A role for presenilin in post-stress regulation: effects of presenilin mutations on Ca^{2+} currents in *Drosophila*

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ABSTRACT **It has been shown that presenilin is involved in maintaining Ca^{2+} homeostasis in neurons, including regulating endoplasmic reticulum (ER) Ca^{2+} storage. From studies of primary cultures and cell lines, however, its role in stress-induced responses is still controversial. In the present study we analyzed the effects of presenilin mutations on membrane currents and synaptic functions in response to stress using an *in vivo* preparation. We examined voltage-gated K^+ and Ca^{2+} currents at the *Drosophila* larval neuromuscular junction (NMJ) with voltage-clamp recordings. Our data showed that both currents were generally unaffected by loss-of-function or Alzheimer's disease (AD) -associated presenilin mutations under normal or stress conditions induced by heat shock (HS) or ER stress. In larvae expressing the mutant presenilins, prolonged Ca^{2+} tail current, reflecting slower deactivation kinetics of Ca^{2+} channels, was observed 1 day after stress treatments were terminated. It was further demonstrated that the L-type Ca^{2+} channel was specifically affected under these conditions. Moreover, synaptic plasticity at the NMJ was reduced in larvae expressing the mutant presenilins. At the behavioral level, memory in adult flies was impaired in the presenilin mutants 1 day after HS. The results show that presenilin function is important during the poststress period and its impairment contributes to memory dysfunction observed during adaptation to normal conditions after stress. Our findings suggest a new stress-related mechanism by which presenilin may be implicated in the neuropathology of AD.—Lu, Y., Lv, Y., Ye, Y., Wang, Y., Hong, Y., Fortini, M. E., Zhong, Y., Xie, Z. A role for presenilin in post-stress regulation: effects of presenilin mutations on Ca^{2+} currents in *Drosophila*. *FASEB J.* 21, 2368–2378 (2007)**

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PRESENILIN PLAYS A CRITICAL ROLE in trafficking and proteolysis of a selected set of transmembrane proteins (1–5). The structure and function of presenilin proteins appear to be highly conserved from *C. elegans* and *Drosophila* to mammals (6, 7). A large number of identified missense mutations in the two human presenilin genes (*PS1* and *PS2*) cause early-onset Alzheimer's disease (AD) (8, 9). One major effect of these *PS* mutations is the hyperproduction of Aß42 peptides generated from the cleavage of amyloid precursor protein (APP) by γ-secretase, a multiprotein protease in which presenilin is the crucial catalytic component (10–13). Increased Aß42 secretion leads to accelerated formation of extracellular amyloid plaques in specific brain regions, potentially providing a mechanistic explanation for the role of this class of presenilin mutations in early-onset AD. However, it is puzzling that so many different mutations that are distributed over the entire length of the presenilin protein all lead to an apparent gain-of-function phenotype, namely, hyperproduction of Aß42 peptides. In fact, in transgenic *C. elegans*, *Drosophila* and mouse models, several of these conserved AD-linked presenilin mutations instead reduce γ-secretase-like activity in the proteolysis of another membrane protein, the Notch receptor (14–16). To gain insight into how presenilin mutations are linked to the pathogenesis of AD, presenilin functions in addition to cleavage of APP and Notch have also been studied extensively.

Presenilin interacts directly with a number of proteins (17) and affects a range of physiological functions, including intracellular Ca^{2+} homeostasis (18–20), capacitative Ca^{2+} entry (20–22), the endoplasmic reticulum (ER) stress response (23–25), and apoptosis (26–28). Some of these effects have been suggested to contribute to hyperproduction of Aß42 or otherwise promote neuronal toxicity that might be relevant to AD pathogenesis (22, 29). However, reported data con-

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cerning the involvement of presenilin in responses to various stresses including oxidative stress, ER stress, and glutamate excitotoxicity have been inconsistent. While some studies have shown that AD-linked presenilin mutations or presenilin deficiency increase neuronal vulnerability to oxidative stress (30–32) and glutamate toxicity (18, 33, 34), other work has shown that overexpression of wild-type presenilin or AD-linked mutant presenilins cause no change in response to oxidative stress (35, 36) or glutamate toxicity (37, 38). The potential role of presenilin in the ER stress response is particularly controversial. Misfolded proteins that accumulate in the ER lumen stimulate expression of molecular chaperones, such as BiP, and transcription factors, such as CHOP (39–41). Some studies claim that this ER stress response is significantly diminished by AD-linked presenilin mutations (23, 42), while others demonstrate that up-regulation of the ER stress-induced signaling pathway and chaperones are independent of presenilin expression (25) and are not altered by knock-in presenilin-1 mutations (43). In addition to these discrepancies, the potential impact of alterations in the stress response caused by presenilin mutations has not been assessed at the synaptic level. Addressing this latter issue is important in understanding the causes of AD because subtle synaptic alterations precede overt neuronal degeneration in AD pathogenesis (44).

Since the above-mentioned studies about the role of presenilin in ER stress were conducted with either cell lines or primary neuronal cultures, we reasoned that it would be worth investigating the functional effects of presenilin mutations in response to stress in the context of an intact organism. We elected to perform an analysis of these mutants in *Drosophila* at two levels: *1)* the synaptic level, where we examined voltage-gated K^+ and Ca^{2+} currents at the neuromuscular junction (NMJ) in *presenilin* mutant larvae, and *2)* the behavioral level, where we evaluated learning and memory in adult flies. The *Drosophila* NMJ has been widely used as a model system to study synaptic functions. It is a glutamatergic synapse with boutons, which in many ways resemble mammalian CNS synapses. The *Drosophila* presenilin homologue is highly conserved (53% amino acid identity), including strong conservation at amino acid residues associated with familial AD mutations (45–47). It is widely expressed in both neuronal and non-neuronal tissues (45). We subjected wild-type and presenilin mutant-expressing *Drosophila* larvae to heat shock (HS) or drug-induced ER stress and recorded voltage-gated currents from the NMJ, which consist of K^+ and Ca^{2+} currents (48, 49). Our analysis showed that voltage-gated K^+ and Ca^{2+} currents were largely intact in larvae expressing loss-of-function and AD-linked presenilin mutations under both normal and stress conditions. However, Ca^{2+} tail currents were altered after stress conditions were halted. Similarly, long-term memory but not learning was reduced in the presenilin mutants only during the recovery period following cessation of stress treatments. These observations lead us to propose that presenilin plays a critical role in maintaining the normal function of Ca^{2+} channels after recovery from stress conditions.

MATERIALS AND METHODS

Fly stocks

2202U was used as the standard wild-type strain. The loss-of-function mutant alleles Psn^{S3} and Psn^{C4} have been described before (50). Both homozygous Psn^{S3} and heterozygous combination of Psn^{S3} and Psn^{C4} (Psn^{S3}/Psn^{C4}) were used in our analyses. Since the two alleles were generated independently from separately mutagenized fly populations, the transheterozygous genotype (Psn^{S3}/Psn^{C4}) can be used to control for any possible background chromosome lesions. The following transgenic lines were also used:

1) w^- (, or ;) $P[w^+, Psn\text{-}prom\text{-}Psn^{L235P}]$; $Psn^{B3}/TM6C, Tb$
2) w^- (, or ;) $P[w^+, Psn\text{-}prom\text{-}Psn^{E280A}]$; $Psn^{B3}/TM6C, Tb$
3) w^- (, or ;) $P[w^+, Psn\text{-}prom\text{-}Psn^{wt}]$; $Psn^{B3}/TM6C, Tb$

To construct these transgenes, various full-length *Psn* cDNAs were subcloned as *Bam*HI/*Xba*I fragments downstream of an ~ 1.5 kb *Psn* promoter *Xho*I/*Eco*RI fragment (*Psn-prom*) amplified from fly genomic DNA by PCR. This fragment contains 5′ flanking and 5′ UTR sequences and supports full rescue of *Psn* mutant flies when used to express wild-type *Psn* cDNAs (Y. Ye and M. E. Fortini, unpublished data). The two wild-type Psn (Psn^{wt}) variants used as control transgenes, *Psn-14a* and *Psn-14b*, are splice variants encoding closely related isoforms that include or lack a 14 amino acid segment in the large cytoplasmic loop domain of the protein, respectively (45). Psn^{L235P} and Psn^{E280A} are transgenes encoding mutant presenilins with AD-associated mutations in residues conserved between *Drosophila* and human presenilin (16). Transgenes were inserted into the *Drosophila* transformation vector pCaSpeR for microinjection to generate the transgenic fly strains. For functional assays, all transgenes were introduced into a homozygous mutant Psn^{B3} background that is genetically null for endogenous *Psn* (50). Flies were grown on standard cornmeal medium at 23°C unless otherwise indicated (*e.g.*, drug treatment regimes; see below). Homozygous loss-of-function mutant and transgenic larvae were selected according to body morphology using the dominant *Tb* marker and were reared under the appropriate conditions as specified for each particular experiment.

Drug treatments

Tunicamycin, thapsigargin, diltiazem hydrochloride, and amiloride hydrochloride were obtained from Sigma Chemical Company. All drugs were stored at −20°C in the dark, diluted to their respective desired concentrations immediately before each experiment, and used under minimal light conditions. Tunicamycin and thapsigargin were dissolved in ethanol. The maximum final ethanol concentration used in the experiments was 0.1%.

Recording from the larval NMJ

Larvae were affixed to a dissection dish with the dorsal side up and dissected in Ca^{2+}-free saline. An incision was made along the dorsal midline, and the cuticle was pulled back and affixed to the dish. All internal organs were removed. Stimulation of the motor nerve in PPF experiments was achieved by 1-ms positive current stimulation of a loop of motor nerve in

a suction electrode. The stimulation threshold was determined by varying stimulation intensity until the amplitude of junction currents did not increase. Stimulation intensity was set 20% above the threshold. Current recordings were obtained from ventral-lateral longitudinal muscle fiber 12 in abdominal segments 3 or 4 (48, 51) using the two-microelectrode voltage-clamp method as described (48, 52). Both the voltage electrode and the current electrode were filled with 3 M KCl, with a resistance in the range of 5–10 MΩ. All electrophysiological recordings were performed at room temperature (19~23°C) and the holding potential for all electrophysiological recordings was set at −80 mV.

I/V curves were generated by 250 ms voltage steps from a holding potential of −80 mV to potentials between −40 and +40 mV in 10 mV increments. To test the tail current kinetics, currents were elicited by 30 ms depolarization to −15 mV and then 160 ms repolarization to voltages between −110 and −70 mV in 20 mV increments. Pulses were given at 5-second intervals. All recordings were conducted at room temperature. K^+ currents were recorded in Ca^{2+}-free saline solution containing (in mM) 70 NaCl, 5 KCl, 10 $NaHCO_3$, 115 sucrose, 5 trehalose, 5 HEPES, 10 $MgCl_2$, at pH 7.1. For Ca^{2+} currents recordings, 10 mM Ba^{2+} was added to block K^+ channels and serve as the charge carrier.

Data were recorded with a Gene Clamp 500B amplifier (Axon Instruments, Foster City, CA, USA), using Clampex software (Axon Instruments) and a Digidata 1320 A/D converter (Axon Instruments). Data were sampled at 50 KHz after Gauss filtering at 500 Hz. All data were leak-subtracted during analysis with the P/4 protocol in Clampex software except for PPF experiments. Cell capacitance was tested to evaluate the current densities. All current traces shown are representative data obtained from larvae as denoted. Current amplitude was measured at the peak of the current. Each tail current was fitted with a single exponential by Clampfit (Axon Instruments) to measure the time constant. Data were expressed as mean ± SEM.

Learning and memory tests

Olfactory associative learning was measured with a Pavlovian conditioning procedure (53). Briefly, a group of ~100 flies was trained by exposing them sequentially to one odor (conditioned stimulus, CS^+) paired with electric shock, then to a second odor (CS^-) without electric shock. Learning was tested by exposing the flies simultaneously to the CS^+ and CS^- in a T-maze for 2 min. Flies trapped in CS^+ and CS^- T-maze arms were counted and a performance index (PI) was calculated as the number of flies avoiding the CS^+ less the number avoiding the CS^-, divided by the total number of flies and multiplied by 100. Hence, a 50:50 random distribution that yields a PI of zero indicates no learning. Twenty-four hour memory was evaluated after massed training, which consists of 10 cycles of one-session training used in the learning test, without any rest between successive training sessions. Flies were subsequently tested for memory as in the learning test in the T-maze after 1 day.

RESULTS

To investigate the potential role of presenilin in the *Drosophila* stress response and poststress recovery, we performed a systematic analysis of larval NMJ activity as well as adult learning and memory under genetic conditions designed to reduce *in vivo* presenilin activity, using both conventional loss-of-function and transgenic approaches. Three different presenilin mutations were analyzed, including a loss-of-function mutation, Psn^{S3}, in the endogenous fly *Presenilin* (*Psn*) gene as well as two AD-linked point mutations, Psn^{L235P} and Psn^{E280A}, expressed as introduced transgenes (16). Two wild-type variants of the *presenilin* gene, *Psn-14a* and *Psn-14b*, were also included in the study as control transgenes (see Materials and Methods). Psn^{S3} is an ethyl methanesulfonate (EMS) -induced hypomorphic mutant derived from w^{1118} (50, 54). *2202U*, an isogenic w^{1118} strain, was used as the wild-type control for Psn^{S3}. Transgenic flies were constructed to express the AD-linked Psn^{L235P} and Psn^{E280A} mutant transgenes and the matching wild-type control transgenes *Psn-14a* and *Psn-14b* (see Materials and Methods). To avoid overexpression artifacts and examine transgene function in the absence of endogenous *presenilin* gene function, the transgenes were placed under the direct transcriptional control of the endogenous *presenilin* promoter in a *presenilin* genetic null background where the endogenous *presenilin* gene was deleted (see Materials and Methods). We confirmed that this approach enables us to express presenilin protein in the transgenic flies at levels similar to that of normal endogenous presenilin in the wild-type *2202U* flies (Supplemental Fig. S1). The homozygous Psn^{S3} and transgenic Psn mutant flies were viable until the third instar larvae stage, but were lethal for Psn^{S3} and semi-lethal for Psn^{L235P} and Psn^{E280A} transgenic flies at later pupal stages due to diminished Notch cleavage activity reflecting complete (Psn^{S3}) or nearly complete (Psn^{L235P} and Psn^{E280A}) elimination of presenilin activity (16, 54). Since the Psn^{S3} mutant contains a second lethal mutation, we also examined the heterozygous combination of this Psn^{S3} allele with another EMS-induced strong hypomorphic mutant allele Psn^{C4} (50 and supplemental data). All larvae used in this study were assayed as homozygotes except for this Psn^{S3}/Psn^{C4} genotype.

Voltage-gated currents were recorded from the late third instar larvae using the two-electrode voltage-clamp method (52; see Materials and Methods). Different components of membrane currents can be isolated *via* physiological and pharmacological manipulations. Since *Drosophila* larval muscle fibers do not have Na^+ channels, the outward and inward currents are carried by K^+ and Ca^{2+}, respectively (52). K^+ currents are recorded in Ca^{2+}-free saline whereas voltage-gated Ca^{2+} currents are recorded in the presence of a high concentration of Ba^{2+}. Ba^{2+} serves as the charge carrier for Ca^{2+} currents and simultaneously blocks K^+ currents (55). Since K^+ currents were not affected significantly by presenilin mutations at all conditions tested (data not shown), only results on voltage-gated Ca^{2+} currents are shown.

We assessed the effects of HS stress on parameters of voltage-gated Ca^{2+} currents. It is known that high temperatures induce stress responses in flies, such as expression of HS proteins (56). Flies were reared under three conditions, including *1)* room temperature (19–23°C; designated as RT), *2)* HS treatment (reared at

29–30°C for 2–3 days prior to recording, designated as HS), and *3)* post-HS treatment (reared at 29–30°C for 2 days, followed by RT for 1 day prior to recording; designated as post-HS). Voltage-gated Ca^{2+} currents were examined at various depolarizing membrane potentials using voltage clamp. In both the wild-type *2202U* flies and flies expressing either wild-type or mutant presenilins, the peak value of the Ca^{2+} current was reached at membrane potentials of approximately −10 to −20 mV (**Fig. 1*A***; for mutants, only Psn^{L235P} and Psn^{S3} data are shown). The current/voltage (I/V) relationship for the Ca^{2+} current was not altered by HS treatment in either wild-type or presenilin mutant larvae.

Voltage-gated Ca^{2+} channels deactivate as their voltage-dependent open gates close on repolarization of the membrane potential. However, the channels continue to carry current for a fraction of a millisecond after repolarization, giving rise to a tail current. The time course of the tail current reflects the kinetics of the channel closing process (deactivation). The tail current in wild-type and mutant presenilin larvae was examined by a 30 ms depolarization to −15 mV, followed by repolarization to different membrane potentials (Fig. 1*B*). Its time constant (τ) was determined by fitting the tail current with a single exponential function. The AD-linked presenilin mutations had no significant effects on Ca^{2+} tail currents at RT. In the loss-of-function mutant Psn^{S3}, we observed a trend that the tail current was slower than in other genotypes, but the differences were not statistically significant (Fig. 1*C*). The tail current was not changed by HS treatment in the wild-type *2202U* strain larvae or in larvae expressing the wild-type or mutant presenilins, including Psn^{S3} and Psn^{E280A} (Fig. 1*B*). An exception was noted in larvae expressing the mutant Psn^{L235P}, in which the tail current was accelerated under HS conditions when measured at the −70 and −110 mV repolarization potentials. To compare the change in tail current decay rate between RT and HS among different genotypes, the ratio of tail current time constant at RT *vs.* at HS was calculated. As shown in Fig. 1*D*, no significant difference was observed between the mutant presenilin flies and controls. Overall, these data indicate that Ca^{2+} channel functions are largely intact in presenilin mutant animals under normal and HS conditions.

We then examined Ca^{2+} currents under the post-HS condition, in which larvae were heat-shocked for 2 days and allowed to recover at RT for 1 day. With this treatment, the I/V curve of the Ca^{2+} current was not changed by presenilin mutations. Compared with wild-type controls, there was no significant difference in the peak Ca^{2+} current or the depolarizing membrane potential at which the peak current was reached in larvae expressing either the loss-of-function or AD-linked mutant presenilins (**Fig. 2*A***). However, the Ca^{2+} tail current in these mutants was noticeably slower under the post-HS condition (Fig. 2*B* and Supplemental Fig. S2*A*). The time constant of the Ca^{2+} tail current was increased at all three membrane potentials tested.

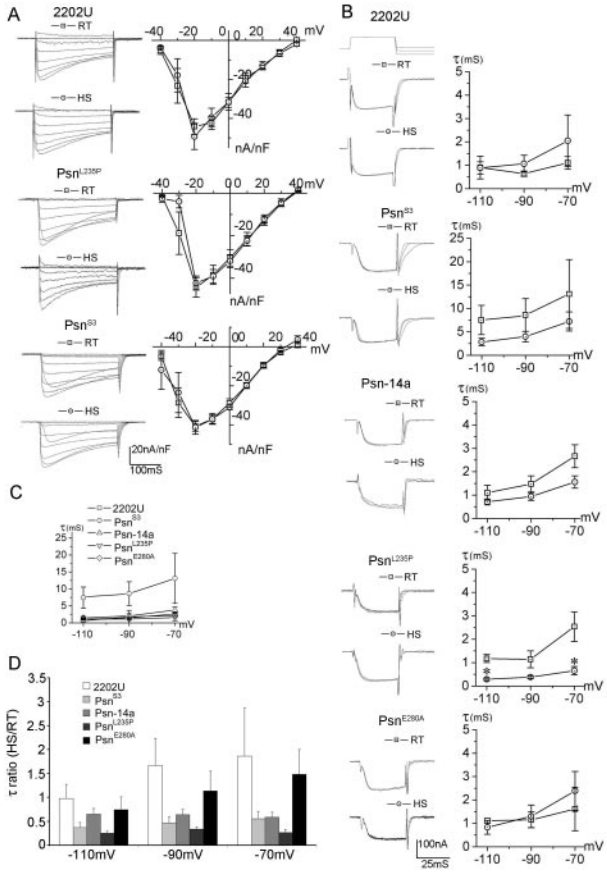

Figure 1. Effects of HS stress on whole-cell Ca^{2+} currents recorded at the larval NMJ. *A)* Current-voltage relationship of Ca^{2+} currents at the NMJ of the wild-type *2202U* and the presenilin mutants, Psn^{L235P} and Psn^{S3}, at room temperature (RT) or after 2 days at 30°C (HS). Square pulses of various voltages (from −40 to +40 mV, 10 mV steps, 250 ms in duration) were applied from the holding potential of −80 mV. Representative current traces (n=5) are shown in each group. For the I/V curve, peak amplitudes of Ca^{2+} currents were plotted against the voltage steps. *B)* Tail currents were recorded at various voltages (from −10 to −70 mV, 20 mV steps) after depolarization at −15 mV for 30 ms. Representative current traces and graphs plotting the tail-current time constant against repolarizing potentials are shown in each group (n=5). The time constant was determined by a single exponential fit to the decay of the tail current. HS treatment was as in panel *A*. *Significant difference compared with the RT control (P<0.05, ANOVA). *C)* Tail currents from different fly genotypes reared at RT. Data were pooled from panel *B*. There were no significant differences among the different genotypes (ANOVA, P>0.05). *D)* Comparison across different genotypes of tail current change between RT and HS. The ratio of tail current time constant at RT *vs.* HS was calculated from data in panel *B*.

When changes in the rate of tail current from RT to post-HS were compared, it was obvious that there was significant slowing of the tail current in the presenilin null mutants and the transgenic flies expressing mutant presenilins compared with corresponding controls (Fig. 2*C, D*).

The HS experiments demonstrated that the Ca^{2+} current was largely unaffected by loss-of-function or AD-linked presenilin mutations under either normal or

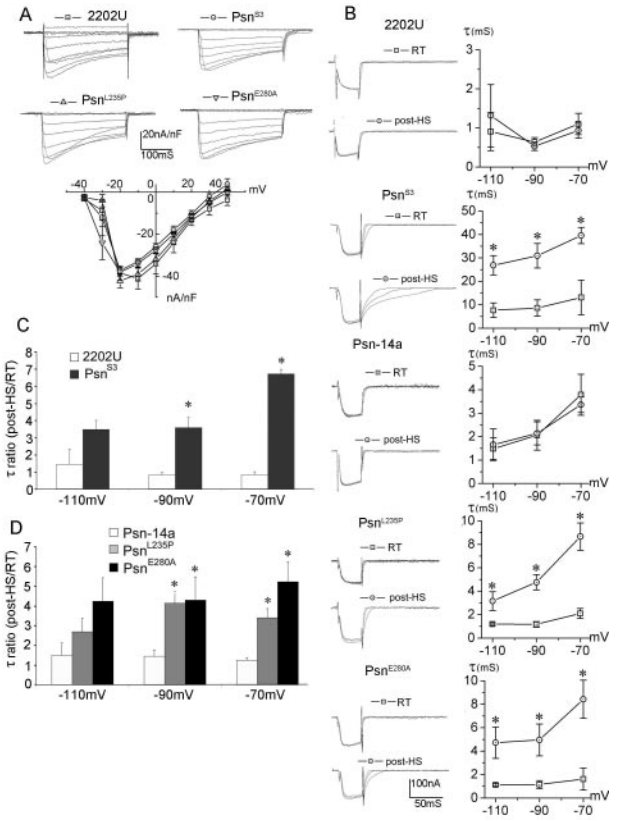

Figure 2. Effects of HS and post-HS treatments on whole-cell Ca^{2+} currents recorded at the larval NMJ. *A)* Current-voltage relationship of Ca^{2+} currents in the wild-type *2202U* and presenilin mutants, Psn^{S3}, Psn^{L235P}, and Psn^{E280A}, after HS at 30°C for 2 days, followed by recovery at RT for 1 day (post-HS). Test pulses were applied as in Fig. 1. Representative current traces (n=5) are shown for each group. The I/V curve shows peak amplitude of Ca^{2+} currents plotted against the voltage steps. *B)* Tail currents were recorded as in Fig. 1. Larvae were reared at room temperature for 3 days (RT) or at 30°C for 2 days, then allowed to recover at RT for 1 day (post-HS). Representative current traces (n=5) and graphs plotting tail current time constants *vs.* repolarizing potentials are shown for each group. Values significantly different from those of the corresponding treatment controls are indicated by asterisks (ANOVA, $P<0.05$). *C, D)* Comparison of tail current change among different genotypes between RT and post-HS. The ratio of tail current time constant at RT *vs.* post-HS was calculated from data in panel *B*. *Significant difference from the corresponding control genotypes (ANOVA, $P<0.05$).

HS conditions. However, the Ca^{2+} tail current was altered after the HS was terminated. To further confirm that presenilin is involved in maintaining homeostasis of the Ca^{2+} tail current during poststress recovery, we treated larvae with another form of stress. We fed larvae with thapsigargin and tunicamycin, which are known inducers of the ER stress response. Tunicamycin is a nucleoside antibiotic that prevents protein glycosylation and thapsigargin is an inhibitor of the ER Ca^{2+}-ATPase. Constant administration of tunicamycin by feeding had no discernable effects on Ca^{2+} tail currents in presenilin null mutant flies (Supplemental Fig. S2*B*) or in transgenic flies expressing the wild-type or AD-linked mutant presenilin Psn^{E280A} (**Fig. 3*A*, *C***). In transgenic Psn^{L235P} flies, there was an apparent weak but not statistically significant slowing in tail current kinetics at −90 and −70 mV after tunicamycin treatment. Consistent with the post-HS findings, 1 day after the larvae were transferred to food without tunicamycin

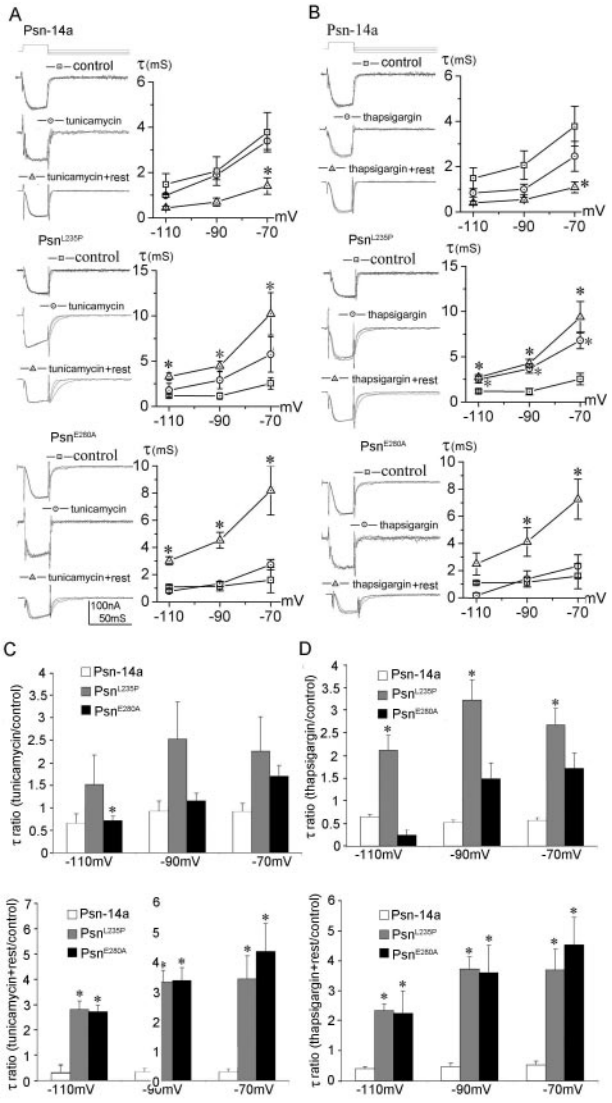

Figure 3. Effects of ER stress on whole-cell Ca^{2+} tail currents recorded at the larval NMJ. ER stress was induced by tunicamycin (*A*) or thapsigargin (*B*), which were added to the food (tunicamycin:5 mg/L; thapsigargin: 2 μM) and thus administered to larvae for 2 days. For controls (□), the same amount of the drug vehicle, ethanol, was added to the food. Tail currents were recorded immediately after drug treatments (○, drug) or 1 day after transferring treated larvae to fresh food (△, drug+rest). Representative current traces (n=5) and graphs plotting the tail current time constants *vs.* repolarizing potentials are shown. *Values significantly different from control treatment values ($P<0.05$, ANOVA). *C, D)* Comparison of tail current changes among different genotypes between drug-treated and control (top panel) or between drug + rest and control (bottom panel). The ratio of tail current time constant was calculated from data in panels *A, B*. *Significantly different from wild-type presenilin control value (ANOVA, $P<0.05$).

significantly slower kinetics in the tail current were observed in the presenilin null mutant (Supplemental Fig. S2*B*) and both transgenic fly strains expressing mutant presenilins, even though the tail current in the wild-type control *Psn-14a* flies exhibited faster kinetics at the −70 mV repolarization potential (Fig. 3*A, C*). Similarly, the Ca^{2+} tail current was not altered by feeding of thapsigargin to the wild-type *Psn-14a* and mutant Psn^{E280A} transgenic lines, but was prolonged in the mutant Psn^{E280A} 1 day after thapsigargin treatment ceased (Fig. 3*B, D*). A prolonged tail current was also observed in the presenilin null mutant 1 day after cessation of thapsigargin treatment (Supplemental Fig. S2*C*). In the mutant Psn^{L235P}, the tail current was slower upon administration of thapsigargin and the slower kinetics persisted after thapsigargin treatments ended (Fig. 3*B*). Overall, these results further demonstrate that presenilin is required for maintaining normal Ca^{2+} tail currents after termination of the stress treatments, and extend our initial findings with heat shock to include other stress inducers.

The Ca^{2+} currents recorded include at least two components: a diltiazem-sensitive one that is similar to the vertebrate L-type Ca^{2+} channel (LTCC) current and an amiloride-sensitive one similar to the vertebrate T-type Ca^{2+} channel current (49). To determine which types of Ca^{2+} channel are affected by presenilin mutations under poststress conditions, we used diltiazem and amiloride to block the L-type and T-type Ca^{2+} channel currents, respectively (49). Under post-HS conditions, the tail current in the presence of amiloride was prolonged in mutant Psn^{S3} larvae (**Fig. 4**). On the other hand, the tail current in the presence of diltiazem was not affected, suggesting that the L-type rather than the T-type Ca^{2+} channel tail current is specifically affected by presenilin mutations during the poststress recovery period (Fig. 4).

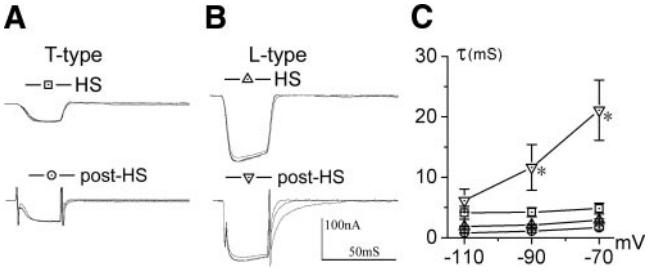

Figure 4. Effects of stress treatments on whole-cell Ca^{2+} tail currents of T- and L-type Ca^{2+} channels recorded at the NMJ of the Psn^{S3} mutant. *A)* Representative traces of T-type Ca^{2+} channel currents recorded from larvae subjected to HS or post-HS treatments. T-type Ca^{2+} channel currents were recorded by blocking L-type channels with diltiazem (1 mM). *B)* Representative traces of L-type Ca^{2+} channel currents recorded from larvae subjected to HS or post-HS treatments. L-type Ca^{2+} channel currents were recorded by blocking T-type channels with amiloride (1 mM). *C)* Time constants of the T- and L-type Ca^{2+} channel tail currents recorded at different repolarizing potentials from larvae treated as in panels *A, B* (n=5). Symbols are as in panels *A, B*. *Values significantly different from the corresponding control HS treatment values (P<0.05, ANOVA).

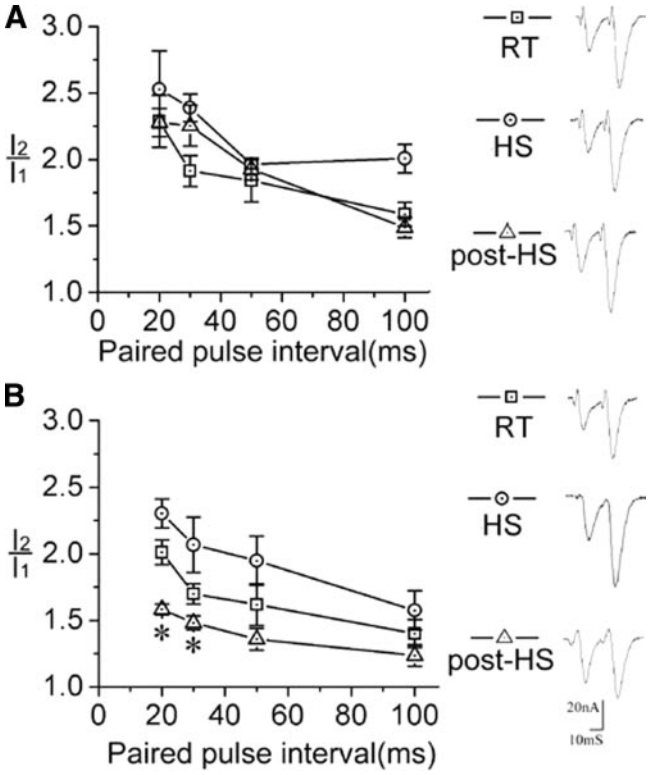

Figure 5. Effects of post-HS treatment on PPF at the larval NMJ of the wild-type *2202U* strain (*A*) and the Psn^{S3} mutant (*B*). Recordings were performed in saline containing 0.2 mM Ca^{2+}. Responses to 5 or 10 consecutively paired stimuli at 20–100 ms interpulse intervals were averaged. Traces show representative responses at 20 ms interstimulus intervals. Facilitation (I_2/I_1) is expressed as the mean amplitude ratio of response 2 to response 1 at each interval. Values significantly different from the corresponding RT control values are indicated by asterisks (ANOVA, P<0.05).

The *Drosophila* larval NMJ exhibits dynamic functional plasticity resembling that found in vertebrate central synapses (57). To investigate whether presenilin mutations affect synaptic plasticity, we examined paired pulse facilitation (PPF), a form of short-term plasticity at the NMJ. PPF is generally believed to reflect enhanced transmitter release during the twin pulse stimulation due to residual increases in presynaptic Ca^{2+} after the first pulse (58). Recently it was proposed that PPF may result from gradual saturation of an endogenous Ca^{2+} buffer (59). We examined PPF with multiple intervals (≤100 ms) that separated the two stimulation pulses. The wild-type larvae showed an ~2-fold facilitation at 20–30 ms interstimulus intervals, and this facilitation was not affected by either HS or post-HS treatment (**Fig. 5*A***). In Psn^{S3} mutant larvae, the PPF was comparable to the wild-type at RT (Fig. 5*B*). The HS treatment resulted in a slight but not statistically significant enhancement of the facilitation. However, the post-HS treatment significantly reduced the PPF at 20–30 ms intervals. We then examined functional synaptic transmission. In a presenilin null mutant, the

amplitude of the excitatory junctional current (EJC) was not affected by HS but was markedly increased in post-HS conditions (Supplemental Fig. S3). The amplitude and frequency of spontaneous miniature excitatory junctional current (mEJC) showed no significant difference between controls and the presenilin null mutant (data not shown), indicating an increased probability of quantal transmitter release.

To gain insight into the potential behavioral consequences of these electrophysiological perturbations, we examined the effects of HS treatments on learning and memory in the presenilin mutants. To overcome the lethality at the pupal stage in the presenilin mutants, we constructed presenilin null background flies carrying a wild-type *Drosophila* presenilin transgene and a second mutant presenilin transgene bearing an AD mutation, expressing both transgenes under the control of the endogenous presenilin promoter as direct transcriptional fusions. This constructed genotype closely mimics the genetics of AD patients carrying an AD-associated presenilin mutation, which is autosomal dominant and therefore involves one copy of a normal presenilin gene together with one copy of an AD mutant presenilin gene. Learning in these flies was not affected by either HS or post-HS treatment, nor was 24 h memory by HS alone (**Fig. 6*A***). However, under post-HS conditions, 24 h memory in these flies expressing the AD mutant presenilin was significantly reduced (Fig. 6*B*). Neither learning nor memory was affected by either treatment in flies expressing a wild-type presenilin control transgene (Fig. 6*A, B*). Taken together, our data indicate that presenilin AD mutations impair a specific aspect of poststress Ca^{2+} channel function that is implicated in long-term memory formation.

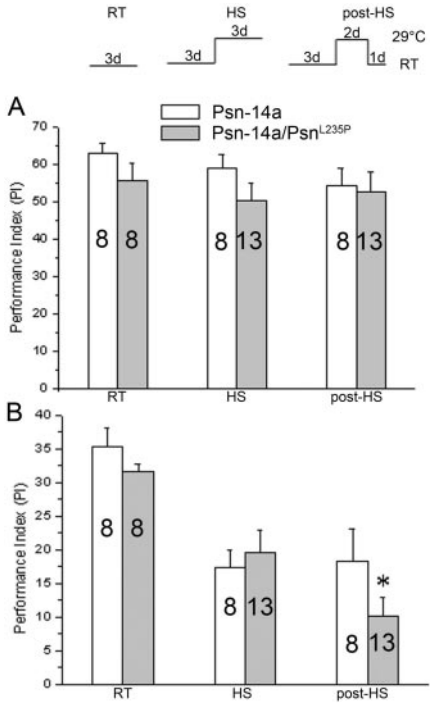

Figure 6. Effects of stress treatments on learning and memory in adult flies. In an endogenous *presenilin* gene null background, transgenic flies were constructed that were homozygous for the wild–type *Psn-14a* transgene (w^-(, or ;) *P[*w^+*, Psn-prom-Psn-14a]*; Psn^{B3}) or heterozygous for the AD-linked Psn^{L235P} transgene (w^-(, or ;) *P[*w^+*, Psn-prom-*Psn^{L235P}*]/P[*w^+*, Psn-prom-Psn-14a]*; Psn^{B3}). *A)* Learning indices for flies treated with the HS stress paradigms as indicated above each bar graph. *B)* 24 h memory assays for flies treated with the HS stress paradigms as in panel *A*. *Significant difference from the homozygous wild-type presenilin control index (ANOVA, $P<0.05$). The number of trials is indicated within each bar.

DISCUSSION

In this study, we examined the effects of presenilin mutations on membrane currents and synaptic functions at the *Drosophila* larval NMJ under stress conditions, including heat shock and ER stress. To ensure appropriate genetic attribution, multiple mutations as well as two normal variants of presenilin were tested. The effects of AD-linked presenilin mutations were analyzed using a transgenic *Drosophila* expression strategy. To circumvent potential problems associated with transgene overexpression and complications from endogenous presenilin gene activity, transgenic presenilin activities were assessed using transgenes driven by the native fly presenilin promoter in a genetic background where the endogenous presenilin gene was inactivated by null mutations.

By performing our studies in an intact whole-animal model, the observed effects of presenilin mutations on the stress response could be correlated with their *in vivo* physiological consequences. At the synaptic level, the effects of presenilin were most obvious after stress treatments had terminated and animals were in the recovery phase. We found that both K^+ and Ca^{2+} currents remained largely unaltered in loss-of-function or AD-linked presenilin mutants under both normal and stress conditions. This outcome is consistent with findings in mice where partial or complete loss of presenilin function does not alter basal neurotransmission at hippocampal area CA1 synapses (60, 61). Under stress conditions, the Ca^{2+} tail current was in general not changed significantly, although some minor variability was detected among the different genotypes. For example, the Ca^{2+} tail current in flies expressing Psn^{L235P} became faster under HS but was prolonged under ER stress. It is uncertain whether this might reflect a possible presenilin involvement in the stress response because of the variability among the different genotypes. However, a consistent finding observed in all the presenilin mutants examined is that the Ca^{2+} tail current was prolonged at 1 day after cessation of the stress treatment; further investigation revealed that this effect was specifically attributable to the LTCC tail current.

In vertebrates, prolonged LTCC tail currents have been observed during development (62) or after strong depolarizations (63–65). The LTCC consists of multiple subunits, and each subunit has multiple isoforms as a result of differential gene expression, alternative

splicing, and post-translational modification (66). Different channel compositions give rise to different gating properties of the LTCC and may influence the different rates of deactivation tail current (67–71). The class $Ca_v1.2$ α_1 subunit has been shown to contribute to forming an LTCC with anomalous gating properties characterized by rare and short openings with very low open probability during depolarization but long openings with high open probability after repolarization (70, 72). Isoforms of β subunits suppress the prolonged tail current, probably by facilitated inactivation of the channel (73, 74). Phosphorylation of the LTCC by the cAMP-dependent protein kinase A (PKA) or protein kinase C (PKC) can also slow down the deactivation tail current (75–77).

How presenilin might be involved in modification of the LTCC tail current after stress termination is not clear. Despite differences in some pharmacological properties compared with their vertebrate counterparts (49), the *Drosophila* larval muscle LTCCs are also modulated by PKA (78) and PKC (79). Perturbations of the PKA or PKC pathways that enhance phosphorylation by pharmacological or genetic means increase the Ca^{2+} current (78, 79). However, whether the tail current might be affected by these phosphorylation pathways has not been examined in these studies. Nevertheless, the effect of presenilin mutations on the LTCC tail current during recovery from stress is unlikely to be merely a result of channel phosphorylation. Instead, we observed a specific effect on the LTCC tail current only, and all other properties of the LTCC current remained unaffected. This effect is distinct from the other known effects of phosphorylation on both vertebrate and *Drosophila* LTCCs in which the current-voltage relationships and/or channel activation and inactivation are also affected (77, 80). It was suggested recently that presenilin also forms ER Ca^{2+} leak channels, a function quite distinct from its recognized activity as an intramembranous aspartyl protease (81). In accordance with this model, altered intracellular Ca^{2+} levels due to the inability of mutant presenilins to conduct Ca^{2+} properly might affect the tail current *via* Ca^{2+}-dependent inactivation of the LTCC. However, the proposed channel function for presenilin is associated with the full-length presenilin holoprotein, which is virtually undetectable at steady-state levels in neuronal and non-neuronal tissues *in vivo* (82) (see also Supplemental Fig. S1). Thus, although an ER-localized Ca^{2+} leak channel function of presenilin cannot be ruled out as a potential explanation for our findings, we believe that this newly proposed function of presenilin is unlikely to account for the effects of loss-of-function and AD-associated presenilin mutants on *Drosophila* LTCC properties.

A more likely explanation for our findings is that the prolonged LTCC tail current is the final outcome of multiple events that occur in the poststress recovery period, possibly involving functions of presenilin in the regulation of gene transcription. This possibility is supported by the observation that the LTCC tail current in presenilin mutants was not affected immediately after cessation of stress (Y. Lu and Y. Zhong, unpublished data). Stresses, including heat, can induce modifications of the Ca^{2+} channels, such as changes in channel composition (83–86). Presenilin might be involved in reversing the changes that occur under stress conditions once these conditions are alleviated, perhaps by transcriptional regulation of specific isoforms of channel subunits, ancillary subunits, and modulatory components. Levels of protein kinases might also be affected, changing the dynamic equilibrium of Ca^{2+} channel phosphorylation. Such precedents for the involvement of presenilin in neuronal transcriptional control mechanisms exist. Indeed, presenilin is essential for release of the nuclearly translocating transcriptional regulatory factor NICD during Notch signal transduction (54, 87), and its processing of APP and APLP is likewise involved in transcriptional activation through release of an analogous membrane-released fragment, termed AICD (88–91). Furthermore, loss of presenilin function has been shown to reduce CREB/CBP-dependent gene expression (61). It remains to be determined whether these or other presenilin-dependent transcriptional mechanisms might underlie the modulatory effects of presenilin on LTCC tail current during recovery from stress.

A reduction in PPF is generally regarded as the result of increased probability of transmitter release (92). The reduced PPF in presenilin mutants after stress termination could be related to the prolonged LTCC tail current seen in these animals. LTCCs have been shown to be involved in the regulation of neurotransmitter release (77, 93, 94) as well as prepulse-induced facilitation (64, 65). A putative neurosteroid, pregnenolone sulfate modulates presynaptic transmission when applied to hippocampal slices: acute EPSP is augmented whereas PPF is decreased (94). This effect has been partly attributed to elevated intracellular Ca^{2+} from activation of the LTCC. In hippocampal and cerebellar Purkinjie neurons and some sensory neurons, LTCCs may also contribute to normal functions of neurotransmitter release (95–99). A slower deactivation LTCC tail current in the presenilin mutants during recovery from stress-inducing treatments will result in elevated Ca^{2+} influx and increased release probability of synaptic vesicles, which is consistent with our observation of the increased EJC amplitude (Supplemental Fig. S3), and therefore a reduced PPF. An alternative explanation for the reduced PPF involves a change in endogenous Ca^{2+} buffer capacity. Alterations in the fast endogenous Ca^{2+} buffer calbindin-D28k affect the magnitude of PPF (59). As discussed above, transcription may be affected in presenilin mutants during stress recovery, which could affect the level of Ca^{2+} buffer proteins.

Our behavioral assay data raise the intriguing possibility that long-term plasticity (LTP) might also be affected after termination of the stress response. It has been shown that the LTCC is involved in NMDA receptor-independent LTP and spatial memory formation in mouse behavioral tests (100). As predicted by

this established role for the LTCC in LTP and memory formation, we found that long-term memory in mutant presenilin flies was specifically affected during recovery from stress. It is interesting that A-kinase anchoring proteins, which are required for PKA phosphorylation-induced prolongation of LTCC tail current in mammals (75), are also involved in long-term memory formation in *Drosophila* (Y. Lv and Y. Zhong, unpublished data), hinting at a potentially conserved function for LTCC function in LTP in the fly and mammalian nervous systems.

Alzheimer's disease-associated amyloid-β (Aβ) peptides selectively up-regulate the LTCC by direct binding to the channel and promoting insertion of the α subunit into the plasma membrane (101). However, flies do not make toxic Aβ peptides (102), so our observed impairment of LTCC function during poststress recovery is an interesting amyloid-independent effect of presenilin mutants on neuronal function, perhaps distinct from its amyloid-related effects on neuronal survival. The poststress Psn mutant effects could thus reflect more subtle perturbations in neuronal function than those caused by massive neurodegeneration. Impaired presenilin function thus might disrupt the synaptic activities of neuronal networks before any overt neurodegeneration occurs, and could also render neurons less able to recover from stress when exposed to toxins, during aging, and in the later neurodegenerative stages of Alzheimer's disease. Furthermore, because the flies we used in the learning and memory studies carry a wild-type presenilin allele and an AD-linked presenilin allele, and thus closely recapitulate the autosomal dominant presenilin mutant genotype found in human AD patients, the specific defect uncovered in long-term memory during stress recovery might be clinically relevant to AD neuropathophysiology. In humans, even among people carrying the identical presenilin mutation, there is significant variability in the age of disease onset (103–106), indicating contributions from other genetic modifiers and/or environmental factors. Our observation of prolonged Ca^{2+} tail currents and impaired plasticity at synaptic and behavioral levels in AD-associated presenilin mutants during recovery from stress raises the possibility that neuronal stress response and recovery mechanisms might be intimately linked to the pathogenesis of Alzheimer's disease and could serve as a potential target in considering therapeutic strategies for prevention and treatment of this dementia. FJ

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