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

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Manuscript received March 5, 2003
Accepted for publication May 2, 2003

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* trangene is sufficient for rescuing various phenotypic 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 Bonifacino 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 $\mu 2$, and a small subunit $\sigma 2$; 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 Bonifacino 2001).

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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 to 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 w^{1118}. The strain used for mapping and balancing transgenes, yw^{67C2}*; Sp/SM5,Cy; Sb/TM3,Ser*, was also obtained from the Brower Lab.

Stoned mutants stn^{c} and $stn^{13\text{-}120}$ were from our collection and stn^{8P1} was obtained from Norbert Perrimon (HHMI, Harvard Medical School). stn^{c} was maintained as a homozygous stock whereas stn^{8P1} and $stn^{13\text{-}120}$ were maintained over the *FM7i* balancer chromosome (Bloomington Stock Center), which carries the *P*-element *P{w[mC} = ActGFP]JMR3*, encoding green fluorescent protein (GFP) under the cytoplasmic *actin* promotor. 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). stn^{8P1} was also maintained over a modified Y chromosome $Dp(1,Y)y^{+}mal^{+}$ (abbreviated *Dp*), which contains a *stoned* duplication.

The neuronal Gal4 driver line $elav^{C155}$ 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 $elav^{C155}$ *stn* flies by genetic recombination. The UAS-synaptotagmin transgenic line *UAS-synaptotagmin I* (autosomal) was obtained from Troy Littleton (MIT) and the *yw;* $P[w^{+} UAS\text{-}Syt^{+}]$ (III) line was a gift of Noreen 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 *Eco*RI fragment of bases 1–1219 of the published sequence. Clone "fused" contained an *Eco*RI/*Pst*I fragment of bases 1220–5808 and the third "4.8z3" contained a *Pst*I fragment of bases 5809–8137. The full-length cDNA, named JC9814, was inserted into pBluescript SK+ by ligating p33 into the fused plasmid with *Eco*RI and ligating the 4.8z3 sequence in using *Pst*I. 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 *Pst*I and *Not*I (from the polylinker) to remove bases 5809–8137. Primers were designed to amplify only from the *Pst*I site at 5809 to base 6714. The reverse primer had an artificial *Not*I 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 *Eco*RI and *Not*I restriction sites.

Separate stonedA and B constructs were prepared in the laboratory of Kathleen Buckley from TM001. The full-length cDNA was digested with *Xba*I and *Not*I, 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 *Xba*I site and a reverse primer replaced the *Acl*I site at 2665 and the first termination codon at 2670 with an additional engineered stop codon and a *Not*I site (tcgaacgt taataagcggcca). We were then able to ligate this entire stonedA sequence directly into pUAST using *Eco*RI and *Not*I. The Buckley lab prepared the stonedB fragment by digesting the full-length cDNA with *Acl*I (2665) and *Sca*I (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 *Eco*RV. We cloned this sequence into pUAST using *Eco*RI and *Not*I 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 *Ppu*MI site (3962). In this case, the forward primer contained an artificial *Eco*RI site so that this new fragment could be cloned directly into the plasmid using the existing *Eco*RI and *Ppu*MI 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 *P*-element-mediated embryonic germline 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: $elav^{C155}$ $stn^{lethal}/FM7i$ or $elav^{C155}$ stn^{C} homozygous virgin females were crossed to *yw/Y; P[stnX]* homozygous transgenic males. To analyze phenotypic rescue by a given transgene, male progeny of the genotype $elav^{C155}$ *stn/Y; P[stn]/+* were selected and studied. In the case of lethal alleles, males carrying the balancer were discarded. These animals were then compared to *ORCB* and $elav^{C155}$ *stn/Y*; +/+ animals obtained from a cross to *yw/Y* males from the background strain used to establish the transgenic lines. To generate stn^{8P1} mutant larvae, stn^{8P1}/Dp males were crossed to *yfC(1)DX/Y* females, yielding males of the genotype stn^{8P1}/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 $elav^{C155}$ stn^{8P1}/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* (Sanyal and Ramaswami 2002) lines, respectively. For each *stoned* allele, *stn/FM7i; Gal4/Gal4* homozyous 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 *DsytI* transgenes for rescue of *stn* lethality: Rescue of stoned lethal phenotypes by neuronal or ubiquitous overexpression of Drosophila synaptotagmin I was assessed in stn^{8P1} and $stn^{13\text{-}120}$. Stoned double mutants containing either $elav^{C155}$ or *shi Gal4* were crossed to either *UAS-synaptotagmin I* (autosomal) males or *yw; P[w$^+$ UAS-Syt$^+$]* (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 (Fergestad 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 Ca^{2+}-free HL3 saline (Stewart *et al.* 1994) containing 0.5 mm EGTA and 21.5 mm $MgCl_2$ 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-conjugated goat anti-rabbit secondary antibodies (ICN Biochemicals, 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 antiserum (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 examining the distribution of synaptotagmin within synaptic boutons, we stained the larval preparation with rabbit anti-syt antibody (DSYT2, 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, 0.5 μm thick, were collected at ×100 power, ×3 zoom.

Identification and analysis of satellite boutons: For analyses of satellite boutons, larval preparations were double labeled with a monoclonal antibody to synaptotagmin (1:500; from Kaushiki Menon and Kai Zinn, California Institute of Technology) 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 typically were observed sprouting from larger boutons rather than from axons and were usually much smaller than these “parent” boutons.

Electrophysiology and data analysis: For electroretinograms, 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 electrodes were between 5 and 10 MΩ. Flies were routinely allowed to recover in the dark for at least 15 min prior to recording. Electroretinograms (ERGs) were induced with flashes of light. Data were acquired using an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA) and digitized with a Digidata 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 (A3) in HL3 saline (in millimolars: 70 NaCl, 5 KCl, 1.5 $CaCl_2$, 20 $MgCl_2$, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, and 5 HEPES, pH 7.3). 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

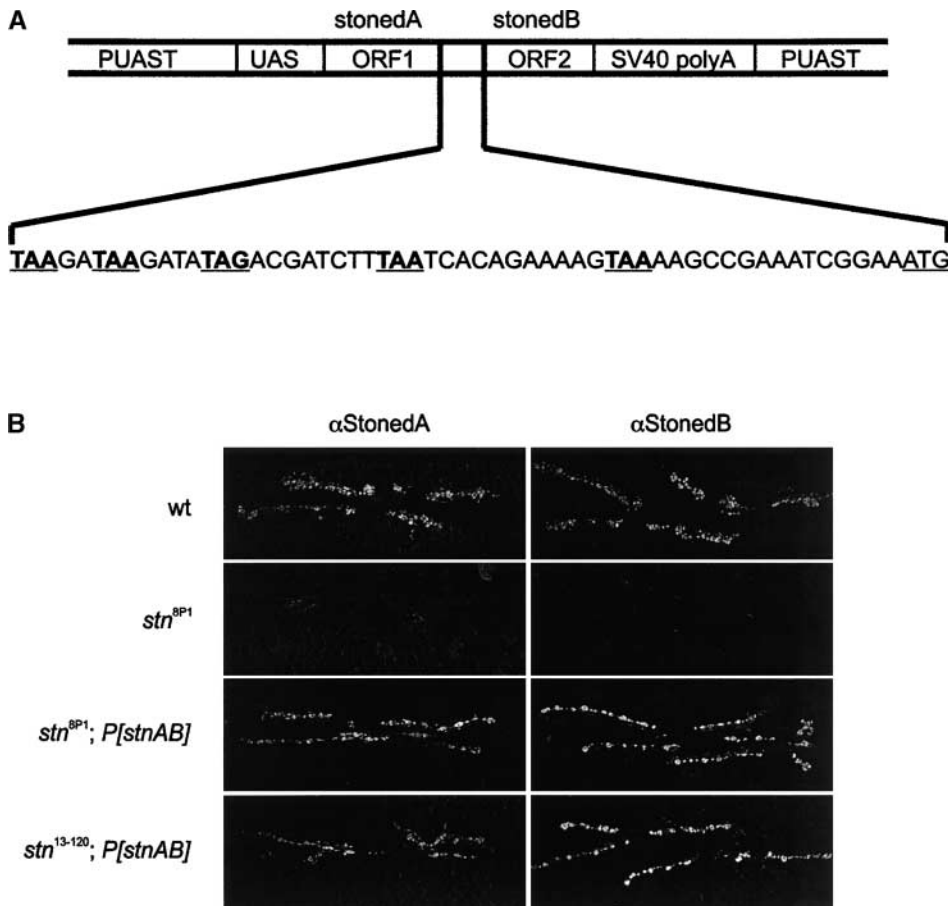

Figure 1.—Neural expression of a full-length dicistronic *stoned* cDNA (*stonedAB*) transgene (*P[stnAB]*) restores both StonedA and StonedB proteins to stn^{8P1} and $stn^{13\text{-}120}$ mutant presynaptic terminals. (A) Organization of the *P[stnAB]* construct with details of the 55-bp intercistronic element that contains a total of five termination codons in all three frames (shown in boldface type). (B) StonedA and stonedB at presynaptic terminals (top), which are missing in terminals of stn^{8P1} "escapers" (second row and Stimson *et al.* 2001), are restored (third row) by expressing the *P[stnAB]* transgene under the control of the neural *elav* promoter. Similar restoration of stoned proteins is seen for the embryonic lethal allele $stn^{13\text{-}120}$ (bottom). All stonedA images and stonedB images are taken at identical gain and aperture settings so the displayed brightness of staining roughly represents the amount of presynaptic protein.

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 Ca^{2+}-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* 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 contruct were generated. Experiments described below indicated that this stonedAB cDNA encodes all essential *stoned* functions.

To test the ability of the Gal4-responsive *P[stnAB]* ("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 ($elav^{C155}$) 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 $stn^{13\text{-}120}$ was analyzed. The $stn^{13\text{-}120}$ 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

TABLE 1

Neural expression of *P[stnAB]* rescues *stn* lethal alleles

Cross	Actual *stn*/Y progeny (% of total)	Expected *stn*/Y assuming normal viability (%)	Total no. progeny scored
C155 stn^8P1^/FM7i × yw	0	25	510
C155 stn^8P1^/FM7i × P[stnAB]	27	25	549
stn^8P1^/FM7I; shi Gal4 × P[stnAB]	22	25	267
C155 stn^13-120^/FM7i × yw	0	25	551
C155 stn^13-120^/FM7i × P[stnAB]	22	25	615
stn^13-120^/FM7i; shi Gal4 × P[stnAB]	32	25	431
stn^13-120^/FM7i; MHC Gal4 × P[stnAB]	0	25	293

embryonic lethality. Although there is no established null allele for *stoned*, existing data indicate that $stn^{13\text{-}120}$ must be at least a strong hypomorph, causing severely reduced expression of both stoned products (Andrews *et al.* 1996). The other allele, stn^{8P1}, molecularly uncharacterized, causes early larval lethality (Miklos *et al.* 1987); it is similar to $stn^{13\text{-}120}$ in reducing presynaptic stonedA and stonedB to levels undetectable at motor terminals of rare, escaper, third instar stn^{8P1} larvae (Stimson *et al.* 2001).

Neural, but not muscle, expression of stonedAB is sufficient to restore complete viability to stn^{8P1} and $stn^{13\text{-}120}$ (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^{8P1} and $stn^{13\text{-}120}$ 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[stnAB]* (*SAB1*) expression on various previously described electrophysiological and immunocytochemical phenotypes of lethal (stn^{8P1} and $stn^{13\text{-}120}$) and viable (stn^{C}) *stoned* mutants. At third instar larval neuromuscular junctions (NMJs), phenotypes in stn^{C} and stn^{8P1} 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 $stn^{13\text{-}120}$ embryonic motor synapses (Fergestad *et al.* 1999). All of these mutant phenotypes are completely rescued by neural expression of the *SAB1* 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^{C} and stn^{8P1}, are increased following *SAB1* 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 *SAB1* expression restores normal levels and distribution of synaptotagmin to stn^{C} and stn^{8P1} nerve terminals (Figure 2B). Also significantly, $stn^{13\text{-}120}$ 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 synaptic 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.

StonedB expression alone, via the *P[stnB]* transgene (*SB5*), was sufficient to rescue all previously organismal and synaptic defects in *stoned* alleles we analyzed (Table 2, Figure 3). Neurally expressed stonedB was as effective as stonedAB in restoring viability to stn^{8P1} and $stn^{13\text{-}120}$ mutants (Table 2). However, in contrast to animals expressing the stonedAB transgene, motor terminals of stn^{C}, stn^{8P1}, and $stn^{13\text{-}120}$ third instar larvae expressing stonedB showed strong immunoreactivity for stonedB, but greatly reduced stonedA compared to the wild type (Figure 3B). We tested whether synapses with this specific deficit in stonedA showed any physiological or morphological defects. Remarkably, evoked transmitter release as well as the levels and distribution of synaptotagmin in stonedB-expressing stn^{C}, stn^{8P1}, and $stn^{13\text{-}120}$ mutant

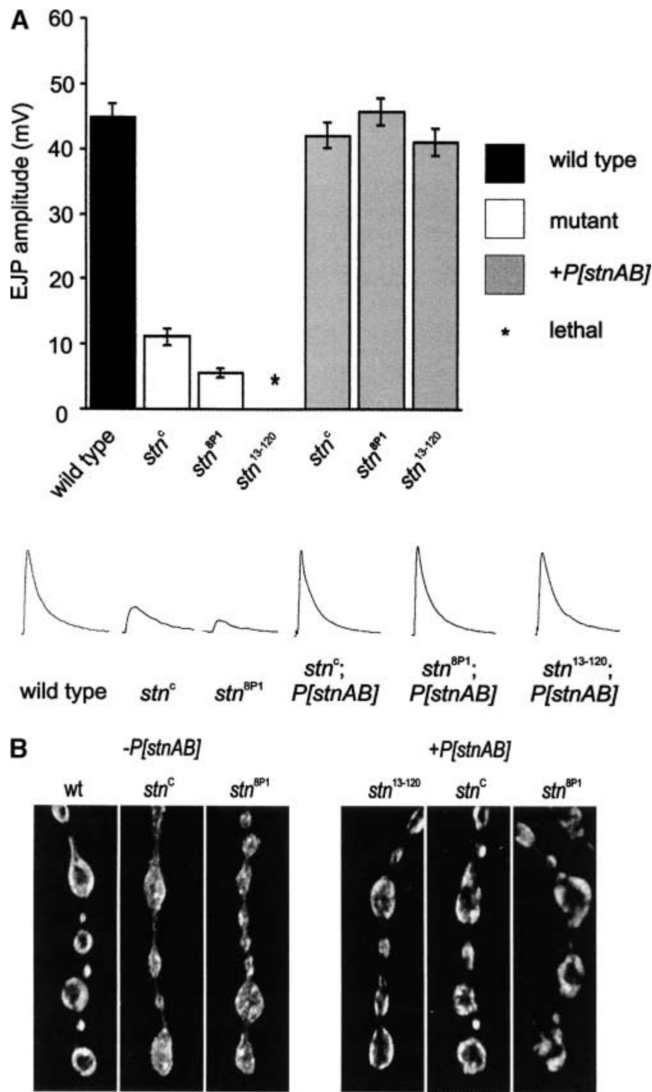

FIGURE 2.—All known synaptic phenotypes of *stn* mutants are rescued by neural (*elav*) expression of *P[stnAB]*. (A) Mean EJP amplitudes and representative traces in third instar larval synapses of mutants with and without a rescuing *P[stnAB]* transgene. Evoked transmission is not shown for $stn^{13\text{-}120}$ that die as late embryos, well before the third instar larval stage. Error bars represent SEMs. (B) Synaptotagmin distribution. stn^{C} and stn^{8P1} boutons show characteristic mislocalization of synaptotagmin staining to the plasma membrane (STIMSON *et al.* 1998, 2001); this defect is rescued by neural expression of *P[stnAB]*. The same result is seen in $stn^{13\text{-}120}$ mutants rescued by the presence of the *P[stnAB]* transgene.

synapses were completely normal and indistinguishable from wild-type controls (Figure 3, A and C). EJP amplitudes were 42.9 ± 2.3, 44.7 ± 1.8, and 42.8 ± 3.8 ($P_{rescue} <$ 0.57, 0.85, 0.63) for stn^{C}, stn^{8P1}, and $stn^{13\text{-}120}$ synapses, respectively, expressing normal levels of stonedB, but not stonedA, immunoreactivity (Figure 2B).

While these data are consistent with stonedB being sufficient to perform all functions of *stoned*, the slight but unequivocal increase in presynaptic stonedA immunoreactivity in mutant animals expressing *SB5* (an issue 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 stn^{8P1} and $stn^{13\text{-}120}$. 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" stn^{8P1} mutant third instar larvae that expressed stonedA. However, stn^{C} larvae expressing *stonedA* transgenes were obtained and their synaptic properties compared to stn^{C} 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 stn^{C} larvae expressing *stonedA* transgenes were 8.3 ± 0.4 mV, not significantly different from the 11.1 ± 1.3 mV observed for appropriate stn^{C} controls (Figure 4B). The exaggerated plasma membrane distribution of synaptotagmin seen in stn^{C} 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 stn^{C} 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 stn^{C} ERG, stonedA expression had no effect (Figure 4D).

TABLE 2

Neural expression of *P[stnB]* alone rescues *stn* lethal alleles

Cross	Actual *stn*/Y progeny (% of total)	Expected *stn*/Y assuming normal viability (%)	Total no. progeny scored
C155 stn^{8P1}/FM7i × *yw*	0	25	510
C155 stn^{8P1}/FM7i × *P[stnB]*	24	25	499
stn^{8P1}/FM7I; shi Gal4 × *P[stnB]*	25	25	242
C155 $stn^{13\text{-}120}$/FM7i × *yw*	0	25	551
C155 $stn^{13\text{-}120}$/FM7i × *P[stnB]*	23	25	500
$stn^{13\text{-}120}$/FM7i; MHC Gal4 × *P[stnB]*	0	25	256

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^{8P1} 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^{8P1} (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 synapse growth (Torroja *et al.* 1999; Zito *et al.* 1999).

Like satellite boutons in APPL-overexpressing strains, those in stn^{8P1} 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^{8P1}/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^{8P1} 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^{8P1} terminals show 5.7 ± 0.51 satellites compared to 2.8 ± 0.47 in $stn^{8P1}/mal^{+}Y$ ($P < 0.007$).

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_{rescue} 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 (gynandromorph) animals carrying both female $stn^{13\text{-}120}$/+ and male $stn^{13\text{-}120}$ tissue, viable mosaic animals with large patches of $stn^{13\text{-}120}$ 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 $stn^{13\text{-}120}$ (Fergestad 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 $elav^{C155}$ and ubiquitous *shi Gal4*-driven expression of *stoned* cDNA completely rescue the lethality of stn^{8P1} and $stn^{13\text{-}120}$. 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 $stn^{13\text{-}120}$ retains some residual stonedA activity that contributes to the ability of neurally expressed stonedB to rescue $stn^{13\text{-}120}$ phenotypes, but two lines of evidence argue against this possibility. First, the $stn^{13\text{-}120}$ 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 $stn^{13\text{-}120}$ results in viable animals with morphologically and functionally normal presynaptic terminals that, importantly,

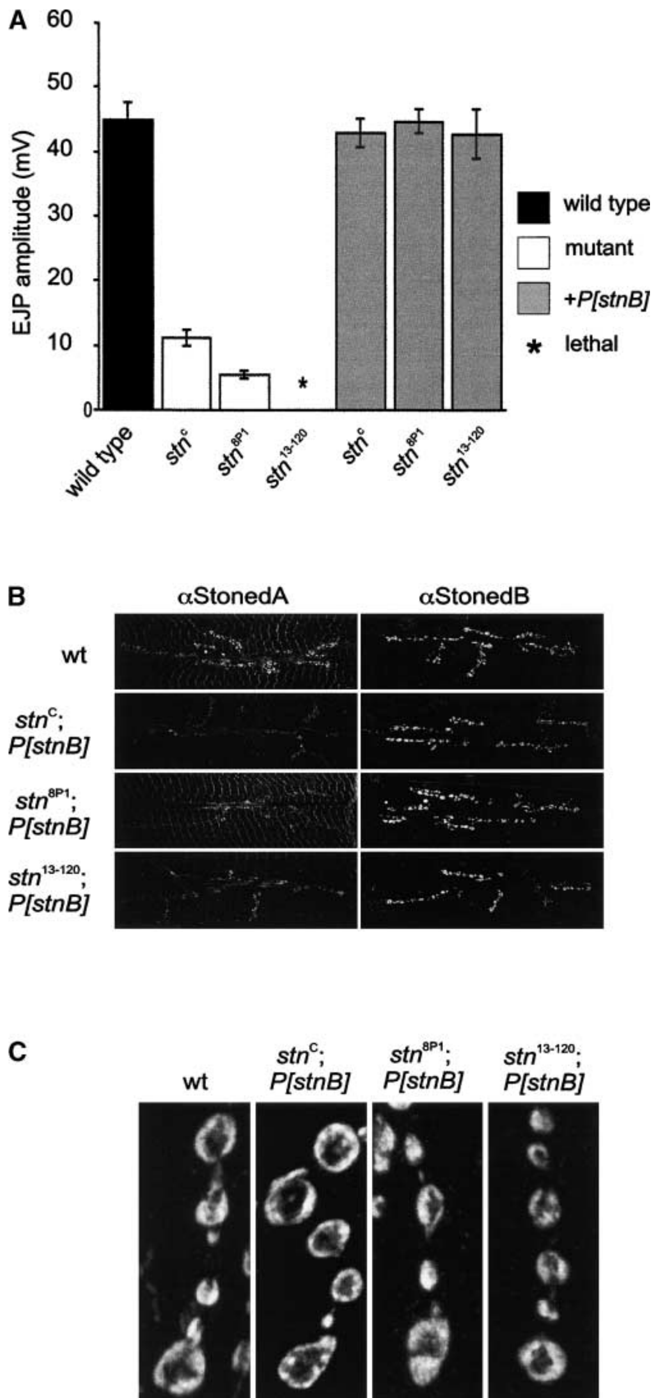

Figure 3.—Neural expression of a truncated cDNA containing only StonedB coding sequences (a *P[stnB]* transgene) is sufficient to rescue mutant synaptic phenotypes in stn^{C}, stn^{8P1}, and $stn^{13\text{-}120}$. (A) Mean EJP amplitudes in mutants before and after rescue with *P[stnB]*. Error bars represent 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[stnB]* expression. (C) Synaptotagmin distribution. Rescue with the *P[stnB]* transgene restores wild-type localization of synaptotagmin at the larval neuromuscular junction (see Figure 2B for prerescued localization).

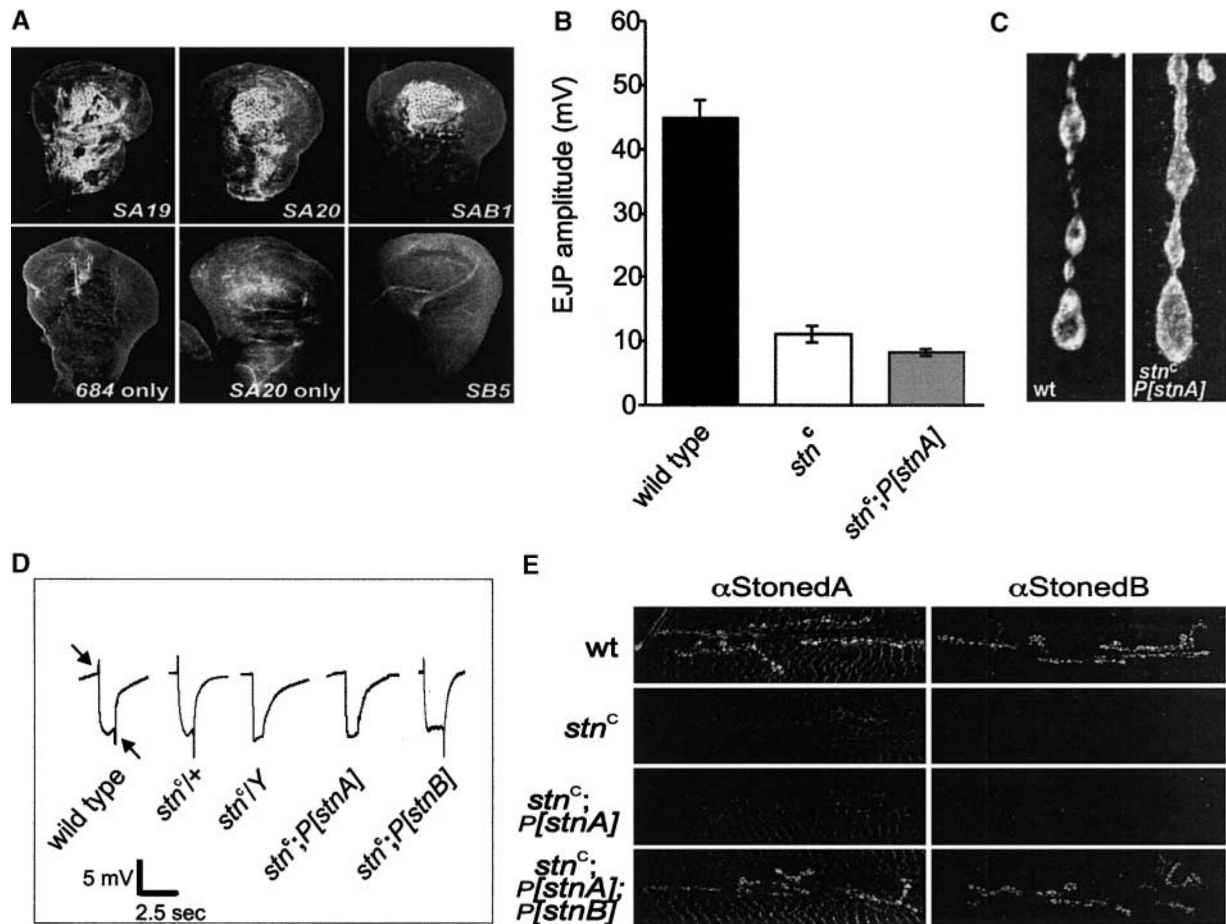

FIGURE 4.—Neural expression of StonedA coding sequences alone does not alleviate any of the known defects in *stoned* mutants. (A) Lethality of the other *stoned* alleles, stn^{8P1} and $stn^{13\text{-}120}$, was not rescued by the *P[stnA]* transgene although it expressed stonedA protein in wing discs when controlled with the Gal4 driver *684* (MATERIALS AND METHODS). (B) Mean EJP amplitudes for wild-type and stn^{C} mutants are shown before and after neural expression of *P[stnA]*. Stn^{C} larvae expressing *P[stnA]* have EJPs with amplitudes indistinguishable from stn^{C}. Error bars represent SEMs. (C) *P[stnA]* does not rescue the synaptotagmin mislocalization phenotype seen in stn^{C}. (D) In electroretinogram recordings, synaptic on/off transients missing in stn^{C} are restored by *P[stnB]* transgenes but not by *P[stnA]*. (E) Barely detectable levels of stonedA observed in stn^{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^{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 congential myasthenic syndromes, a class of human genetic diseases that interrupt neuromuscular transmis-

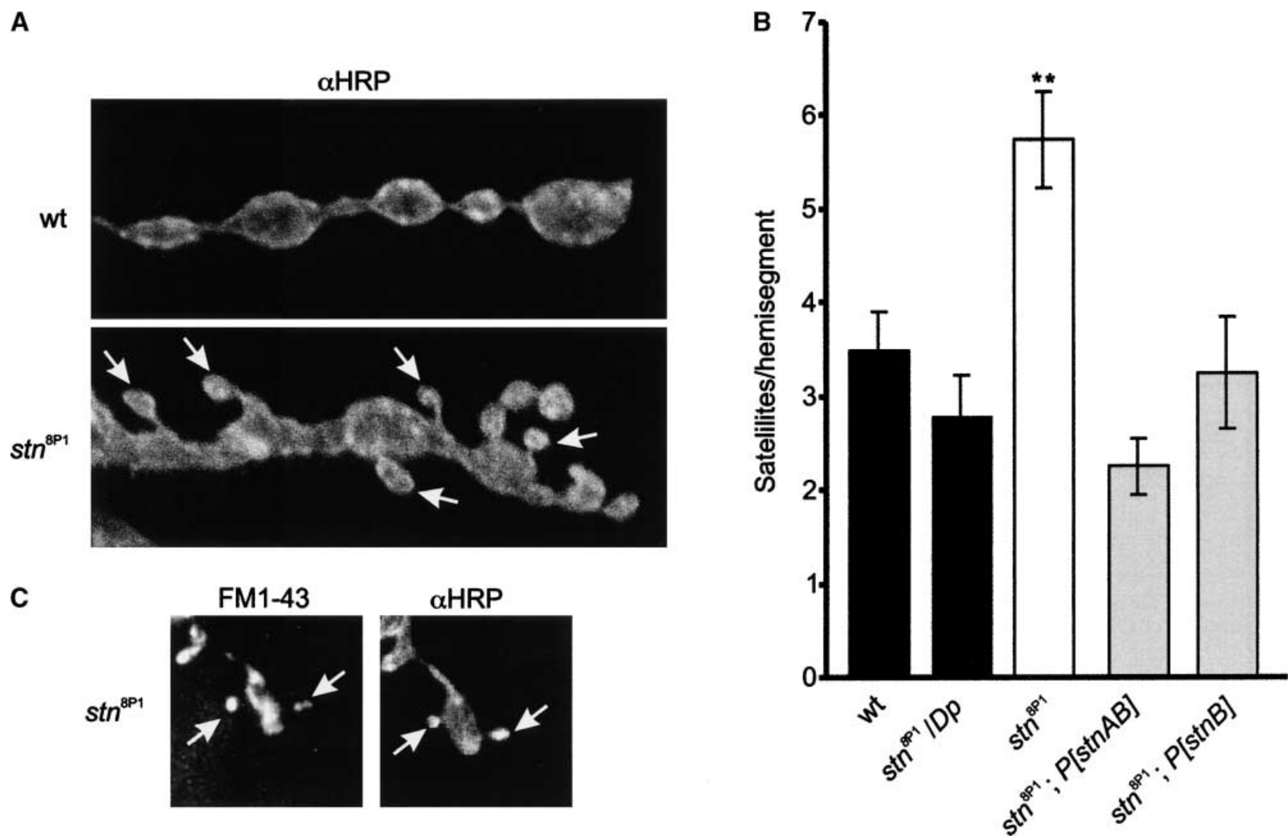

Figure 5.—A function for the *stoned* locus in synaptic development is revealed by unique morphological defects in stn^{8P1}. (A) "Satellite boutons" (arrows) in stn^{8P1} synapses are revealed by anti-HRP staining. (B) Histogram of quantified data shows that stn^{8P1} larvae have significantly more 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).

sion. Some of these have been associated with morphological defects at the NMJ that are similar to those of *stoned* mutants (Fergestad *et al.* 1999; Maselli *et al.* 2001; Stimson *et al.* 2001). The underlying mechanism of stonin function at synapses is likely to involve known molecular interactions of stonins with synaptotagmin, Eps15, and intersectin (Phillips *et al.* 2000; Martina *et al.* 2001). A particularly attractive idea is that it serves as a "pseudoadaptin" that, at a certain stage of vesicle formation, competes for the AP2-binding sites on vesicle proteins and, by displacing AP2, facilitates large-scale, sequential changes in the assembly state of endocytic proteins that underlie the ordered progression of events in the endocytic pathway (Walther *et al.* 2001). However, this model is not easily reconciled with the observation that stonedB remains associated with a vesicle fraction isolated from heads of *shibire* flies depleted of synaptic vesicles (Phillips *et al.* 2000).

A major issue to be addressed is whether stonedB in particular and stonins in general participate in a wide range of endocytic events or only in the relatively rapid and specialized process of synaptic-vesicle endocytosis. 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 stn^{8P1} 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) maintainance 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 mosquitos 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.

We thank Leona Mukai, Charles Hoeffer, and other members of the Ramaswami lab for technical assistance, useful discussions, and comments on the manuscript. We acknowledge Patty Jansma and Carl Boswell for expert assistance with confocal microscopy performed using microscopes belonging to the ARL Division of Neurobiology and the MCB Department, respectively, Jodi Carter for full-length stoned cDNA (JC9814), Tom McCormack for work on TM001, and Kathy Buckley for stonedA and B constructs. The work was funded by grants (nos. NS34889 and KO2-NS02001) to M.R. from the National Institutes of Health (NIH), by the McKnight and Alfred P. Sloan Foundations, by a Human Frontiers Science Program grant to M.R., L.E.K. (and four others), a National Science Foundation predoctoral fellowship to T.C.J., NIH and Flynn Foundation Developmental Neurobiology Training Grants to the University of Arizona (D.T.S.), and by grant no. 960117 from the NH and MRC (Australia) to L.E.K.

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Communicating editor: T. C. Kaufman