Functional Dissection of a Eukaryotic Dicistronic Gene: Transgenic stonedB, but Not stonedA, Restores Normal Synaptic Properties to Drosophila stoned Mutants

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ABSTRACT

The dicistronic Drosophila stoned mRNA produces two proteins, stonedA and stonedB, that are localized at nerve terminals. While the stoned locus is required for synaptic-vesicle cycling in neurons, distinct or overlapping synaptic functions of stonedA and stonedB have not been clearly identified. Potential functions of stoned products in nonneuronal cells remain entirely unexplored in vivo. Transgene-based analyses presented here demonstrate that exclusively neuronal expression of a dicistronic stoned cDNA is sufficient for rescue of defects observed in lethal and viable stoned mutants. Significantly, expression of a monocistronic stonedB transgene is sufficient for rescuing various pheno-typeic deficits of stoned mutants, including those in organismal viability, evoked transmitter release, and synaptotagmin retrieval from the plasma membrane. In contrast, a stonedA transgene does not alleviate any stoned mutant phenotype. Novel phenotypic analyses demonstrate that, in addition to regulation of presynaptic function, stoned is required for regulating normal growth and morphology of the motor terminal; however, this developmental function is also provided by a stonedB transgene. Our data, although most consistent with a hypothesis in which stonedA is a dispensable protein, are limited by the absence of a true null allele for stoned due to partial restoration of presynaptic stonedA by transgenically provided stonedB. Careful analysis of the effects of the monocistronic transgenes together and in isolation clearly reveals that the presence of presynaptic stonedA is dependent on stonedB. Together, our findings improve understanding of the functional relationship between stonedA and stonedB and elaborate significantly on the in vivo functions of stonins, recently discovered phylogenetically conserved stonedB homologs that represent a new family of “orphan” medium (μ) chains of adaptor complexes involved in vesicle formation. Data presented here also provide new insight into potential mechanisms that underlie translation and evolution of the dicistronic stoned mRNA.

The Drosophila stoned locus generates an unusual dicistronic message with two open reading frames, ORF1 and ORF2, that are separated by a 55-nucleotide interval containing termination codons in all alternative reading frames. ORF1 encodes an 850-residue protein termed stonedA and ORF2 contains a 1260-residue protein termed stonedB (Andrews et al. 1996). The locus has received considerable attention not only for its curious dicistronic organization, but also because stonedA and stonedB proteins appear to have important functions in regulating synaptic-vesicle trafficking at the presynaptic terminal (Blumenthal 1998; Fergestad and Broadie 2001; Robinson and Bonifacio 2001; Stimson et al. 2001).

At nerve terminals, stimulus-evoked calcium entry triggers transmitter release through rapid, regulated exocytosis of readily releasable synaptic vesicles. Vesicle proteins, including fusion proteins and neurotransmitter pumps, so deposited on plasma membrane are then retrieved via endocytosis and recycled locally to form new synaptic vesicles. During membrane retrieval from the plasma membrane, adaptor proteins bind cytosolic tails of synaptic-vesicle proteins such as synaptotagmin and cluster them into microdomains from which nascent endocytic vesicles first bud and then detach via sequential and concerted actions of several proteins including clathrin, intersectin/DAP160, Eps15, dynamin, and others (Zhang and Ramaswami 1999; Slepnev and De Camilli 2000). The classical plasma membrane adaptor complex AP2 involved in initial recognition of internalized molecules contains two large subunits, α and β, a medium subunit μ2, and a small subunit ω2; three other homologous adaptor complexes, AP1, AP3, and AP4, are similarly organized as tetramers, each containing subunits homologous to the large, medium, and small chains of AP2 (Hirst and Robinson 1998; Robinson and Bonifacio 2001).
The involvement of stoned proteins in membrane retrieval at the nerve terminal is indicated by several observations. First, stonedA and stonedB are highly enriched at presynaptic nerve endings (Stimson et al. 1998, 2001; Fergestad et al. 1999). Second, they bind the synaptic-vesicle protein synaptotagmin (Phillips et al. 2000). Third, mutations in stoned that alter expression of both stonedA and stonedB cause substantial defects in synaptic-vesicle recycling: thus, stoned mutations cause defective, delayed retrieval of synaptic-vesicle proteins, aberrant synaptic-vesicle size, and defective synaptic transmission (Stimson et al. 1998, 2001; Fergestad et al. 1999; Fergestad and Broadie 2001). The mechanism by which stoned proteins participate in vesicle cycling is less clear. While both stonedA and stonedB sequences contain motifs consistent with direct roles in vesicle traffic (Andrews et al. 1996; Stimson et al. 1998), only stonedB, with orthologs in Caenorhabditis elegans and mammals (Upadhyaya et al. 1999; Martina et al. 2001; Walther et al. 2001), is strongly conserved across phylogeny; stonedA is not obviously conserved outside insects and perhaps noninsect arthropods. Together with evolutionary conservation, the very strong homology of stonedB homologs to μ-subunits (medium chains) of adaptor proteins has led to an economical, but untested, hypothesis that synaptic defects observed in viable and lethal stoned mutants predominantly reflect cellular functions of stonedB.

StonedB and its homologs, together termed the “stonin” family, are orphan adaptor μ-chains. Eukaryotic genomes do not encode obvious partner subunits (similar orphan large and small chains) with which stonins can assemble into a new class of adaptor (Robinson and Bonifacino 2001). Thus, stonedB and its homologs are likely to function by a mechanism different from conventional μ-chains. Stonins contain extended C-terminal domains conserved among μ-subunits of tetrameric AP complexes. In addition, they contain an N-terminal proline and serine-rich domain and share a unique central 140-residue “stonin homology domain” not found in μ-chains of the four known adaptor complexes (Martina et al. 2001). Like the C. elegans ortholog encoded by gene C27H6.1 and stonedB, mammalian stonin2 (but not the second homologous stonin1) contains multiple NPF motifs that may mediate its documented binding to EH domains of Eps15, Eps15R, and intersectin (Martina et al. 2001). Like Drosophila stonedB, stonin2 also binds to synaptotagmin. Some insight into the mechanism of stonin function in endocytosis is suggested by the observation that human stonin2 facilitates the uncoating of clathrin-coated vesicles in vitro, potentially by displacing the adaptor AP2 from its binding sites on vesicle proteins (Walther et al. 2001).

Several issues remain to be resolved to better understand functions of the stoned gene in particular and of stonins in general. First, because stonins may be ubiquitously expressed in all cell types (Martina et al. 2001), it remains unclear whether they are general components of endocytosis or proteins required only for specific forms of neuronal endocytosis. Second, the specific stoned product(s) whose deficiency underlies physiological and morphological defects observed in stoned mutant synapses (Stimson et al. 1998, 2001; Fergestad et al. 1999; Fergestad and Broadie 2001) has yet to be identified. Finally, the origin, significance, and regulation of the dicistronic stoned mRNA remains mysterious. Experiments and observations discussed here address these issues.

MATERIALS AND METHODS

Cultures and stocks: Drosophila cultures were maintained at 21°. Wild type was Oregon-R (ORCB; from Danny Brower, University of Arizona). The strain used for the germline transformation was w1118. The strain used for mapping and balancing transgenes, yw;Sm5,Cy; Sh/TM3,Ser, was also obtained from the Brower Lab.

Stoned mutants stro and stro1357 were from our collection and stro81 was obtained from Norbert Perrimon (HHMI, Harvard Medical School). stro was maintained as a homozygous stock whereas stro81 and stro1357 were maintained over the FM7i balancer chromosome (Bloomington Stock Center), which carries the P-element P[w]mC = ActGFP]JM3R, encoding green fluorescent protein (GFP) under the cytoplasmic actin promoter. For larval dissections, stoned males could be distinguished from FM7i males by lack of green fluorescence using a Leica MZ6 stereo microscope outfitted with a GFP fluorescence illuminator (Kramer Scientific, Elmsford, NY). stro81 was also maintained over a modified Y chromosome Dp(1,Y)y balancer (Bloomington Stock Center), which contains the P-element P[w]mC = ActGFP]JM3R, encoding green fluorescent protein (GFP) under the cytoplasmic actin promoter. For larval dissections, stoned males could be distinguished from FM7i males by lack of green fluorescence using a Leica MZ6 stereo microscope outfitted with a GFP fluorescence illuminator (Kramer Scientific, Elmsford, NY).

The neuronal Gal4 driver line elav1135 and the muscle driver line MHC Gal4 were from Corey Goodman’s lab (University of California, Berkeley). The 684 wing disc driver (Manseau et al. 1997) was from Danny Brower’s lab. We isolated elav1135 stn flies by genetic recombination. The UAS-synaptotagmin transgenic line UAS-synaptotagmin I (autosomal) was obtained from Troy Littleton (MIT) and the yw; P[w]mC = ActGFP]JM3R, was also obtained from Norreen Reist (Colorado State University).

Construction of stoned transgenes: The complete stoned cDNA was obtained by ligation of fragments from three different cDNA clones. Clone “p33” contained an EcoRI fragment of bases 1–1219 of the published sequence. Clone “fused” contained an EcoRI/PstI fragment of bases 1229–5808 and the third “4.8z3” contained a PstI fragment of bases 5809–8137. The full-length cDNA, named JC9814, was inserted into pBluescript SK+ by ligating p33 into the fused plasmid with EcoRI and ligating the 4.8z3 sequence in using PstI. For use in transgene rescue, 1.4 kb of the original 1.6-kb 3’ untranslated region (UTR) was removed. Briefly, JC9814 was digested with PstI and NcoI (from the polylinker) to remove bases 5809–8137. Primers were designed to amplify only from the PstI site at 5809 and to base 6714. The reverse primer had an artificial NcoI site included for cloning purposes. This amplified product was ligated into the digested JC9814 and was named TM001. This stoned cDNA, containing the entire coding sequence but only 211 bases of the 3’ untranslated sequence, was cloned into the transformation vector pUAST (Brand and Perrimon 1993) in a two-step process using the EcoRI and NcoI restriction sites.

Separate stonedA and B constructs were prepared in the laboratory of Kathleen Buckley from TM001. The full-length cDNA was digested with XbaI and NcoI, removing all of the
stonedB sequence and all sequences 3' of base 2446 in stonedA. Two primers were designed to amplify a product to replace the missing 3' fragment of stonedA. The forward primer incorporated the 2446 XbaI site and a reverse primer replaced the AcI site at 2665 and the first termination codon at 2670 with an additional engineered stop codon and a NotI site (ttagaagt taataaggggca). We were then able to ligate this entire stonedA sequence directly into pUAST using EcoRI and NotI. The Buck- ley lab prepared the stonedB fragment by digesting the full-length CDNA with AclI (2665) and ScaI (6550), a region that included the five intercistronic termination codons and the entire stonedB coding sequence with 46 bases of 3' UTR. The sites were then filled to make blunt ends and were cloned using EcoRV. We cloned this sequence into pUAST using EcoRI and NotI sites in the polylinker. We removed the intercistronic region of stop codons from the 5' end of this construct by amplifying a region from the ATG start site of stonedB (2724) to the PpomI site (3962). In this case, the forward primer contained an artificial EcoRI site so that this new fragment could be cloned directly into the plasmid using the existing EcoRI and PpomI sites. Both the stonedA and stonedB constructs in pUAST were sequenced to confirm that no errors were introduced during PCR amplification or cloning.

The stonedAB, stonedA, and stonedB constructs, respectively, were used to create transgenic P(SAB), P(SB), and P(SA) lines using Pelement-mediated embryonic germine transformation. Two independent SAB lines were obtained along with 23 SA lines and 22 SB lines. SAB1, SB5, and SA20 were used for the majority of the experiments.

Experimental and control animals for transgene rescue analysis: elav1350 stn86A/Sm130 or elav1350 stn1 homozygous virgin female larvae were crossed to yw/Y; P(stnX)/+ males of the genotype elav1350 stn1 homozygous transgenic males. To analyze phenotypic rescue by a given transgene, male progeny of the genotype elav1350 stn1/Y; P(stn1)/+ were selected and used. In the case of lethal alleles, males carrying the balancer were discarded. These animals were then compared to ORCB and elav1350 stn1/Y; +/+ animals obtained from a cross to yw/Y males from the background strain used to establish the transgenic lines. To generate stn86I mutant larvae, stn86I/Dp males were crossed to yC1DX/Y females, yielding males of the genotype stn86I/Y. These males survive at a very low frequency (~5%) and are developmentally delayed and smaller than their female siblings. (We were not able to generate elav1350 stn86I/Y males because escapers were never seen.)

To assess rescue using ubiquitous or muscle-specific enhancers, we used the shi Gal4 line (Staples and Ramaswami 1999) and the muscle-specific driver MHC Gal4 (Sanuval and Ramaswami 2002) lines, respectively. For each stoned allele, stn/FM7i; Gal4/Gal4 homozygous lines were generated and crossed to males homozygous for the transgene of interest. Again, efficiency of rescue was determined by comparing stn progeny from this cross with progeny obtained when similar females were crossed to transgene-free yw/Y males.

Analyzing Dysf transgenes for rescue of stn lethality: Rescue of stoned lethal phenotypes by neuronal or ubiquitous over-expression of Drosophila synaptotagmin I was assessed in stn86I and stn852. Stioned double mutants containing either elav1350 or shi Gal4 were crossed to either UAS-synaptotagmin I (auto- somal) males or yw; P[w+]UAS-SytI f (III) males and raised at 25°. The progeny of these crosses were examined for the presence of stn/Y males. No adult rescue was detected with either transgene, with either driver, in either mutant background (total n = 1862). Contrasting observations were previously reported when a different Gal4 driver was used (Ferguson and Broadie 2001).

Immunocytochemistry and confocal microscopy: Wandering third instar larvae were dissected to expose the abdominal body wall muscles, as described previously (Estes et al. 1996; Stimson et al. 1998, 2001). In brief, larval dissections were performed in Ca2+-free HL3 saline (Stewart et al. 1994) containing 0.5 mM EGTA and 21.5 mM MgCl2 to prevent muscle contraction. Analyses were restricted to synapses of muscles 6 and 7 of abdominal segments 2–3 (A2–A3). Dissected larvae were fixed in 3.5% paraformaldehyde and processed for antibody staining. Wing discs were prepared from larvae processed as above, except that they were visualized using FITC-conju- gated goat anti-rabbit secondary antibodies (ICN Biochemi- cals, Costa Mesa, CA) at a dilution of 1:200. They were removed from the preparation just prior to imaging and placed on slides treated with a diluted polylysine solution (Sigma Chemical, St. Louis). A PCM 2000 laser-scanning confocal microscope (Nikon, Melville, NY) and SimplePCI software (C Imaging, Cranberry Township, PA) was used for image acquisition.

StonedA antisera (Andrews et al. 1996; Stimson et al. 1998) and StonedB antiserum (3500; Stimson et al. 2001) were used at a final dilution of 1:1000. Anti-stonedB and anti- stonedA were visualized using an Alexa 568 goat anti-rabbit antibody (Molecular Probes, Eugene, OR) at a dilution of 1:1000. For examination of the distribution of synaptotagmin within synaptic boutons, we stained the larval preparation with rabbit anti-syt antibody (DSVT2, from Hugo Bellen, Baylor College of Medicine, Houston) at a concentration of 1:200–300 and visualized with Alexa 488 goat anti-rabbit (Molecular Probes). Confocal sections, 0.5μm thick, were collected at ×100 power, ×5 zoom.

Identification and analysis of satellite boutons: For analyses of satellite boutons, larval preparations were double labeled with a monoclonal antibody to synaptotagmin I (1:500; from Kaushiki Menon and Kai Zinn, California Institute of Technol- ogy) visualized with Alexa 568 goat anti-mouse antibody and FITC-conjugated anti-HRP (1:100; ICN). Any single bouton that was not included in a chain of boutons, but instead appeared to be a lateral sprout, was counted as a satellite bouton. Although not regarded as criteria in this study, satellites typi- cally were observed sprouting from larger boutons rather than from axons and were usually much smaller than these “parent” boutons.

Electrophysiology and data analysis: For electoretinograms, flies were anesthetized by cooling them briefly on ice and then mounted upright in modeling clay such that the right eye was exposed. The ground electrode, a heat-pulled glass capillary filled with 3 M KCl, was inserted into the back of the fly’s head. The recording electrode, a similarly pulled glass capillary, was advanced until it lightly touched the surface of the fly’s compound eye. Electrode resistances for both elec- trodes were between 5 and 10 MΩ. Flies were routinely allowed to recover in the dark for at least 15 min prior to recording. Electoretinograms (ERGs) were induced with flashes of light. Data were acquired using an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA) and digitized with a Digitdata 200 board. All traces were filtered and analyzed using the pClamp6 software (Axon Instruments) and assembled using Adobe Photoshop.

Electrophysiological recordings were made from muscle 6 in the third abdominal segment (A5) in HL3 saline (in milli- molars: 70 NaCl, 5 KCl, 1.5 CaCl2, 20 MgCl2, 10 NaHCO3, 5 trehalose, 115 sucrose, and 5 Hepes, pH 7.5). Excitatory junctional potentials (EJPs) were measured by stimulating the motor nerve with a glass suction electrode. An isolated pulse stimulator (A-M Systems, Everett, WA) delivered 1-msec pulses at 1 Hz at a voltage above threshold to stimulate both motor neurons innervating muscle 6. Recordings were taken using an Axoclamp 2B amplifier and pClamp6 software (Axon Instruments). Intracellular glass electrodes were pulled using a
Sutter Instruments (Novato, CA) electrode puller. Electrodes were filled with 3 M KCl and had resistances of 15–30 MΩ. After electrode insertion into muscle 6, resting membrane potential of muscles measured −60 to −80 mV. EJP amplitude was measured by Mini Analysis software (Synaptosoft, Decatur, GA), which averaged amplitudes from at least 20 evoked responses.

**FM1-43 loading:** For FM1-43 loading of stn^{8P1}/Y synaptic boutons, dissected larvae were placed in normal HL3 saline containing 4 μM FM1-43. The segmental motor nerve was then stimulated at 5 V, 30 Hz for 2 min. Immediately following the end of stimulation, noninternalized FM1-43 was rinsed away by several washes in Ca²⁺-free HL3 saline. Stained boutons were viewed using a water immersion lens with a Zeiss Axioscope fluorescence compound microscope (Frankfurt, Germany). Digital images were acquired with a cooled CCD camera (Princeton Instruments, NJ) controlled by MetaMorph Imaging software (Universal Imaging, West Chester, PA). Immediately after imaging, the preparation was fixed and processed for anti-HRP immunohistochemistry.

**Data analysis and statistics:** The error measurements are reported as standard error of the mean (SEM). Statistical significance was determined by Student’s t-test.

**RESULTS**

**Defining a dicistronic stoned transgene, stonedAB, that rescues stoned lethality:** To identify minimal coding sequences and promoter elements required for providing essential stoned functions, we initially created a dicistronic stoned cDNA by appropriately ligating stoned cDNA by appropriately ligating stoned coding sequences isolated from partial cDNA clones or amplified cDNA fragments (MATERIALS AND METHODS; Figure 1A). This dicistronic cDNA, termed stonedAB, was cloned into the Drosophila transformation vector pUAST, under control of the yeast transcription factor Gal4 (BRAND and PERRIMON 1993). Through germline transformation, multiple transgenic lines carrying this construct were generated. Experiments described below indicated that this stonedAB cDNA encodes all essential stoned functions.

To test the ability of the Gal4-responsive P[stenAB] (“responder”) transgenes to provide stoned functions in vivo, they were crossed into stoned mutant backgrounds either alone or in the presence of “driver” transgenes that drive Gal4 expression ubiquitously (shi Gal4), specifically in neurons (elav^{C359}), or specifically in muscles (MHC Gal4; LIN and GOODMAN 1994; STAPLES and RAMASWAMI 1999; ESTES et al. 2000). The ability of the transgenes to rescue lethality of mutants stn^{8P1} and stn^{13-120} was analyzed. The stn^{5120} allele carries a large insertion in the vicinity of stonedA coding sequences (ANDREWS et al. 1996); this mutation disrupts the normal stoned transcript (ANDREWS et al. 1996), eliminating all detectable stonedA and stonedB protein in the embryonic nervous system (FERGESTAD et al. 1999), and causes late...
embryonic lethality. Although there is no established null allele for stonel, existing data indicate that stn$^{15-120}$ must be at least a strong hypomorph, causing severely reduced expression of both stoned products (Andrews et al. 1996). The other allele, stn$^{P}$, molecularly uncharacterized, causes early larval lethality (Mirkos et al. 1987); it is similar to stn$^{15-120}$ in reducing presynaptic stonedA and stonedB to levels undetectable at motor terminals of rare, escape, third instar stn$^{P}$ larvae (Stimson et al. 2001).

Neural, but not muscle, expression of stonedAB is sufficient to restore complete viability to stn$^{P}$ and stn$^{15-120}$ (Table 1, data shown for line SAB1). This indicates that essential functions of stoned revealed by these mutations are limited to the nervous system. Synapses of stn$^{P}$ and stn$^{15-120}$ larvae rescued by neural stonedAB expression reveal the presence of wild-type levels of both stonedA and stonedB proteins (Figure 1B). Together with the observation that our artificial stonedAB transcript lacking native 5′ and most of the 3′ untranslated mRNA sequences can functionally replace stoned, this suggests that correct translation of the native dicistronic transcript does not depend on unique noncoding elements present in these sequences of mRNA.

**Neural expression of stonedAB rescues synaptic defects of stoned mutants:** To determine cellular functions of stoned provided by neuronal stonedAB expression, we analyzed in detail the effects of P[stonAB] (SABI) expression on various previously described electrophysiological and immunocytochemical phenotypes of lethal (stn$^{P}$ and stn$^{15-120}$) and viable (stn$^{8P1}$) stoned mutants. At third instar larval neuromuscular junctions (NMJs), phenotypes in stn$^{8P1}$ and stn$^{P}$ associated with altered synaptic-vesicle recycling include: (i) reduced evoked transmitter release, (ii) increased synaptotagmin immunoreactivity on the axonal membrane, and (iii) reduced levels of stonedA and/or stonedB proteins (Stimson et al. 1998, 2001). Similar phenotypes have been described at stn$^{15-120}$ embryonic motor synapses (Fergestad et al. 1999). All of these mutant phenotypes are completely rescued by neural expression of the SABI transgene. Under appropriate conditions, EJPs are a good measure of transmitter release (see MATERIALS AND METHODS). EJP amplitudes, 11.1 ± 1.3 mV and 5.5 ± 0.7 mV in stn$^{8P1}$ and stn$^{P}$, are increased following SABI expression to 42.1 ± 2.0 mV and 45.8 ± 2.2 mV, respectively (P rescue < 0.40, 0.63), values indistinguishable from the 44.8 ± 2.1 mV of the wild-type controls (Figure 2A). Similarly, neuronal SABI expression restores normal levels and distribution of synaptotagmin to stn$^{8P1}$ and stn$^{P}$ nerve terminals (Figure 2B). Also significantly, stn$^{15-120}$ animals rescued by neural expression of stonedAB develop into third instar larvae (subsequently to adults) with normal viability and synaptic physiology and morphology (Table 1, Figure 1B, Figure 2). These data indicate, first, that stonedAB encodes all functions required for previously described functions of stoned and, second, that potential stoned expression in postsynaptic muscle is not required for regulating essential aspects of synaptic function.

**Neural stonedB expression restores viability and synaptic transmission to stoned mutants:** Because the rescuing stonedAB transgene encodes both stoned products, it was of particular interest to determine if one or both stoned polypeptides were required for organismal and presynaptic functions of stoned. To address this question, we generated transgenic flies expressing either stonedB or stonedA under Gal4 control (MATERIALS AND METHODS) and used them to analyze effects of neurally expressing individual stoned products on phenotypes of various stoned alleles.

**TABLE 1**

Neural expression of P[stonAB] rescues stn lethal alleles

<table>
<thead>
<tr>
<th>Cross</th>
<th>Actual stn/Y progeny (% of total)</th>
<th>Expected stn/Y assuming normal viability (%)</th>
<th>Total no. progeny scored</th>
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<tr>
<td>C155 stn$^{P}$/FM7i × wu</td>
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<td>510</td>
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<tr>
<td>C155 stn$^{P}$/FM7i × P[stonAB]</td>
<td>27</td>
<td>25</td>
<td>549</td>
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<tr>
<td>stn$^{P}$/FM7i; shi Gal4 × P[stonAB]</td>
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<td>25</td>
<td>267</td>
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<tr>
<td>C155 stn$^{15-120}$/FM7i × wu</td>
<td>0</td>
<td>25</td>
<td>551</td>
</tr>
<tr>
<td>C155 stn$^{15-120}$/FM7i × P[stonAB]</td>
<td>22</td>
<td>25</td>
<td>615</td>
</tr>
<tr>
<td>stn$^{15-120}$/FM7i; shi Gal4 × P[stonAB]</td>
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<td>431</td>
</tr>
<tr>
<td>stn$^{15-120}$/FM7i; MHC Gal4 × P[stonAB]</td>
<td>0</td>
<td>25</td>
<td>293</td>
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</tbody>
</table>

Neural expression of P[stonAB] rescues stn lethal alleles.
discussed in more detail in Figure 4) is equally consistent with a model in which small amounts of stonedA are also required. Also important, these data alone do not exclude the possibility that stonedA and stonedB have overlapping, redundant functions.

Neural expression of stonedA is not sufficient for rescuing stoned phenotypes: To identify potential synaptic functions of stonedA, we generated stoned mutants expressing stonedA transgenes in the nervous system. In contrast to stonedB expression, stonedA did not alter the lethal phenotype of stn8P1 and stn13-120. This lack of rescue was a common property of four independent stonedA transgenes that we tested (SA1, SA5, SA19, SA20). Furthermore, ERG recordings (see below) were used as a rapid screen for rescue for 14 additional lines with the same result. The transgenes expressed stonedA protein efficiently in wing discs when crossed to the wing Gal4 driver 684 (Figure 4A). Strong stonedA immunoreactivity was observed in a reticulate pattern in expressing cells of wing imaginal discs (Figure 4A shows data for the SA19 and SA20 transgenes). Similar levels of stoned immunoreactivity, absent in the 684 driver control discs, were also observed when the rescuing SAB1 transgene was crossed to the 684 driver. These data support the conclusion that, in the absence of stonedB, the stonedA protein is translated from its monocistronic mRNA, but thereafter unable to provide essential synaptic functions of stoned.

To determine potential contributions of stonedA expression to synaptic functions, we further examined effects of stonedA expression on synaptic physiology and synaptotagmin distribution at stn mutant larval synapses. For reasons likely involving specific genetic backgrounds, we were unable to obtain any viable “escaper” stn8P1 mutant third instar larvae that expressed stonedA. However, stnC larvae expressing stonedA transgenes were obtained and their synaptic properties compared to stnC mutants alone. In contrast to stonedB, which completely restored mutant nerve terminals to wild-type function and morphology, stonedA expression had no effect on either the mutant EJP or plasma-membrane distribution of synaptotagmin. EJP values for stnC larvae expressing stonedA transgenes were 8.3 ± 0.4 mV, not significantly different from the 11.1 ± 1.5 mV observed for appropriate stnC controls (Figure 4B). The exaggerated plasma membrane distribution of synaptotagmin seen in stnC mutants was also seen in the presence of neurally expressed stonedA (Figure 4C). To test whether stonedA might encode a function required at central synapses, which may differ from the NMJ, we compared effects of neural stonedA and stonedB expression on the synaptic on-and-off transient components of the stnC ERG, an extracellularly recorded ensemble response of the adult visual system to a brief light flash (PETROVICH et al. 1993). While stonedB expression completely restored the missing synaptic components of the stnC ERG, stonedA expression had no effect (Figure 4D).
These experiments could indicate that either stonedA at nerve terminals cannot restore synaptic functions missing in stn^C mutants or stonedB is required for the transport, localization, or stability of stonedA. To address this issue, we examined synapses of stn^C mutants with a neurally driven SA transgene for stonedA and stonedB immunoreactivity. Unexpectedly, presynaptic stonedA was not obviously increased following neural expression of stonedA alone via SA5, SA19, or SA20 transgenes. However, when stonedA was coexpressed with stonedB by combining the SA5 and SB15 transgenes in an elav^{C155} stn^C background, substantially elevated levels of presynaptic stonedA were apparent (Figure 4E). Thus, the stable presence of stonedA at nerve terminals requires stonedB. For this reason, our studies of stonedA transgenes provide only limited insight into functions of presynaptic stonedA. However, because essential stoned functions occur under conditions of highly reduced stonedA, it appears more likely that stonedA functions at synapses are either modest or redundant.

**A novel function for stoned in synaptic growth is also provided by stonedB:** While examining the effect of stoned transgenes on various phenotypes associated with altered synaptic-vesicle recycling, we observed an unexpected consequence of the stn^P1 mutation on the structure of presynaptic motor terminals. Dramatic alterations in presynaptic architecture, specifically the abundance and occasional proliferation of small, bud-like boutons from a morphologically normal bouton, are obvious in stn^P1 (Figure 5). Similar unusual “satellite” boutons emanating from “parent boutons” have been recently observed in Drosophila strains overexpressing specific forms of the Alzheimer’s amyloid precursor protein ortholog APPL and are hypothesized to represent early stages of branch formation and activity-dependent synaptic growth (Torroja et al. 1999; Zirro et al. 1999).

Like satellite boutons in APPL-overexpressing strains, those in stn^P1 contain components required for active neurotransmitter release, including synaptotagmin (Figure 5C) and csp (data not shown). To directly examine whether satellite boutons are functional in stn^P1/Y terminals, we used the fluorescent dye FM1-43 that labels actively cycling synaptic vesicles. Both parent and satellite boutons are labeled with FM1-43 in response to nerve stimulation, indicating that all components required for evoked vesicle fusion and subsequent recycling are present in these unusual varicosities (Figure 5C). These morphological defects observed in the stn^P1 mutant strain map to a mutation in the same region as stoned, as they are complemented by the duplication mal^Y (Figure 5B). Mutant stn^P1 terminals show 5.7 ± 0.51 satellites compared to 2.8 ± 0.47 in stn^P1/mal^Y (P < 0.007).

To determine the stoned product(s) involved in regulating bouton morphology, we tested the ability of neurally driven SAB1 and SB5 expression to rescue this phenotype (Figure 5B). As for other defects in stn mutants, the stonedAB and stonedB transgenes completely rescued the aberrant bouton phenotype (satellite bouton frequency 2.3 ± 0.30 and 3.3 ± 0.61, corresponding to P_{reca} values of <0.002 and <0.03, respectively). As considered below, this provides support for a model in which the stoned locus, and stonedB in particular, has previously unappreciated functions in membrane traffic events distinct from synaptic-vesicle recycling.

**DISCUSSION**

A dicistronic mRNA from the Drosophila stoned locus is translated to produce two proteins, stonedA and stonedB. The first is a poorly conserved molecule with no obvious homolog in *C. elegans* and mammals; the second is a founding member of a new, widely conserved family of proteins called stonins. Previous analyses have demonstrated that at least one or both stoned products are required for regulating normal synaptic-vesicle recycling and, thereby, synaptic transmission and the distribution of synaptic-vesicle proteins. Experiments described here make three important points. First, stoned function is essential only in the nervous system. Second, while stonedA is presynaptically localized, its stable presence at nerve terminals is not only largely dispensable, but also dependent on the expression of stonedB. In contrast, transgenically provided stonedB provides all essential molecular activities missing in viable and lethal stoned alleles. Finally, the stoned locus, and likely stonedB, has a previously unrecognized function in regulating the structure of synaptic boutons. These points...
are discussed below in the context of the genetics of stoned, cellular functions of stonins, and the evolution and regulation of the dicistronic stoned mRNA.

**Insights into molecular functions of stoned and its products:** Complete rescue of stoned lethal alleles by neuronal expression of a stoned cDNA strongly argues that the major function of stoned is in the nervous system. This result is consistent with two previous observations that suggest a neural-specific function for stoned. First, in an elegant genetic scheme for generating mosaic (gyn-andromorph) animals carrying both female $stn^{15-129}$/+ and male $stn^{15-120}$ tissue, viable mosaic animals with large patches of $stn^{15-120}$ nervous tissue were never obtained under conditions where mosaics with large mutant patches of nonneuronal tissue were frequent (Petrovich et al. 1993). The apparently normal development of nonneuronal mutant tissue argued that stoned functions in these cell types must be modest or dispensable (Petrovich et al. 1993). Second, a recent study observes that overexpression of a synaptotagmin (DsytI) transgene in neurons restores partial viability to $stn^{15-120}$ (Ferguson and Broadie 2001). This suppression, however, is either limited or dependent on the specific transgenes and/or the mutant strain backgrounds that were utilized. Both neural-restricted elav-Gal4 and ubiquitous shi Gal4-driven expression of stoned cDNA completely rescue the lethality of $stn^{15-11}$ and $stn^{15-120}$. In contrast, similar expression of DsytI transgenes (Littleton et al. 1999; Mackler and Reist 2001) has no effect on viability of $stn$ mutants under conditions used in our experiments (materials and methods). Our demonstration that stoned cDNA expression in neurons is sufficient for restoring normal viability and synaptic function to stoned lethal alleles thus confirms and extends previous studies of this locus.

A more detailed analysis of artificial monocistronic stoned cDNAs encoding either stonedA or stonedB reveals that neural expression of stonedB alone is sufficient to reproduce all of the effects observed with a full-length dicistronic cDNA. Our favored interpretation, that the second cistron of stoned encodes all vital and important stoned functions, is limited by the absence of a well-defined stoned null background in which the transgene analyses should ideally be performed. It could be argued that $stn^{15-120}$ retains some residual stonedA activity that contributes to the ability of neurally expressed stonedB to rescue $stn^{15-120}$ phenotypes, but two lines of evidence argue against this possibility. First, the $stn^{15-120}$ mutation comprises an insertion in the 3' end of the stonedA-encoding cistron; thus, the mutation should substantially reduce ORF1 function (Andrews et al. 1996). Second, immunofluorescence analysis (Figure 3B) demonstrates that stonedB transgene expression in $stn^{15-120}$ results in viable animals with morphologically and functionally normal presynaptic terminals that, importantly,

![Figure 3](image-url)

**Figure 3.—** Neural expression of a truncated cDNA containing only StonedB coding sequences (a P[stenB] transgene) is sufficient to rescue mutant synaptic phenotypes in $stn^{15-11}$, $stn^{15-120}$, and $stn^{15-120}$. (A) Mean EJP amplitudes in mutants before and after rescue with P[stenB]. Error bars represent SEMs. (B) StonedB protein levels are restored to wild type in $stn$ mutants where they were previously undetectable (see Figures 1B and 4E for prerescue levels). Small but unambiguous increase in levels of presynaptic stonedA are also seen in these mutants following neural P[stenB] expression. (C) Synaptotagmin distribution. Rescue with the P[stenB] transgene restores wild-type localization of synaptotagmin at the larval neuromuscular junction (see Figure 2B for prerecued localization).
Figure 4.—Neural expression of StonedA coding sequences alone does not alleviate any of the known defects in stoned mutants. (A) Lethality of the other stoned alleles, stn\textsuperscript{8P1} and stn\textsuperscript{13-120}, was not rescued by the P[stnA] transgene although it expressed stonedA protein in wing discs when controlled with the Gal4 driver 684 (MATERIALS AND METHODS). (B) Mean EJP amplitudes for wild-type and stn\textsuperscript{C} mutants are shown before and after neural expression of P[stnA]. Stn\textsuperscript{C} larvae expressing P[stnA] have EJPs with amplitudes indistinguishable from stn\textsuperscript{C}. Error bars represent SEMs. (C) P[stnA] does not rescue the synaptotagmin mislocalization phenotype seen in stn\textsuperscript{C}. (D) In electroretinogram recordings, synaptic on/off transients missing in stn\textsuperscript{C} are restored by P[stnB] transgenes but not by P[stnA]. (E) Barely detectable levels of stonedA observed in stn\textsuperscript{C} larval synapses (top two rows; STIMSON et al. 1998) are not perceptibly increased after P[stnA] transgene expression in neurons (third row). Remarkably, presynaptic stonedA protein is restored to wild-type levels when both P[stnB] and P[stnA] transgenes are expressed simultaneously in the nervous system.

are still substantially deficient in stonedA. The same is true of stn\textsuperscript{8P1} animals rescued by a stonedB transgene. Thus, our data are more consistent with a model in which stonedB alone performs all identified presynaptic and organismal functions of stoned.

What then might be the function of stonedA? Our data indicate that stonedA expression alone is not sufficient to rescue any documented mutant phenotype of stoned and that stonedA is largely dispensable for organismal viability and presynaptic function. However, a direct analysis of stonedA function is limited by our observation that the stable presence of stonedA at presynaptic terminals requires stonedB (Figures 3B and 4C). Thus, we were unable to assess stonedA functions in the absence of stonedB. Our current analysis does not exclude the possibility that stonedA has molecular functions that overlap with or facilitate those of stonedB. This possibility is consistent with previous coimmunoprecipitation experiments indicating association of stonedA and stonedB in a common molecular complex and shared association of both stonedA and stonedB with the synaptic-vesicle protein synaptotagmin (PHILLIPS et al. 2000).

The issue of stonedA function is further considered in the last section of the DISCUSSION.

Functions of the stonin family of proteins: Our analysis of stonedB function is particularly relevant as it constitutes the first in vivo functional analysis of a member of the stonin family of proteins. Our data predict that the stonins in general will be found to regulate endocytosis of synaptic-vesicle proteins and that stonin-deficient synapses will display phenotypes of stoned mutants. Indeed stonin genes may be good candidates for certain congenital myasthenic syndromes, a class of human genetic diseases that interrupt neuromuscular transmis-
Figure 5.—A function for the stoned locus in synaptic development is revealed by unique morphological defects in stn8P1. (A) “Satellite boutons” (arrows) in stn8P1 synapses are revealed by anti-HRP staining. (B) Histogram of quantified data shows that stn8P1 larvae have significantly more satellite boutons per A3 hemisegment than do wild type. This phenotype is rescued by a duplication on the Y chromosome that carries a wild-type copy of stn. The phenotype is similarly rescued by the neural expression of either the P[stnAB] or the P[stnB] transgene. (C) Satellite boutons contain functional synaptic vesicles and other components of the synaptic-vesicle machinery as they are labeled with an endocytic tracer FM1-43 dye following nerve stimulation. A bouton with two satellites in a live terminal imaged after loading with FM1-43 (left) has been fixed and stained with anti-HRP (right).

Our experiments address this issue in two ways. First, the observation that stonedB expression in the nervous system restores normal viability to otherwise lethal alleles of stoned argues for a neural, if not synapse-specific, function for the protein. Nonneuronal functions of stonedB, if any, must be dispensable. However, the second observation that stonedB is also required for regulating morphological changes in boutons associated with synaptic growth (Torroja et al. 1999; Zito et al. 1999; Estes et al. 2000; Roos et al. 2000) suggests a role for stonedB in events not limited to synaptic-vesicle recycling. Satellite boutons similar to those we describe in stn8P1 are found in synapses of Drosophila overexpressing the wild type, but not in an endocytosis-defective form of the Drosophila amyloid precursor protein homolog appl (Torroja et al. 1999). Thus, it is possible that stonedB influences endocytosis of APPL or other growth-related cell surface molecules that are part of a normal pathway for structural synaptic change.

Given the reported ubiquitous expression of mammalian stonins in multiple cell types and the ability of an overexpressed dominant-negative stonin to interfere
with endocytosis in nonneuronal cells, it is possible that mammalian stonins have wider functions (Martina et al. 2001). Perhaps stonins, initially selected for a specialized task like synaptic-vesicle recycling, have since evolved and diversified to be capable of broad, general functions in endocytosis. The concurrent proliferation of synaptotagmin-encoding genes in mammals (Sudhof 2002) may have contributed to diversification of stonin functions in mammalian species.

Evolution and significance of dicistronic organization of stoned: The stoned dicistronic mRNAs in eukaryotes are a genetic oddity whose functions and evolution are poorly understood (Blumenthal 1998). Unlike most polycistronic mRNAs that are processed to yield individual monocistronic mRNAs, the mature stoned transcript exists in a dicistronic form (Andrews et al. 1996; Blumenthal 1998; Blumenthal et al. 2002). Potential reasons suggested for this organization of the stoned mRNA include (a) maintenance of stoichiometry and (b) facilitation of dimer formation between the two proteins because of spatially associated translation of the two proteins. Biochemical experiments demonstrating that the two proteins may be found in a single complex provide some support for these hypotheses (Phillips et al. 2000).

Neither of these hypotheses are supported by our observations. First, our experiments clearly demonstrate that stoichiometry is not an important factor in stoned function. Animals in which stonedA-stonedB stoichiometry is severely altered show completely normal viability and synaptic function. Second, we show that splitting the two cistrons of stoned into the two constituent ORFs encoding stonedA and stonedB separately allows stonedB-dependent localization of stable stonedA at nerve terminals. This argues that selective pressure to maintain the dicistronic organization of stoned is not particularly strong and may not be driven by the two previously suggested mechanisms.

Additional data pertinent to the evolution of this dicistronic mRNA are provided by analyzing the conservation of stonedA and stonedB coding sequences in other species. While stonedB is conserved across metazoa, the only clear stonedA homolog known is found encoded in the genome of the mosquito *Anopheles gambiae* (~45% identical). Like its fruit fly counterpart, mosquito stonedA has five conserved DPF motifs plus a sixth DPF not found in the fruit fly. However, the potential leucine zipper motif of fruit fly stonedA (Stimson et al. 1998) is not conserved. In mosquito, the stonedA coding cistron lies no more than 39 bases upstream of an identically oriented stonedB coding cistron; thus, the data are consistent with the existence of a conserved dicistronic organization in insects. Because nematode and mammalian genomes have monocistronic orthologs for stonedB but not for stonedA, it is possible that the dicistronic stoned mRNA originated in arthropods some time after divergence from the vertebrate lineage, but before the divergence of Drosophila from Anopheles. Combined with our data, these observations suggest that there may not be strong functional reasons for the evolutionary conservation of stonedA.

One remarkable conserved feature of stonedA sequence both in mosquitoes and in Drosophila is the complete absence of internal methionine residues in the coding sequence. In a single 900-amino-acid protein the probability of such an absence occurring by chance alone is \( \sim 7 \times 10^{-7} \), if one makes the simplistic assumption that all codons occur at an equal frequency (63/64). Given its conservation in mosquito, it appears likely that this unusual feature of stonedA coding sequences is relevant to the mechanism by which the dicistronic mRNA is translated into two different proteins. While our experiments do not address this mechanism, the definition of a single dicistronic cDNA including intercistronic sequences sufficient to direct translation of the two stoned proteins should facilitate, in future, the detailed analysis of molecular mechanisms that allow the unusual translation of this mRNA.

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**LITERATURE CITED**


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