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Mutations in the Drosophila *pushover* Gene Confer Increased Neuronal Excitability and Spontaneous Synaptic Vesicle Fusion

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Manuscript received August 15, 1995
Accepted for publication January 4, 1996

ABSTRACT

We describe the identification of a gene called *pushover* (*push*), which affects both behavior and synaptic transmission at the neuromuscular junction. Adults carrying either of two mutations in *push* exhibit sluggishness, uncoordination, a defective escape response, and male sterility. Larvae defective in *push* exhibit increased release of transmitter at the neuromuscular junction. In particular, the frequency of spontaneous transmitter release and the amount of transmitter release evoked by nerve stimulation are each increased two- to threefold in *push* mutants at the lowest external [Ca^{2+}] tested (0.15 mM). Furthermore, these mutants are more sensitive than wild type to application of the potassium channel-blocking drug quinidine: following quinidine application, *push* mutants, but not wild-type, display repetitive firing of the motor axon, leading to repetitive muscle postsynaptic potentials. The *push* gene thus might affect both neuronal excitability and the transmitter release process. Complementation tests and recombinational mapping suggest that the *push* mutations are allelic to a previously identified *P*-element-induced mutation, which also causes behavioral abnormalities and male sterility.

IN multicellular organisms, information is transmitted through the neuromuscular system by propagation of action potentials in neurons and by release of neurotransmitter at synapses. Action potentials result from the transient opening of sodium channels, leading to an inward current that depolarizes the neuron. This depolarization is followed by the transient opening of potassium channels, leading to an outward current that repolarizes the neuron. The arrival of an action potential at the nerve terminal triggers the opening of the calcium channels, thus allowing influx of calcium into the terminals. This calcium influx evokes fusion of transmitter-containing synaptic vesicles with the nerve terminal membrane and release of transmitter into the synapse. This process is termed synaptic transmission. In fast excitatory transmission, as occurs at neuromuscular junctions, interactions of neurotransmitter with receptors in the postsynaptic cell leads to an inward current and consequent depolarization of the postsynaptic cell.

Synaptic transmission is dependent on and regulated by calcium influx from the extracellular space. Calcium influx, in turn, is controlled partly by the duration of nerve terminal depolarization, because calcium enters nerve terminals through channels that are voltage gated. The duration of nerve terminal depolarization reflects the relative magnitudes of the inward currents, carried by sodium and calcium ions, and the outward currents, carried by potassium ions. Thus the time course and amount of transmitter release can be controlled by the activities of ion channels present. In addition, synaptic transmission is dependent on the large number of proteins that control the synaptic vesicle cycle (reviewed recently by SUDHOF 1995). These components include those that affect vesicle docking, maturation, exocytosis and re-uptake. Such components determine the sensitivity of the synaptic vesicles to calcium influx.

In several organisms, mutations in a number of genes have been identified that affect ion channel activity or synaptic vesicle fusion. Mutations in any of these genes perturb synaptic transmission in particular ways. For example, mutations in the sodium channel structural gene *paralytic* (*para*) lead to reduced neuronal excitability: *para* mutants can exhibit action propagation failure at elevated temperature, and adults can exhibit temperature-sensitive paralysis (SUZUKI *et al.* 1971; WU and GANETZKY 1980; LOUGHNEY *et al.* 1989). In addition, certain disorders in humans, such as long QT syndrome or hyperkalemic periodic paralysis, can result from mutations in cardiac or skeletal muscle sodium channel genes (BARCCHI 1995; WANG *et al.* 1995). In contrast, mutations in the potassium channel structural gene *Shaker* (*Sh*) lead to increased neuronal excitability: *Sh* mutants respond to single nerve stimulations with repetitive action potentials, and adults exhibit leg shaking while under ether anesthesia (KAPLAN and TROUT 1969; JAN *et al.* 1977; TEMPEL *et al.* 1987). Mice, flies or nematodes defective in the gene encoding the synaptic vesicle protein *synaptotagmin* (*syt*) exhibit greatly reduced synaptic function (NONET *et al.* 1993; LITTLETON *et al.* 1993; DIANTONIO and SCHWARZ 1994; GEPPERT *et al.*

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1994). From these and other results it was proposed that *syt* encodes the calcium sensor that triggers transmitter release following calcium influx (reviewed recently by LITTLETON and BELLEN 1995). Finally, mutations in the Drosophila *kinesin heavy chain* (*khc*) gene or the nematode *unc-104* gene, which encode kinesin or a kinesin-like protein, respectively, lead to defects in synapse structure or function, most likely as a consequence of defective transport of ion channels or synaptic vesicle components to the nerve terminal (HALL and HEDGECOCK 1991; GHO *et al.* 1992; T. HILLMAN and M. STERN, unpublished observations). Thus, mutations such as these identify the genes responsible for controlling synaptic transmission and also enable the functions of these genes to be inferred.

Here we describe the isolation of two mutations in a gene termed *pushover (push),* which affect both behavior and neuronal function. Flies defective in *push* are sluggish and uncoordinated, and exhibit a defective escape response and male sterility. Furthermore, *push* mutants exhibit several defects in synaptic transmission at the larval neuromuscular junction. We find that *push* mutants exhibit an increased frequency of spontaneous fusion of synaptic vesicles and an increase in the amount of transmitter released following nerve stimulation. In addition, in the presence of the potassium channel-blocking drug quinidine, *push* mutants exhibit an increase in neuronal excitability: the motor neuron of the *push* mutant fires several times in response to a single nerve stimulation, leading to repetitive depolarizations in the muscle. We localized the *push* gene by recombinational mapping to position 27 on the left arm of the second chromosome, which corresponds approximately to cytological position 28 DE. This location is the site of a *P*-element enhancer trap insertion (called 3420) that causes expression of the reporter gene in larval and adult olfactory organs as well as male sterility (RIESGO-ESCOVAR *et al.* 1992). We find that flies homozygous for the 3420 *P* element exhibit the same behavioral, but not electrophysiological, defects as *push* mutants. Furthermore, the *push* mutations fail to complement both the behavioral and male fertility defects of this *P* element. Hence we conclude that the *push* mutation affects the same gene disrupted by the 3420 *P* element.

MATERIALS AND METHODS

All genes, mutants, or special chromosomes not described in this section can be found in LINDSLEY and ZIMM (1992).

Isolation of EMS-induced mutations in *push*: The *push* mutations were isolated as described previously (STERN and GANETZKY 1992). In brief, Sh^{133} mutant males carrying an isogenic second chromosome marked with *brown* (*bw*) were mutagenized with EMS, and ~5000 lines carrying independently mutagenized *bw* chromosomes were isolated. About 2500 lines carried homozygous viable second chromosomes, and flies homozygous for the second chromosome from each of these lines were screened for behavioral abnormalities. Flies from two lines, termed *push* and $push^2$ displayed uncoordination, sluggish behavior, defects in flight and male sterility. Although these mutant lines were induced in a Sh^{133} background, all of the phenotypes of these mutants are expressed in a Sh^+ background as well. Sh^+ derivatives of push and $push^2$ were used for all experiments described in this paper.

Behavioral tests: Flies for behavioral tests were grown and tested at 21–22° in uncrowded bottles. Both *push bw* flies (collected from a *CyO/push bw* stock) and flies from the isogenic wild-type parent line were collected up to 2 days following eclosion and aged individually in vials for 2 days before testing. Flies were tested behaviorally in four different ways. (1) Motility tests. Flies were placed at the bottom of vials, and the time required to run upwards to a height of 5 cm was determined. (2) Coordination tests. Flies were subjected to five trials of mechanical shock, accomplished by agitation of the vials, and it was determined if any of the mechanical shocks led to overturning of the fly onto its dorsal surface. If so, the time required for the fly to right itself was determined. (3) Flight/escape response. Flies were placed on a horizontal surface and agitated with a paintbrush at 15-sec intervals. The time required to fly off the surface was determined. (4) Flight ability. Flies that could not escape from a horizontal surface were tested for flight ability as follows: flies were dropped from a height of 1 meter, if the fly landed within a circle of 15 cm radius, centered directly under the drop point, then it was scored as flight defective, whereas if the fly did not land within this circle, then it was scored as flight competent.

Electrophysiology: Larval dissections, nerve and muscle recordings, and muscle voltage clamping were performed as described previously (JAN and JAN 1976; GANETZKY and WU 1982a; STERN and GANETZKY 1992). Saline solution used for dissections and recordings was 5 mM HEPES pH 7.1, 128 mM NaCl, 2 mM KCl, 4 mM $MgCl_2$, 35.5 mM sucrose and $CaCl_2$ as specified in the text. Because the *push* mutations confer male sterility, it was not possible to generate a homozygous stock of either *push* mutant. Therefore to obtain larvae homozygous for *push*, $push^2$ or the *P*-element-induced mutation 3420, we established lines heterozygous for the appropriate *push* allele and a chromosome carrying a translocation between two balancer chromosomes, *CyO* and *TbTM6B*, called *CyO-TbTM6* (kindly supplied by R. KREBER and B. GANETZKY). Larvae homozygous for the *push* allele were recognized by their nontubby appearance.

Muscle cell 6 from abdominal segments 4 or 5 were used for data collection. Quinidine (Sigma), if used, was bath applied from a 20 mM stock solution immediately before experimentation. Unless otherwise noted, the wild-type larvae described were obtained from the parental stock (called *iso bw*, also in a Sh^+ background) from which the *push* and $push^2$ mutations were generated. Data were collected, digitized and analyzed with the MacAdios and Superscope systems from GW Instruments.

Complementation and recombination tests: Complementation tests for behavioral phenotypes among *push* mutations were performed by constructing males transheterozygous for two *push* alleles and scoring them for behavior and male fertility. Complementation tests for electrophysiological phenotypes were performed by placing each allele over the *CyO-TbTM6* balancer chromosome, as described above, and crossing two lines carrying the alleles to be tested. Nontubby larval progeny were scored for the Push electrophysiological phenotype.

Cosegregation of the *push* electrophysiological and behavioral phenotypes were accomplished by generating 17 chromosomes carrying recombination events between *Sp* (map position 2-22) and a Pw^+ element located at 30BC (approximate map position 2-37). These recombinants were scored

TABLE 1

Behavioral defects of *push* mutants

Genotype	Climbing	Coordination	Escape response	Flight
Wild type (n = 44)	12 ± 3 sec	21/44 (48%, 2.3 ± 0.5 sec)	42/44 (95%)	44/44 (100%)
push (n = 39)	49 ± 5 sec	21/39 (54%, 46 ± 35 sec)	2/39 (5%)	8/39 (21%)

Wild-type and *push* flies were selected and tested as described in MATERIALS AND METHODS. *Climbing:* Mean time required for a fly to climb 5 cm. Seven *push* flies and one wild-type fly were not able to climb 5 cm even within 90 sec; a climbing time of 90 sec was thus applied to these flies. *Coordination:* The number of flies that could withstand each of five mechanical shocks (vigorous agitation of vial) without overturning onto its dorsal surface was determined. For flies that could be overturned, the mean time required for the fly to right itself was determined. *Escape response:* The number of flies that were able to fly off of a horizontal surface within 90 sec of placement was determined. Flies were prodded with a paintbrush at 15-sec intervals if applicable. *Flight:* Flies that could not escape from a horizontal surface within 90 sec were then dropped from a height of 1 meter, and their ability to fly was determined as described in MATERIALS AND METHODS. Flies that could escape from a horizontal surface were scored as flight competent. Number of flies are indicated in parentheses. Values are means ± SEMs.

behaviorally, and then seven of these chromosomes were crossed to the CyO-TbTM6 translocation balancer chromosome (described above) and lines were established. Males from each line were then crossed to females heterozygous for the tester *push* allele and the *CyO-TbTM6* translocation balancer chromosome. Nontubby larvae from these crosses were tested for the Push electrophysiological defects.

Cosegregation of the *push*^{2} electrophysiological and behavioral phenotypes were tested in a similar manner, except that recombination events were collected between *Sp* and an uncharacterized recessive lethal mutation (carried by the *Sp* mapping chromosome) at map position 2-31. Seven recombinant chromosomes were collected and lines established. Flies from each line were tested for the *push*^{2} behavioral defects of coordination and male fertility, and males from each line were then crossed to females heterozygous for the tester *push* allele and the *CyO-TbTM6* translocation balancer chromosome. Nontubby larvae from these crosses were tested for the Push electrophysiological defects.

RESULTS

Isolation of two new behavioral mutants: To identify additional genes affecting neuronal excitability, we mutagenized the Drosophila second chromosome with EMS (see MATERIALS AND METHODS) and screened for mutants exhibiting behavioral defects. Two mutagenized lines of particular interest displayed similar behavioral defects including sluggishness, uncoordination, and defects in flight (see Table 1). Although both wild-type flies and these mutant flies could sometimes be pushed onto their dorsal surfaces by mechanical agitation, the mutant flies required an average of 46 sec to right themselves, which is 20-fold more time than required for wild-type flies (Table 1). Therefore we named this gene *pushover* (*push*). In addition, *push* mutant males, but not females, are sterile. Because males defective in *push* were observed to mate, and because virgin females placed with *push* males quickly laid copious numbers of eggs that failed to hatch, we surmise that the sterility was not the result of defective mating behavior but rather of a downstream effect, such as sperm function. These mutations failed to complement for each of these phenotypes and thus most likely represented two alleles in the same gene, termed *push* and *push*^{2}.

Mapping: We localized the *push* mutations by recombinational mapping as follows. First, each mutation was localized to a similar region, between *Sp* (2-22) and *J* (2-41). Then, the *push* mutation was localized more precisely between *Sp* and *P* element carrying w^+ located at cytological region 30BC (kindly supplied by R. LEVIS). Of 17 chromosomes carrying recombination events between *Sp* and this *P* [w^+] element, six were push and 11 were *push*$^+$. This mapping placed *push* to map position 2-27, which corresponds approximately to cytological region 28-29.

Several *P*-element insertions causing male sterility were also located in this region. One such insertion line, termed 3420 and located at cytological position 28DE, was identified from a screen of enhancer traps directing reporter gene expression to the adult and larval olfactory organs (RIESGO-ESCOVAR *et al.* 1992; J. CARLSON, personal communication). We found that flies homozygous for the 3420 insertion exhibited behavioral defects very similar to those exhibited by the *push* and *push*^{2} mutants, including a "Pushover" phenotype and defects in flight. Furthermore, the 3420 insertion failed to complement the behavioral and male fertility defects of the *push* mutation. Because of the failure of complementation and the close genetic linkage observed, we believe that the 3420 insertion is a mutant allele of *push*.

Effects of the *push* mutations on synaptic transmission: We used the larval neuromuscular preparation (JAN and JAN 1976) to test the possibility that the *push* mutation would confer defects in motor neuron function. Muscle depolarization resulting from evoked transmitter release, termed excitatory junctional potentials (ejps), were monitored at three different external [Ca^{2+}]: 0.15, 0.4 and 1.0 mM. We found that *push* mutants exhibited a twofold increase in ejp amplitude over wild type at an external [Ca^{2+}] of 0.15 mM (1.6 ± 0.3 mV ejp amplitude in wild-type larvae, 3.1 ± 0.5 mV ejp amplitude in *push* larvae, $P < 0.05$), but that there was

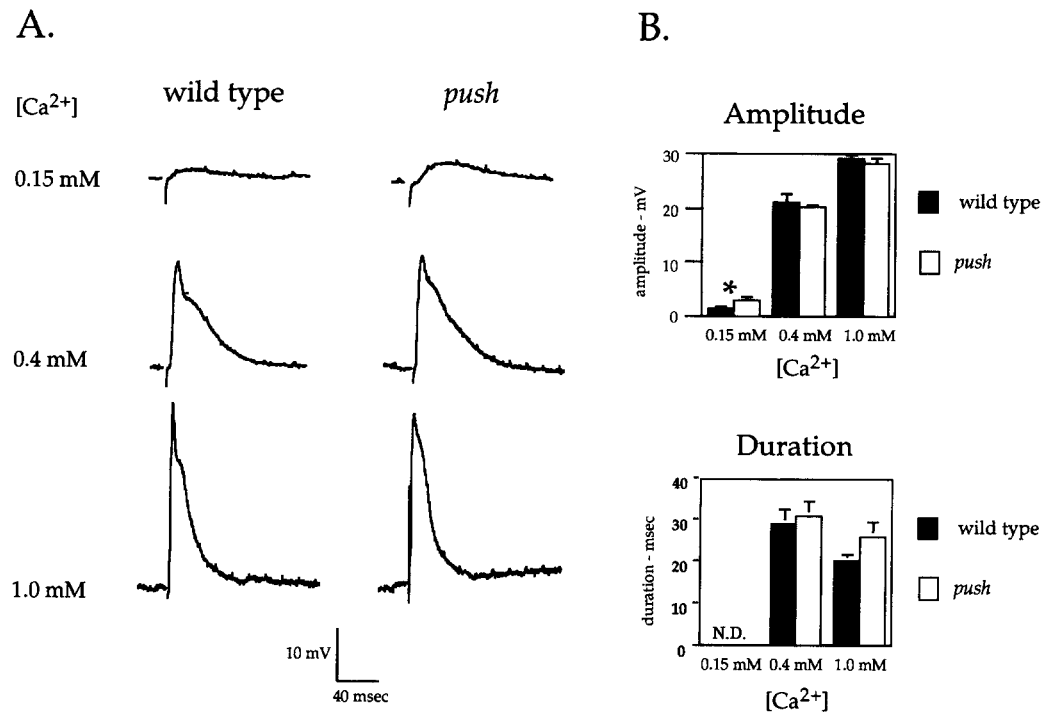

FIGURE 1.—*push* mutants display increased transmitter release at the neuromuscular junction. (A) Intracellular recordings of muscle ejps in response to nerve stimulation at the indicated external [Ca^{2+}]. Representative traces are shown. (B) Mean amplitude and duration of ejps in wild-type and *push* mutant larvae at the indicated external [Ca^{2+}]. Duration was measured as the ejp width at half maximal amplitude. N.D., not determined. Average amplitudes and durations from each larva were pooled to determine means and SEMs (represented by error bars). $*P < 0.05$ by Student's *t*-test. At an external [Ca^{2+}] of 0.15 mM, $n = 12$. At an external [Ca^{2+}] of 0.4 mM, $n = 7$. At an external [Ca^{2+}] of 1.0 mM, $n = 5$.

no significant differences either in ejp amplitude or duration between *push* and wild type at the higher external [Ca^{2+}] tested (Figure 1). In addition, we monitored muscle depolarizations resulting from the spontaneous fusion of single vesicles (termed miniature excitatory junction potentials or mejps). We found that the *push* mutant displayed an increased frequency of mejps: mejps occurred at a frequency of 2.3 ± 0.3 per second in the isogenic wild-type larvae, but 4.6 ± 0.6 per second in the *push* mutant ($P < 0.05$) (Figure 2).

Two additional results demonstrate that these increases in ejp amplitude and mejp frequency are each specifically the result of the mutation in the *push* gene, rather than the result of genetic changes unassociated with *push*. First, both the increased ejp amplitude and mejp frequency cosegregated with the *push* mutation following free recombination. As described in the MATERIALS AND METHODS, 17 chromosomes were obtained that carried recombination events between *Sp* and a *P* w^+ element at 30BC. Both of the recombinants scored as *push* on the basis of behavioral and fertility phenotypes exhibited approximately a twofold increase in ejp amplitude and mejp frequency over the two recombinants scored as $push^+$. Second, the $push^2$ mutation confers a similar increase in ejp amplitude and mejp frequency over wild type, and these increases also cosegregate with the *push* locus following recombination. In particular, seven lines carrying recombination events between *Sp* and a recessive lethal mutation at position 2-31 were generated and analyzed. Each of the three lines scored as $push^2$ on the basis of behavioral and fertility phenotypes exhibited a two- to threefold increase in ejp amplitude and mejp frequency over the four lines scored as $push^+$. Data from these seven lines were pooled, and we found that the $push^2$ recombinants conferred a 2.7-fold increase in ejp amplitude ($P < 0.01$, $n = 15$) and a 2.0-fold increase in mejp frequency ($P < 0.001$, $n = 15$) over the $push^+$ recombinants.

Interactions of *push* with quinidine: The effects of many mutations that affect synaptic transmission as a result of an increase in neuronal excitability are enhanced by application of the potassium channel-blocking drug quinidine. These mutants include *Shaker* (*Sh*), *Hyperkinetic* (*Hk*), *inebriated* (*ine*) and *bemused* (*bem*) (WU *et al.* 1989; STERN and GANETZKY 1992; STERN *et al.* 1995). We tested to see if the effects of the *push* mutation would also be enhanced by 100 μM quinidine, which completely and specifically blocks the delayed rectified potassium channel in Drosophila larval muscle (SINGH and WU 1989). As shown in Figure 3, application of quinidine had little effect on ejp amplitude or duration in wild-type larvae at any of the external [Ca^{2+}] tested ($P > 0.1$), or in *push* larvae at an external [Ca^{2+}] of 0.15 mM ($P > 0.1$). However, quinidine application to *push* larvae at an external [Ca^{2+}] of 0.4 or 1.0 mM led to muscle depolarizations that were extremely pro-

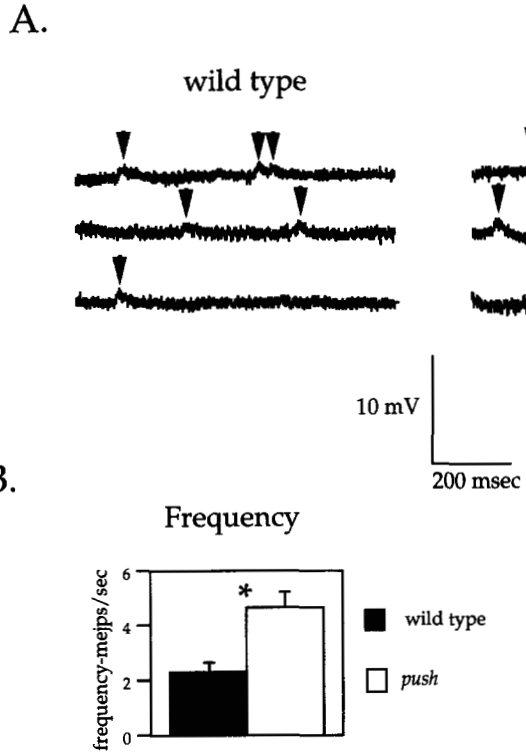

FIGURE 2.—*push* mutants display increased spontaneous transmitter release. (A) Intracellular recordings of muscle potentials, showing the spontaneous muscle depolarizations, termed mejps (indicated by arrowheads). Recordings were collected at an external [Ca^{2+}] of 0.15 mM. Representative traces are shown. (B) Mean frequency of mejps in wild-type and *push* mutant larvae. mejps were measured for 5 sec from each larva, counted for a determination of frequency, and an average of the amplitudes of each mejp during the 5 sec was determined. These values were pooled from 12 larvae of each genotype and used as the basis of the Figure 2B histogram. SEMs are represented by error bars. $*P < 0.05$ by Student's *t*-test; $n = 12$.

longed in duration compared to wild type (compare Figures 1 and 3). This phenotype is statistically significant ($P < 0.01$ at external [Ca^{2+}] of 0.4, $P < 0.02$ at external [Ca^{2+}] 1.0 mM). These defects result specifically from defects in the *push* gene, because they cosegregate with the *push* mutation in each of seven recom-

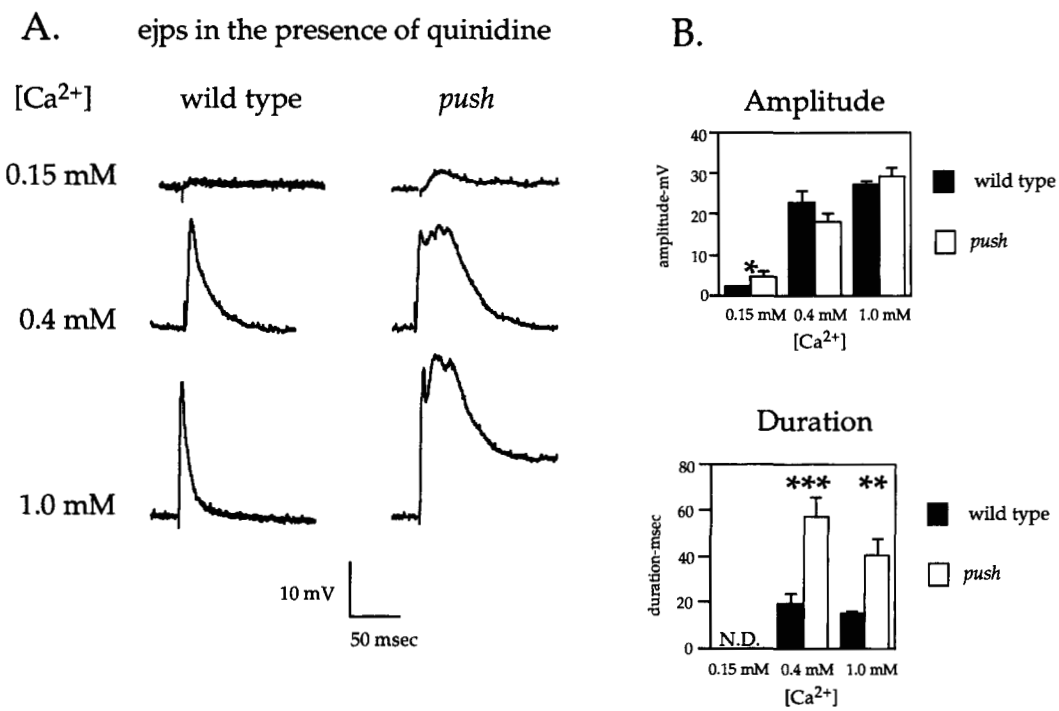

FIGURE 3.—Quinidine enhances the electrophysiological defects in *push* mutants. (A) Intracellular recordings of muscle ejps in response to nerve stimulation at the indicated external [Ca^{2+}]. Quinidine (100 μM) was present for all data collection. Representative traces are shown. (B) Mean amplitude and duration of ejps in wild-type and *push* mutant larvae at the indicated external [Ca^{2+}]. Duration was measured as the ejp width at half maximal amplitude. N.D., not determined. Average amplitudes and durations from each larva were pooled to determine means and SEMs (represented by error bars). $*P < 0.05$ by Student's *t*-test; $**P < 0.02$ by Student's *t*-test; $***P < 0.01$ by Student's *t*-test; $n = 6$.

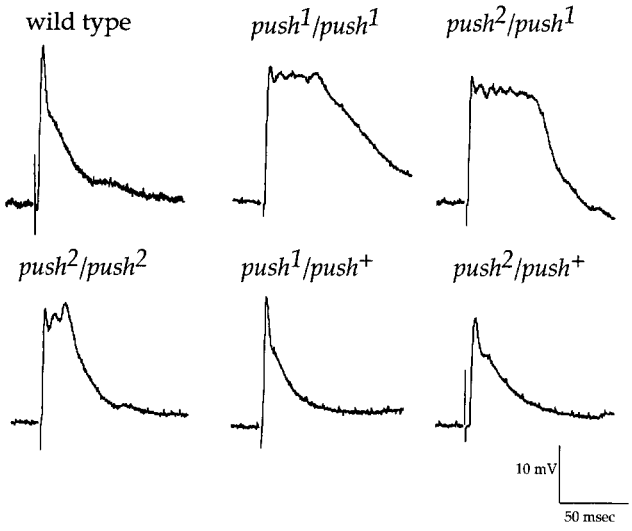

FIGURE 4.—The electrophysiological phenotype of *push* is recessive. Intracellular recordings of muscle ejps in response to nerve stimulation at an external [Ca^{2+}] of 0.4 mM in the presence of 100 μM quinidine. Genotypes as listed above each trace. Representative traces are shown.

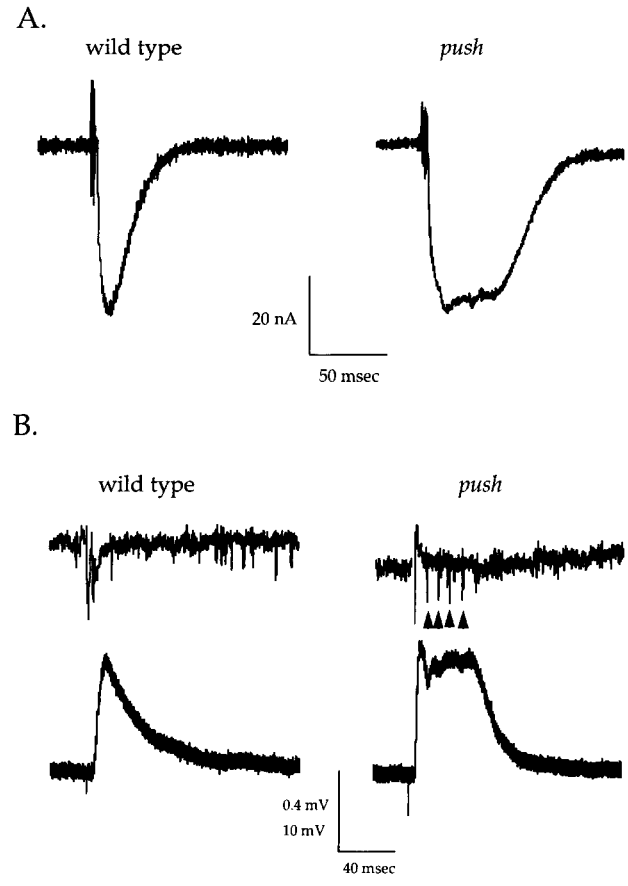

FIGURE 5.—The *push* mutation acts presynaptically. (A) Excitatory junctional currents measured in response to nerve stimulation at an external [Ca^{2+}] of 0.4 mM in the presence of 100 μM quinidine. Holding potential was −60 mV. (B) Simultaneous intracellular recordings from muscle (lower traces) and the nerves that innervate them (upper trace) at an external [Ca^{2+}] of 0.4 mM in the presence of 100 μM quinidine. The burst of extra action potentials that accompany the extra muscle depolarizations are visible in the *push* mutant upper trace and are marked by arrowheads. The extra action potentials that are not associated with ejps most likely result from the firing of sensory neurons present in the nerve. The motor axon action potentials are the largest single unit activities in the nerve.

binants tested. Furthermore, extremely prolonged muscle responses were also observed in the *push*^{2} mutant following quinidine application and in larvae transheterozygous for *push* and *push*^{2} (Figure 4). The mutant phenotype exhibited in the *push* and *push*^{2} transheterozygote was not the result of dominance of either mutation, because both *push* and *push*^{2} were fully recessive for this phenotype (Figure 4).

Evidence that the *push* mutation acts presynaptically: In Figure 1 we showed that the *push* mutant exhibited twofold increase in ejp amplitude when measured at an external [Ca^{2+}] of 0.15 mM. This increased ejp could result from increased transmitter release or increased sensitivity of the muscle to transmitter. We found that the amplitude of the mejps was unchanged between *push* and wild type (0.8 ± 0.1 mV for both *push* and wild type), which demonstrates that the response of the muscle membrane to transmitter was unchanged in the *push* mutant and thus that increased transmitter release accounted for the increase in ejp amplitude observed.

Similarly, the extremely prolonged muscle depolarizations that we observed in *push* mutants in the presence of quinidine (Figure 3) could be a result of prolonged transmitter release or an aberrant muscle response to normal transmitter release. To distinguish between these possibilities, synaptic transmission in the *push* mutant was measured with the muscle held under voltage clamp. If the prolonged depolarizations in the *push* mutant muscle resulted from increased transmitter release, then this increased release should lead to excitatory junctional currents (ejcs) of increased duration. Figure 5A shows that the ejc in the *push* mutant is indeed greatly prolonged compared to the ejc of the *push*$^+$ larva, which is consistent with the possibility that the prolonged ejp in the *push* mutant results from prolonged transmitter release.

The shape of the prolonged muscle response in the *push* mutant suggested that this response resulted from several sequential ejps, each of normal duration. If so, then these repetitive ejps could result from repetitive firing of the motor neuron. Such repetitive firing has been observed in other genotypes that cause increased neuronal excitability, such as mutants defective in *Sh*, *Hk*, *bss* and *ine* (JAN and JAN 1979; GANETZKY and WU 1982b; STERN and GANETZKY 1989, 1992). To test this possibility, nerve activity and muscle responses were simultaneous recorded in the *push* mutant in the presence of quinidine. As shown in Figure 5B, the prolonged ejps observed in the *push* mutant were associated

in the nerve with several extra nerve spikes. No extra nerve spikes were observed in $push^+$ larvae even in the presence of quinidine. These extra nerve spikes most likely result from extra electrical activity in the motor axon (JAN and JAN 1979; GANETZKY and WU 1982) and demonstrate that the prolonged muscle response in *push* mutants result from increased excitability of the motor neuron.

The 3240 line shows no electrophysiological abnormalities: Unlike the *push* and $push^2$ mutants, larvae of the 3420 line display normal synaptic transmission. Muscle responses were normal even in the presence of quinidine, both in larvae homozygous for the 3420 *P*-element insertion and in larvae transheterozygous for *push* and the 3420 insertion (not shown). Further, 3420 larvae display ejps of normal amplitude and mejps of normal frequency at low external $[Ca^{2+}]$ (not shown).

DISCUSSION

We have isolated two new mutant alleles in a gene that we have termed *pushover (push)* and have shown that each *push* mutant displays defects in behavior, male fertility and synaptic transmission. Adults defective in either *push* mutation exhibit uncoordination, sluggishness, a defective escape response, and male sterility. Larvae defective in either *push* mutation exhibit an increase in transmitter release from the larval motor neuron and in the frequency of mejps, which result from the spontaneous fusion of synaptic vesicles. In addition, *push* mutants exhibit increased excitability of the motor neuron, which is revealed in the presence of the potassium channel-blocking drug quinidine. Whereas in a wild-type motor neuron, a single nerve stimulation gives rise to a single neuronal action potential and a single ejp, in a *push* mutant motor neuron, a single nerve stimulation elicits multiple neuronal action potentials and multiple ejps.

There appears to be no clear causal relationship between the electrophysiological abnormalities that we observe at the larval neuromuscular junction and the various behavioral defects we observe in adults. However, we speculate that neurons in the adult central nervous system (CNS) are not functioning properly in *push* mutants, perhaps in similar ways to the malfunctions that we observe in the motor neuron. Such functional defects in CNS neurons could lead directly to the observed behavioral defects. This possibility cannot be tested because these CNS neurons are not accessible to electrophysiological measurement.

Allelism between *push* and *P*-element insertion 3420: The *push* mutations appear to be allelic to a gene previously identified on the basis of insertion of a *P*-element enhancer trap vector (RIEGSO-ESCOVAR *et al.* 1992). This insertion, called 3420, directs reporter gene expression to larval and adult olfactory organs and the testis. The 3420 insertion confers the same behavioral and fertility defects as the *push* mutations, fails to complement these mutations, and is located at the same map position as *push*. Surprisingly, the 3420 insertion fails to confer any of the abnormalities in synaptic transmission conferred by *push* or $push^2$, and complements the *push* mutations for these defects. This result might occur if the insertion affects expression of the *push* gene in tissues that require *push* for behavior and fertility, but not in the larval motor neuron. The observation that the insertion mutation confers similar behavioral phenotypes to the EMS-induced mutations described here suggests that the EMS-induced mutations act by causing partial or complete loss of function.

Interactions between *push* mutations and quinidine: The *push* mutants display increased neuronal excitability: a single nerve stimulation elicits repetitive firing of neuronal action potentials. However, this phenotype is observed only in the presence of the potassium channel-blocking drug quinidine. In the absence of quinidine, this repetitive firing does not occur. Such an enhancement by quinidine is widely observed among Drosophila mutations that confer increased neuronal excitability. These include mutations in the potassium channel structural genes *Sh* and *Hk* (TEMPEL *et al.* 1987; WU *et al.* 1989; CHOUINARD *et al.* 1995), mutations in *inebriated* and *bemused* (STERN and GANETZKY 1992; STERN *et al.* 1995) and increased expression of the sodium channel structural gene *para* (M. STERN, unpublished observations). The synergistic interactions between quinidine and mutations in the potassium channel structural genes might be the result of functional redundancy of potassium channels, as was described previously (STERN and GANETZKY 1992). In this view, quinidine application or the *push* mutation would each produce a modest increase in excitability, insufficient to generate repetitive neuronal action potentials. However, when quinidine application and the *push* mutation are combined, a more extreme increase in excitability is produced, leading to the repetitive action potentials observed.

The relationships among the *push* electrophysiological phenotypes: Mutations in several other genes in Drosophila confer a phenotype of increased neuronal excitability, leading to repetitive firing of neuronal action potentials and repetitive ejps. These include mutations in the potassium channel structural genes *Sh, Hk* and *eag,* and genotypes causing overexpression of *para,* the sodium channel structural gene, and *frequenin,* a negative regulator of potassium channels (JAN *et al.* 1977; GANETZKY and WU 1983; STERN and GANETZKY 1989; STERN *et al.* 1990; MALLART *et al.* 1991). Thus the *push* mutation might also exert its effects via neuronal ion channels. However, any effect of *push* mutations on ion channels might be indirect (described in more detail below): mutations in *push,* unlike mutations in ion channel structural genes, confer defects in mejp frequency and sperm function in addition to increased excitability.

It is possible that the twofold increases in ejp amplitude and mejp frequency each result from the same underlying mechanism, such as a twofold increase in the number of synaptic boutons, or a twofold increase in the number of docked synaptic vesicles. Although no gene has yet been identified in Drosophila that simultaneously increases both ejp amplitude and mejp frequency, such factors have been identified in other systems. For example, in the rat hippocampus increases in both evoked and spontaneous transmitter release can be elicited by the combined application of forskolin and IBMX, which increase intracellular [cAMP] (CHAVEZ-NORIEGA and STEVENS 1994). A similar phenomenon could be occurring in the *push* mutant; however increases in [cAMP] in the Drosophila motor neuron increase evoked transmitter release but does not effect the frequency of spontaneous transmitter release (ZHONG and WU 1991).

The relationship between the increase in neuronal excitability and the increase in ejp amplitude and mejp frequency is not clear. In other systems, conditions that increase excitability can also increase spontaneous vesicle fusion by increasing the influx of extracellular [Ca^{2+}] (DEL CASTILLO and KATZ 1952). However, the *push* mutation causes a twofold increase in mejps frequency even in the absence of extracellular [Ca^{2+}] (L. WARBINGTON, personal communication); thus it is unlikely that the mutations in *push* increase mejp frequency by this mechanism. However, in the sensorimotor synapse of Aplysia it has been observed that application of serotonin causes both increased excitability and increased mejp frequency by the activation of two distinct pathways, one involving protein kinase A and the other involving protein kinase C (BRAHA *et al.* 1990; GHIRARDI *et al.* 1992). Conceivably, the push protein could be involved in a similar type of regulation.

The relationship between synaptic transmission and male fertility: The male sterility of *push* mutants does not appear to result of inability to mate; *push* males have been observed to mate with females. In addition, virgin females placed with *push* males quickly lay large quantities of eggs, which also suggests that the *push* males have mated successfully. The observation that *P*-element insertion 3420 drives *lacZ* expression in the testes (RIESGO-ESCOVAR *et al.* 1992) supports the idea that sperm function might be defective in *push* mutants. The relationship between neuronal excitability, synaptic transmission and sperm function is not clear. However, sperm and neurons share at least three regulatory mechanisms. First, fluxes of potassium and calcium control neuronal function, and in addition, accompany the initiation of sperm motility in fish (see TANIMOTO *et al.* 1994). Furthermore, application of potassium channel blockers inhibits the initiation of motility, which suggests that potassium efflux is required for motility. Second, both sperm and neuronal function are modulated by phosphorylation and dephosphorylation events, mediated by regulatory molecules such as protein kinase A (GHIRARDI *et al.* 1992; WALCZAK and NELSON 1994). Third, both sperm and neurons require the cytoskeleton for proper function. The sperm flagella is centered around microtubules, whereas the transport and localization of synaptic components require cytoskeletal elements including microtubules and actin filaments (VALE *et al.* 1985; GREENGARD *et al.* 1993). The *push* gene could be required for any of these cellular events. Further experiments will be required to distinguish among these possibilities.

We are grateful to MIKE GUSTIN for comments on the manuscripts, KATHY MATTHEWS at the Bloomington, Indiana Stock Center, JOHN CARLSON and BOB LEVIS for fly stocks, LACHELLE WARBINGTON, JOHN CARLSON and DARIA HEKMAT-SCAFE for permission to cite unpublished data, NEAL ZONDLO for assistance in various aspects of this project and BARRY GANETZKY, in whose lab this work was begun. This work was supported by grant GM-46566 from the National Institutes of Health to M.S.

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Communicating editor: A. CHOVNICK