuron-specific pattern recapitulated results obtained with panneuronal expression. Thus, stronger motor neuron expression of either Sgg[DN] or wild-type Sgg obtained with the OK6-GAL4 driver, resulted in significantly reduced neurotransmitter release. A loss-of function mutant of *sgg* (sgg^{e6}) did not produce any significant change in EJC amplitude as compared to controls. (*P*-values relative to C155-GAL4 as control: Fbz, $<1.3 \times 10^{-5}$; AP-1, $<2 \times 10^{-5}$; sgg[DN], $<1.5 \times 10^{-5}$; sgg[WT], <0.08; sgg[DN]-Fbz, $<2.2 \times 10^{-7}$; sgg[WT]-AP-1, <0.46). (*P*-values relative to OK6-GAL4: sgg[e6], <0.1; sgg[DN], $<5.4 \times 10^{-8}$; sgg[WT], <0.0007).

late neural plasticity in general and *sgg*-mediated control of plasticity in particular. To the best of our knowledge, these are the first direct demonstrations that GSK-3β-activity controls transmitter release in Drosophila neurons. Given the reduction of transmitter release in both of these cases, it is conceptually complicated to investigate the involvement of AP-1 in this process. However, to try and test the relationship between *sgg* and AP-1 in the regulation of evoked release, we performed genetic interaction experiments as described in the previous section. We find that coexpression of Sgg[DN] and Fbz reduces synaptic strength beyond either transgene alone (Figure 5, A and B). Panneuronal expression of wild-type sgg, while not reducing synapse strength significantly, completely inhibits the increase in synapse strength observed in AP-1 synapses. Interpretation of these results is complicated by the fact that Sgg inhibition increases synapse size but reduces synapse strength. Hence, we chose to focus on Sgg-mediated regulation of synapse size for further experiments.

***shaggy* acts upstream of the JNK signaling pathway to regulate synapse size:** The somewhat contradictory effects of *sgg* on synaspse size and strength are reminiscent of mutations in *highwire*, a Drosophila E3 ubiquitin ligase (COLLINS *et al.* 2006). Hiw has also been shown to act on a MAPKKK, *wallenda*, to attenuate a JNK signaling cascade that finally impinges on AP-1. Upon scanning the Wnd protein sequence, using the program NetPhosK, we noticed several conserved Gsk-3β-phosphorylation motifs (S36, S77, S547, S788, and S792) suggesting that Wnd might be a target of Sgg *in vivo*. We hypothesized that maybe Sgg acts to downregulate Wnd similar to Hiw leading to the prediction that an expansion of synapse size observed through Sgg inhibition would be suppressed by reducing Wnd activity. Removing a single copy of *wnd* using either the wnd^1 or wnd^2 mutations completely abolishes the synapse growth seen through Sgg inhibition (Figure 6A). The converse experiment to test if a small synapse generated through Sgg overexpression is rescued to normalcy through Wnd overexpression could not be done since Wnd expression using the $elav^{C155}$-GAL4 resulted in lethality. The loss-of-function experiments, however, strongly suggest that Sgg acts on a MAPKK cascade to influence synapse growth. Given that Wnd functions as a

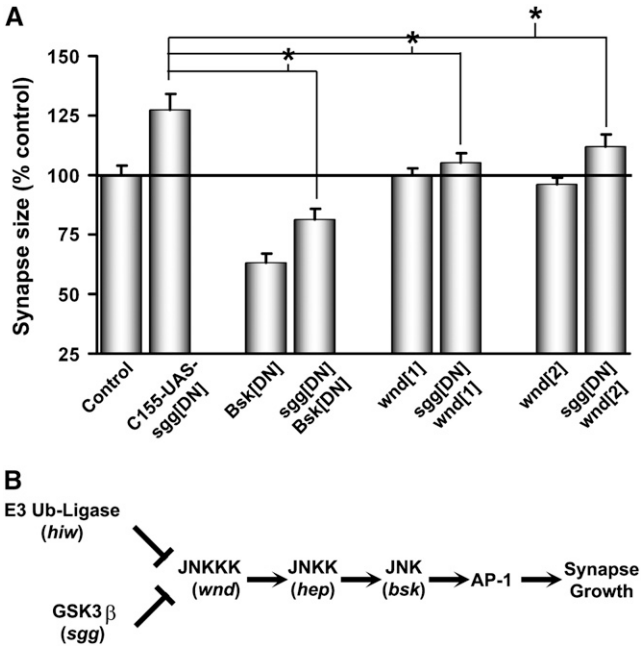

FIGURE 6.—*shaggy* attenuates the JNK signaling cascade to control synapse growth. (A) Inhibition of the Drosophila JNK, *bsk*, suppresses synapse expansion due to Sgg inhibition. Similar suppression is observed through the loss of one copy of the *wnd* gene (a JNKKK) using either the wnd^1 or wnd^2 mutations. (*P*-values compared to C155-GAL4-UAS-sgg[DN]: sgg[DN]-bsk[DN], $<2.5 \times 10^{-7}$; sgg[DN]-wnd[1], <0.0003; sgg[DN]-wnd[2], <0.02). (B) Experiments outlined in this report suggest a model where Sgg attenuates a JNK signal at the synapse to restrict synapse growth. The E3 ubiquitin ligase, *hiw*, has been shown previously to affect this signaling pathway and is shown in this model for comparison.

kinase for the JNK pathway, we next tested if the effect of Sgg inhibition can also be suppressed by directly reducing JNK signaling in neurons. As shown in Figure 6A, when we inhibited JNK (*basket*) signaling, using a dominant-negative Basket transgene, in the background of Sgg inhibition, we observed a small synapse similar to that observed through Bsk inhibition alone (SANYAL *et al.* 2002). These two experiments lend support to our hypothesis that *sgg* acts to inhibit the JNK cascade thereby influencing synapse growth (Figure 6B).

DISCUSSION

The transcription factor AP-1 is a key regulator of neuronal growth, development, and plasticity, and in addition to cAMP response element binding (CREB) protein, it controls transcriptional responses in neurons during plasticity (KAMINSKA *et al.* 1994; SWANK and BERNSTEIN 1994; ROBERTSON *et al.* 1995; MORRIS *et al.* 2000; SANYAL *et al.* 2002). Acute inhibition of Fos attenuates learning in mice and in invertebrate models such as Drosophila; AP-1 positively regulates developmental plasticity of motor neurons (SWANK and BERNSTEIN 1994; SANYAL *et al.* 2002; YASOSHIMA *et al.* 2006). Essential to the understanding of AP-1 activity in neurons is the knowledge of other proteins that influence AP-1 function or are downstream transcriptional targets. Here, we describe a forward genetic screen for modifiers of AP-1 in Drosophila and provide evidence for the validity and success of our strategy.

An overexpression/misexpression screen to isolate genetic modifiers of AP-1: Using a conveniently scored AP-1-dependent adult-eye phenotype, we screened 4307 EP and EPgy2 lines for genes that modified this phenotype. Several advantages of this screen include: (i) the ease and rapidity of screening as compared to the neuromuscular junction, (ii) immediate gene identification, (iii) the potential to analyze *in vivo* phenotypes that arise from overexpression/misexpression, and finally (iv) the scope for rapidly generating loss-of-function mutations through imprecise excision of the same *P*-element. We isolated a total of 249 known genes of which 73 can be directly implicated in eye development. We next prioritized our selection using several criteria, to derive a short list of 13 final candidates that we then tested at the NMJ. Future work will focus on other predicted but as yet unstudied genes (CG no.) that are likely to have important functions at the NMJ.

We believe our prescreening strategy using the adult eye was successful because (i) almost all the genes selected did not result in eye phenotypes when expressed on their own, but selectively modified an Fbz dependent phenotype; (ii) we identified several genes that are known to interact with AP-1 in regulating synaptic phenotypes (these include *ras* and *bsk*); (iii) multiple alleles of some genes were recovered confirming the sensitivity of our screening technique; (iv) several genes involved in eye development were isolated (including *cyclinB*, which was shown recently to be a downstream target of Fos in the regulation of G2/M transition in the developing eye) (HYUN *et al.* 2006); (v) a large number of putative interactors have connections with neural physiology and/or AP-1 function in other cell types; (vi) some candidates with strong phenotypes have previously been shown to play important roles in motor neurons; and finally (vii) the majority of candidates (but not all) isolated as enhancers or suppressors of Fbz in the eye exerted a similar effect on AP-1 at the synapse.

Although the relative success and merits of a functional screen are considerable, there are a few disadvantages. First, the use of *P*-element transposons naturally excludes a large fraction of genes that are refractory to *P*-element transposition events. Second, insertions of EP elements within or in inverse orientation to the gene make it difficult to assign phenotypes to specific genes. Even in instances where we predict overexpression, we need to verify that this is indeed the case and also test if the phenotypes derive from hypomorphic mutations that result from the insertion of the *P*-element close to the target gene. Third, although we expect to recover

genes that play conserved roles in AP-1 biology, those genes that specifically affect synaptic physiology and play no role in the eye will be excluded by this scheme. Finally, this screen will not discriminate between genes that function upstream or downstream of AP-1 in neurons. In spite of these deficiencies, we believe that candidates identified in our screen provide strong impetus for the investigation of additional factors that are involved in the regulation of synaptic plasticity and development by AP-1.

Synaptic phenotypes of AP-1 interactors: Following their identification, we found that several candidates had synaptic functions since several of these genes resulted in significant differences in synaptic size when compared to appropriate controls (Figure 2). This provided the first confirmation of our screening strategy. Next, experiments to determine genetic interaction with AP-1 showed that expression of four genes (*pigeon*, *lbm*, *Cnx99A*, and *sty*) suppressed the Fbz-dependent small synapse phenotype. Of these, *sty* had been isolated as an enhancer while the other three similarly suppressed the Fbz-derived eye phenotype, suggesting potentially conserved functions of these genes in the two tissues.

Four genes isolated as enhancers, similarly enhanced an Fbz-mediated small synapse (*cnk*, *pde8*, *fkbp13*, and *sgg*). Notably, expression of these genes also suppressed an AP-1-dependent synapse expansion at the NMJ. These two lines of evidence indicate that these genes are negative regulators of AP-1 function in these neurons. Together with the fact that all four have previously described functions in the nervous system, these observations confirm the validity of our screen and highlight the utility of genetic screens to uncover novel molecular interactions. Further studies will provide a more comprehensive understanding of the interplay between these genes and AP-1 in the regulation of neuronal development and plasticity. For instance, more careful analysis needs to be carried out to discern whether synaptic phenotypes in each of these cases are due to overexpression or potential insertional mutagenesis of specific genes.

GSK-3β, AP-1, and regulation of synaptic properties: Although GSK-3β-signaling has been implicated in several neurological disorders such as Alzheimer's disease, it is only recently that neuronal roles for this important kinase have come to light. For instance, several studies have demonstrated the role of GSK-3β in the regulation of long-term potentiation (LTP) in vertebrate hippocampal synapses (Hooper *et al.* 2007; Peineau *et al.* 2007; Zhu *et al.* 2007). In particular, these reports highlight the negative regulatory role of GSK-3β in the induction of LTP or in one case, the switching of long-term depression (LTD) into LTP. Interestingly, LTP induction leads to GSK-3β-inhibition thus precluding LTD induction in the same neurons (Peineau *et al.* 2007). In flies, *sgg* mutations have defects in olfactory habituation, circadian rhythms and synaptic growth (Martinek *et al.* 2001; Franco *et al.* 2004; Wolf *et al.* 2007). These observations point to a conserved and central role for GSK-3β in neuronal physiology.

GSK-3β-dependent modulation of transcriptional responses is widely acknowledged. Among several transcription factors that are known to be regulated by this kinase, are AP-1, CREB, NFAT, c/EBP, and NF-κB (Jope 2003). In the context of neuronal function, for instance, RNA interference-based experiments in cultured rat cortical neurons have shown that GSK-3β-activity influences CREB and NF-κB-dependent transcription. Additionally, two other transcription factors, early growth response 1 and Smad3/4 have been identified in DNA profiling experiments in the same study (Liang and Chuang 2006). Significantly, GSK-3β is also a primary target of lithium, a drug used extensively to treat mood disorders (Jope 2003; Zhang *et al.* 2003). Lithium treatment has been reported to result in an upregulation of AP-1-dependent transcription, though a role for GSK-3β in this phenomenon has not been tested directly (Ozaki and Chuang 1997; Hongisto *et al.* 2003).

In Drosophila, recent experiments have described the negative regulation of synaptic growth by the GSK3β-homolog *shaggy* (Franco *et al.* 2004). These studies demonstrate that *sgg* controls synaptic growth through the phosphorylation of the Drosophila MAP1B homolog *futsch*. Our current studies suggest that Sgg-dependent regulation of synapse size occurs through the immediate-early transcription factor AP-1. GSK-3β is believed to inhibit transcriptional activity of AP-1 in cultured cells by direct inhibitory phosphorylation of c-Jun (Boyle *et al.* 1991). Circumstantial evidence also suggests that GSK-3β provides an inhibitory input into AP-1 function in neurons.

It was intriguing to find that Sgg inhibition leads to an expanded synapse with reduced presynaptic transmitter release, similar to *highwire* mutants (Wan *et al.* 2000). Given that in several instances, Sgg-dependent phosphorylation targets a protein for ubiquitination, and that Highwire encodes an E3 ubiquitin ligase, it is conceivable that *sgg* and *hiw* function in the same signaling pathway (Wan *et al.* 2000; Collins *et al.* 2006). Consistent with this hypothesis, both *hiw* and *sgg* function at the synapse seem to impinge on AP-1-dependent transcription through modulation of the JNK signaling pathway (Figure 6). Considering previous reports of GSK-3β-involvement in multiple signaling cascades (Harwood 2001), it will be interesting to study how *sgg* controls multiple aspects of cellular physiology to regulate neural development and plasticity, particularly in the context of brain function and action of widely used drugs such as lithium.

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