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Presynaptic residual calcium and synaptic facilitation at hippocampal synapses of mice with altered expression of SNAP-25

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Abstract

Paired pulse facilitation (PPF) is a form of short-term synaptic plasticity that results from an interaction of residual presynaptic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{res}}$), number of release-competent vesicles, and the sensitivity of the vesicle release mechanisms to Ca^{2+} . While PPF is predominant at hippocampal Schaffer collateral-CA1 (SC-CA1) synapses, facilitation is greater in adult mice (designated Tkneo) that over express an isoform of the plasma membrane-targeted SNARE protein, SNAP-25a, which is normally predominantly expressed in juvenile animals. SNAP-25 is essential for action potential-dependent neuroexocytosis, yet the significance of the shift between the alternatively spliced variants SNAP-25a and SNAP-25b is not fully understood. This alteration of a key component of the protein machinery required for neurotransmitter release in Tkneo mice, therefore, provides a useful tool to further investigate presynaptic mechanisms that influence short-term plasticity. To explore this link between SNAP-25 and PPF, we simultaneously measured postsynaptic potentials and presynaptic $[\text{Ca}^{2+}]_{\text{res}}$ during paired-pulses in adult Tkneo, heterozygote null (HET), and wild type (WT) mice. We demonstrate that enhanced PPF is maintained at mature hippocampal synapses of Tkneo mice that predominantly express SNAP-25a, and that $[\text{Ca}^{2+}]_{\text{res}}$ kinetics are altered at synapses of Tkneo and HET mice, both of which exhibit reduced levels of total SNAP-25 expression. To evaluate the role of SNAP-25 in short-term plasticity and $[\text{Ca}^{2+}]_{\text{res}}$ regulation, we applied a vesicular release probability model for neurotransmission. Our results suggest that the isoform expression and total level of SNAP-25 affect both $[\text{Ca}^{2+}]_{\text{res}}$ dynamics and the ability of releasable vesicles to enter into a facilitated state.

Keywords

SNAP-25; synaptic plasticity; residual Ca^{2+} ; facilitation

1. Introduction

Paired pulse facilitation (PPF) is a form of synaptic plasticity that results primarily from presynaptic mechanisms, which include the actions of presynaptic residual Ca^{2+} ($[\text{Ca}^{2+}]_{\text{res}}$), the number of release-competent vesicles, and the sensitivity of vesicle release mechanisms to Ca^{2+} (Zucker and Regehr, 2002). The plasma membrane-targeted protein, SNAP-25, is a

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key component of the exocytotic machinery that mediates neurotransmitter release (Nagy et al., 2005) and it can act directly on voltage-gated Ca^{2+} channels (VGCCs) to alter Ca^{2+} influx (Catterall, 1999). SNAP-25 is thus well positioned to govern changes of short-term plasticity. The growing evidence that the *Snap25* gene and protein are associated with neuropsychiatric and neurological disorders (Corradini et al., 2009), emphasizes the importance of understanding how SNAP-25 participates in modulating synaptic function. Nevertheless, the precise role of this protein in the physiology of neurotransmission remains controversial (Corradini et al., 2009).

Vesicular release of neurotransmitter requires the formation of a ternary complex comprised of members of the SNARE (soluble N-ethylmaleimide factor attachment receptor) protein family (Fukuda et al., 2000). The t-SNARE, SNAP-25, plays an essential role in action potential-dependent exocytosis at neuronal synapses (Washbourne et al., 2002). This protein is encoded as two isoforms generated by alternative splicing of tandemly arranged, divergent copies of exon 5 (Bark, 1993; Bark et al., 1995). In neonatal brain, SNAP-25a is the predominant isoform, but after the first postnatal week the ratio of the two isoforms is reversed such that SNAP-25b constitutes roughly 90% of the total in most regions of the adult rodent nervous system (Bark et al., 1995; Boschert et al., 1996; Catsicas et al., 1991; Jacobsson et al., 1999).

The *Snap25*^{Tkneo} mutation, generated by the insertion of the HSV thymidine kinase neomycin phosphotransferase reporter gene downstream from the alternatively spliced exons 5a and 5b, suppresses the transition of splicing that produces the two isoforms and also reduces the overall expression of SNAP-25 (Bark et al., 2004). Consequentially, homozygous mutant mice (Tkneo) exhibit isoform and expression level profiles typical of juvenile (P4 – P7) mice (Bark et al., 2004). Electrophysiological analysis has shown that PPF is enhanced at Schaffer collateral to CA1 pyramidal neuron (SC-CA1) synapses of adolescent Tkneo mice compared to wild type (WT) littermates and heterozygote null (HET) mice. Since SNAP-25 has been suggested to modulate the Ca^{2+} dependency of vesicular release (Bronk et al., 2007; Graham et al., 2002), the increased PPF of Tkneo mice could result from an alteration of this process. We carried out simultaneous presynaptic Ca^{2+} imaging and field excitatory postsynaptic potential recording of SC-CA1 synapses in adult *Snap25* Tkneo, HET, and WT mice in order to investigate the influence on PPF of the relationship between SNAP-25 isoforms and presynaptic $[\text{Ca}^{2+}]_{\text{res}}$ dynamics. We fit our data with a model that describes the $[\text{Ca}^{2+}]_{\text{res}}$ -dependent recruitment of vesicles into a subpopulation of release-competent vesicles that has a higher release probability (Bark et al., 2004; Schiess et al., 2006). This analysis suggests that the expression of a specific SNAP-25 isoform can modify the influence of $[\text{Ca}^{2+}]_{\text{res}}$ on the facilitation of vesicular release thereby supporting a role for the composition of the exocytosis machinery in sculpting neuronal plasticity.

2. Results

To characterize the relationship between SNAP-25 and Ca^{2+} -dependent mechanisms of enhanced short-term synaptic plasticity, we evaluated the dynamics of presynaptic residual Ca^{2+} ($[\text{Ca}^{2+}]_{\text{res}}$) and plasticity in Tkneo, HET, and WT mice. We previously reported that Tkneo mutant mice exhibit about a 50% reduction in total SNAP-25 protein expression in cortex, hippocampus, and cerebellum and exhibit an isoform profile characteristic of juvenile (P4 – P7) mice with greatly increased levels of SNAP-25a and reduced levels of SNAP-25b RNA transcripts (Bark et al., 2004). Furthermore, HET mice, which bear the targeted ablation of one *Snap25* WT allele, also exhibit a 50% reduction in total SNAP-25 protein (Pozzi et al., 2008; Washbourne et al., 2002) (see Figure S2F). Quantitative rtPCR analysis of the relative level of SNAP-25a RNA compared to total SNAP-25 RNA

transcripts expressed in the hippocampus of adult mice, demonstrate that relative SNAP-25a expression in HET mice is very similar to that in WT mice, but greatly increased in the Tkneo mice (WT, $5.8 \pm 0.64\%$; HET, $5.0 \pm 0.52\%$; Tkneo, $45.8 \pm 2.22\%$, see Figure S1A). Consistent with this quantitative assay of mRNA expression, Western blots were performed using SNAP-25 isoform-specific antibodies and a monoclonal antibody that recognizes both isoforms (Fig. S2A-F). These demonstrated that the relative expression of SNAP-25b is greatly reduced in TKneo compared to WT mice (13 fold in cortex and 8 fold in hippocampus, Figure S2A, B), whereas SNAP-25a expression was increased by about 50% in cortex and hippocampus of TKneo compared to WT mice (Figure S2C, D). Taken together, these results are consistent with our earlier results (Bark et al., 2004; Bark, 2009) and show that while SNAP-25b is the predominant hippocampal isoform of SNAP-25 in both WT and HET mice, the relative expression of these isoforms is significantly altered in TKneo mice leading to a greatly increased contribution of the SNAP-25a isoform in Tkneo when compared to WT and HET control mice. Since the level of total SNAP-25 expression is comparably reduced in Tkneo and HET mice (Bark et al., 2004) (see Figure S1E), we used HET mice as controls to distinguish between effects due to the decreased level of total SNAP-25 and those due to the dramatically increased relative expression of the SNAP-25a isoform compared to SNAP-25b in Tkneo mice.

We chose to investigate short-term plasticity at a 50 ms interpulse interval because this is near the average natural frequency recorded in freely moving rodents (Klyachko and Stevens, 2006). Previous studies (e.g. Pozzi et al., 2008), however, have compared short-term facilitation at hippocampal SC-CA1 synapses between WT and HET mice at shorter interpulse intervals to reveal enhanced facilitation in SNAP-25-deficient synapses. In preliminary studies, therefore, we measured PPF at varying interpulse intervals for all three genotypes and found no significant difference in PPF between WT and HET slices at intervals of 20, 50, and 100 ms. However, when the interpulse interval was shortened to 10 ms, PPF in both HET and Tkneo slices was significantly greater than that recorded from WT slices (Fig 1A). These measurements were made from records of fEPSP slopes recorded in CA1 *stratum radiatum* following surgical isolation of Schaffer collateral axons from their somata in CA3. In our previous studies (Schiess et al., 2006; Scullin and Partridge, 2010; Scullin et al., 2010), we have used both fEPSP slope ratios and population spike (PS) amplitude ratios to determine PPF at the SC-CA1 synapse with results that are comparable to those observed in figures 1A and C. As discussed in Experimental Procedures, due to our interest in the presynaptic $[Ca^{2+}]_{res}$ we chose to use measurements of PS amplitude for the remaining analyses in this study.

There was no difference in amplitude of the response to the first stimulus (R1) at half maximum stimulus intensity among the three genotypes (Figure 1B & 1C). We reported previously that synaptic short-term facilitation at SC-CA1 synapses of adolescent (P21 – P24) Tkneo mice is greater than that of either similarly aged WT littermates or HET mice (Bark et al., 2004). As shown in Figure 1D, we found that the paired pulse ratio (PPR) was enhanced in adult ($\approx$ P100) Tkneo mice compared to WT and HET mice. Thus, the phenotype of enhanced plasticity in Tkneo mice persists from post-weaning adolescence through adulthood and is not purely a developmental phenomenon

2.1 Relationship of paired-pulse responses

The magnitude of PPF is stimulus dependent and we examined the relationship between stimulus strength and the ratio of R1 to R2 (Figure 2). For all three genotypes, at lower stimulus strengths, this ratio was greater than 1, indicative of PPF. However, as R1 increased, this ratio was reduced and in some cases became less than 1, reflecting the onset of paired-pulse depression. Because presynaptic $[Ca^{2+}]_{res}$ is hypothesized to be involved in short-term plasticity (Zucker and Regehr, 2002), we have previously interpreted this non-

linear relationship between R1 and R2 to indicate that a mechanism downstream from presynaptic $[Ca^{2+}]_{res}$ contributes to changes in plasticity (Schiess et al., 2006). We therefore sought to examine the role of the level of SNAP-25 expression and its isoforms on synaptic plasticity by taking advantage of differences among the three genotypes.

In order to begin to assess how differences in SNAP-25 expression might lead to the greater $[Ca^{2+}]_{res}$ -dependent facilitation observed in Tkneo mice, we fit the non-linear relationship between R1 and R2 amplitudes (Figure 2) with our established model that accurately reflects PPF and is based on parameters that define Ca^{2+} -dependent vesicle release pools (Schiess et al., 2006). Overall, our modeling analysis (see Supplementary Material) suggests that decreasing the amount of SNAP-25 increases the sensitivity of the facilitatory mechanism, which could be due to an increase in the effectiveness of $[Ca^{2+}]_{res}$ in this process. In particular, this could occur through a difference in saturation of the $[Ca^{2+}]_{res}$ clearance mechanism among the three genotypes perhaps as a result of a SNAP-25-dependent increase in cytoplasmic buffering or in an active Ca^{2+} transport process.

2.2 Ca^{2+} regulation

As synaptic facilitation has been shown to be correlated with presynaptic $[Ca^{2+}]_{res}$ and our model (see Supplementary Material) suggests an increase in the Ca^{2+} sensitivity of this process (Regehr and Tank, 1991; Schiess et al., 2006; Wu and Saggau, 1994), we measured presynaptic $\Delta F/F_0$ after filling SC-CA1 presynaptic terminals with the fluorescent Ca^{2+} -sensitive indicator, MgGreen-AM. Because our modeling studies indicated an influence of *Snap25* genotype on $[Ca^{2+}]_{res}$ clearance, we measured the kinetics of the $\Delta F/F_0$ transients that accompanied PPF (Schiess et al., 2006; Schiess et al., 2010; Scullin et al., 2010; Wu and Saggau, 1994). As expected from our previous measurements in rats (Schiess et al., 2006), we found a linear relationship between the transient $\Delta F/F_0$ response of the first (F1) and that of the second (F2) of paired stimuli with a greater than unity slope. Furthermore, we found no difference in this slope among the three genotypes (Figure 3A, $P = 0.98$), suggesting that these *Snap25* genotypes have little effect on the summation of presynaptic $[Ca^{2+}]_{res}$ during paired pulses.

In order to assess effects of SNAP-25 isoform or level on basal probability of release (P_R) rates, we next determined the dependence of R1 amplitude on extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) for the three genotypes. As shown in Figure 3B, the amplitude of R1 increased as expected in response to increasing $[Ca^{2+}]_o$ for all three genotypes (Blatow et al., 2003; Wasling et al., 2004), consistent with our previous MK-801 studies that showed no change in R1 release probability in the Tkneo mice (Bark, 2009). Thus, alteration of the initial P_R in Tkneo mice when compared to either HET or WT could not alone account for the observed differences in $[Ca^{2+}]_i$ saturation or PPF. We therefore sought to investigate additional factors that might contribute to the SNAP-25 isoform- or level-dependent enhanced plasticity.

As a further indication of the role of presynaptic Ca^{2+} kinetics in synaptic efficacy, we calculated the integral of $\Delta F/F_0$ for single pulses (grey traces in Figure 4B) and pairs of pulses (black traces in Figure 4B) after normalization to the $\Delta F/F_0$ peak value ($\Delta F/F_0$ at approximately 50 ms in Figure 4A) following a single pulse or the first of paired pulses (Scullin et al., 2010). This value, $\int v\Delta F/F_0$ (Figure 4B & D), has units of time and, as $\Delta F/F_0$ decays to baseline, $\int v\Delta F/F_0$ approaches a value that is proportional to the weighted summed time constants of $[Ca^{2+}]_{res}$ clearance reflecting the multiple $[Ca^{2+}]_{res}$ elimination kinetic processes (Scullin et al., 2010). Importantly, $\int v\Delta F/F_0$ is distinct from $\int \Delta F/F_0$, which also has units of time proportional to the weighted time constant of $[Ca^{2+}]_{res}$ decay; however, the latter integral is additionally proportional to the amplitude of $\Delta F/F_0$ and thus it reflects cumulative $[Ca^{2+}]_{res}$. While the peak $\Delta F/F_0$ response to a single stimulus was not significantly different among the three genotypes (Figure 4C, $P = 0.39$), the value of $\int v\Delta F/F_0$

F_0 for both Tkneo and HET mice was significantly less than that of WT mice (Figure 4D). This suggests that the level of SNAP-25 expression affects the timecourse of $[Ca^{2+}]_{res}$ clearance and further suggests a SNAP-25-dependent difference of the effectiveness of $[Ca^{2+}]_{res}$ in facilitating release.

Because some proportion of the $[Ca^{2+}]_{res}$ signal during paired-pulses results from the summation of $[Ca^{2+}]_{res}$ between F1 and F2, it is difficult to accurately measure $\Delta F/F_0$ caused by the influx of Ca^{2+} during F2. However, if we assume that the values of $\int \Delta F/F_0$ for successive pulses are independent, then the sum for a pair of pulses should be twice the size of that for a single pulse. We have previously demonstrated that there is significant supra-linear summation of $[Ca^{2+}]_{res}$ during paired-pulses in the adult rat hippocampus such that the ratio of $\int \Delta F/F_0$ resulting from paired-pulses is more than twice that elicited by a single pulse (Schiess et al., 2006). While our $\Delta F/F_0$ measurement does not directly assess the Ca^{2+} that is responsible for vesicular release, it is representative of the saturation of the mechanisms that determine the timecourse of presynaptic $[Ca^{2+}]_{res}$ (Scullin and Partridge, 2010). Furthermore, we and others have shown $[Ca^{2+}]_{res}$ to be a critical determinant of the facilitation of the post-synaptic response (Schiess et al., 2010; Wu and Saggau, 1994; Zucker and Regehr, 2002). When the same analysis was performed on the data derived from slices of Tkneo, HET, and WT mice, we found that the ratio of $\int \Delta F/F_0$ resulting from paired-pulses to that from a single pulse was larger than two for all three genotypes and significantly greater for Tkneo than for either HET or WT mice (Figure 4E). Thus the increased accumulation of $[Ca^{2+}]_{res}$ in Tkneo presynaptic terminals was comparable to the greater synaptic facilitation observed in this genotype. Therefore, although the initial $[Ca^{2+}]_{res}$ accumulation following a single pulse is less in SNAP-25-deficient (HET and Tkneo) synapses, the accumulation during the second of paired responses shows significant additional enhancement only in the Tkneo synapses. As discussed below, one possible explanation for this observation is that the SNAP-25a isoform influences the saturation of the $[Ca^{2+}]_{res}$ clearance mechanisms thereby influencing the $[Ca^{2+}]_{res}$ accumulation after repeated stimuli, whereas the total amount of SNAP-25 influences accumulation after a single stimulus.

2.3 $[Ca^{2+}]_{res}$ sensitivity of PPF

To further elucidate differences in the $[Ca^{2+}]_{res}$ dependence of facilitation among the three genotypes, we used the well-established method of measuring PPR at different values of $[Ca^{2+}]_o$ (Blatow et al., 2003; Schiess et al., 2006; Wasling et al., 2004). As expected, in SC-CA1 synapses from WT mice, we observed a significantly negative slope of -0.8393 ($F_{1,42} = 4.21$, $P = 0.047$) for this relationship (Figure 5B). In contrast, we observed a clearly different response to increasing $[Ca^{2+}]_o$ in slices from Tkneo and HET mice (Figure 5A & B). For both of these genotypes, this relationship had a significantly positive slope between 1 and 2 mM $[Ca^{2+}]_o$ (overall significance $F_{2,72} = 3.86$, $P = 0.026$; Tkneo: $F_{1,29} = 7.96$, slope = 0.86 , $P = 0.0085$; HET: $F_{1,19} = 4.92$, slope 1.27 , $P = 0.039$). Between 2 and 3 mM $[Ca^{2+}]_o$, the slope for the Tkneo and HET mice was not significantly different from 0 (Tkneo: $F_{1,51} = 0.46$, $P = 0.67$; HET: $F_{1,33} = 0.46$, $P = 0.50$). As expected from our previous studies and those from other laboratories (Blatow et al., 2003; Schiess et al., 2006; Wasling et al., 2004), at low $[Ca^{2+}]_o$, the marked sensitivity of PPF to small variations in the reduced amplitude of R1 led to the considerable variability in the PPF observed in WT mice at 1 mM $[Ca^{2+}]_o$ (Figure 5B).

2.4 Potential Mechanisms of Genotype Differences

We have shown previously that uptake into and release from endoplasmic reticulum (ER) are primary mechanisms regulating $[Ca^{2+}]_{res}$ clearance in the SC presynaptic terminal, and that these mechanisms undergo developmental changes between P3 and P15 (Scullin and

Partridge, 2010; Scullin et al., 2010). Because our $\int v\Delta F/F_0$ data (Figure 4D) were consistent with a SNAP-25-dependent change in $[Ca^{2+}]_{res}$ clearance, we assessed the differences among the genotypes in the contribution of the endoplasmic reticulum to presynaptic Ca^{2+} regulation. Since the ratio of SNAP-25a to SNAP-25b in adult Tkneo mice is similar to that of P4 - P7 mice, we investigated two important mechanisms of $[Ca^{2+}]_{res}$ regulation that are known to change between P2 - P6 and adult (Scullin et al., 2010). First, we assessed the contribution of the SERCA pump to the uptake of presynaptic $[Ca^{2+}]$ into the ER (Schiess et al., 2010) by using thapsigargin to block SERCA pump activity (Verkhratsky, 2005). As expected, application of 3 μM thapsigargin slowed $[Ca^{2+}]_{res}$ clearance as indicated by a significantly increased $\int v\Delta F/F_0$ in thapsigargin to that in ACSF following a single pulse in the WT and, importantly, it led to a similar increase in Tkneo mice (Student's t-test, $P = 0.32$, Figure 6A). This suggested that differences in SERCA uptake, which are characteristic of developing SC-CA1 synapses, do not play a major role in shaping the difference in $[Ca^{2+}]_{res}$ clearance between Tkneo and WT mice. Second, we determined the role of Ca^{2+} -dependent Ca^{2+} release from the ER by applying 10 mM caffeine. As we previously reported (Scullin et al., 2010), caffeine, which induces Ca^{2+} release from cytoplasmic stores in young mice, did not result in significant Ca^{2+} release ($\Delta R/R_0$) in the presynaptic terminals of adult WT mice (paired Student's t-test, $P = 0.13$, Figure 6B); however, there was also no increase of $\Delta R/R_0$ following caffeine application in slices from adult Tkneo mice (paired Student's t-test, $P = 0.84$, Figure 6B). Taken together with our findings with thapsigargin, this suggests that SNAP-25 isoform or amount are not the primary factors in the observed changes between juvenile and adult synapses in the developmental maturation of the role of cytoplasmic stores in $[Ca^{2+}]_{res}$ clearance.

Finally, we extended our findings of the differences of $[Ca^{2+}]_{res}$ clearance among the 3 genotypes during paired-pulses by measuring $[Ca^{2+}]_{res}$ accumulation during longer stimulus trains. We used Fura-2 ratiometric imaging and a paradigm of repeated 2 s, 10 Hz pulse trains to approximate physiological θ burst firing and long-term synaptic plasticity paradigms (Grover et al., 2009). While there was not a significant difference in the initial $\Delta R/R_0$ response among the three genotypes ($F_{2,9} = 1.09$, $P = 0.38$; Tkneo = 0.82 ± 0.07 ; HET = 0.72 ± 0.017 ; WT = 0.73 ± 0.062), there was a significantly greater increase in this response with successive stimulus trains in Tkneo slices suggesting an increase in background $[Ca^{2+}]_i$ early in the stimulus train when compared to HET or WT mice. This difference, however, diminished later during the repetitive stimulus train (Figure 6C). These data suggest a diminished degree of negative feedback modulation on Ca^{2+} influx in Tkneo mice.

3. Discussion

3.1 SNAP-25 isoform in adult SC-CA1 synapses

We report here that the level of SNAP-25 expression affects distinct properties of presynaptic $[Ca^{2+}]_{res}$ in adult SC-CA1 synapses and that differential expression of the two SNAP-25 isoforms is involved in determining the amount of facilitation at these synapses. This indicates that the regulated expression of SNAP-25 is likely to be essential in sculpting the properties of specific synapses as they contribute to nervous system circuits. The shift in expression between SNAP-25a and SNAP-25b isoforms may underlie a refinement of the Ca^{2+} -dependent vesicular fusion process underlying the mature phenotype of synaptic plasticity (Bark et al., 2004; Johansson et al., 2008; Pozzi et al., 2008). Indeed, detailed investigation of the kinetics of neuroexocytosis in reconstituted null mutant chromaffin cells and cultured neurons has suggested that SNAP-25b is more effective than SNAP-25a or the constitutive cellular homologue SNAP-23 in maintaining vesicles in the primed state, which leads to a greater size of the readily releasable pool (RRP) (Delgado-Martinez et al., 2007; Sorensen et al., 2003). In addition, SNAP-25 is phosphorylated by protein kinase C

(Kataoka et al., 2006b), which correlates well with the developmentally-regulated expression of SNAP-25b mRNA (Bark et al., 1995) and protein (Prescott and Chamberlain, 2011; Yamamori et al., 2011) that increases until about 3 weeks of age. Moreover, there is evidence that the level of this phosphorylation in turn modulates Ca^{2+} flux through VGCCs (Pozzi et al., 2008). This suggests that SNAP-25b may be a preferential target for phosphorylation and a reduction in this phosphorylation may contribute to the differences seen in presynaptic facilitation and Ca^{2+} dynamics observed in the Tkneo and HET synapses. It is interesting, therefore, that Takahashi and colleagues have recently reported that a mouse mutant designed to eliminate phosphorylation at Ser187 shows behavioral impairments as well as enhanced seizure activity (Kataoka et al., 2011).

3.2 SNAP-25 and the regulation of PPF

We have reported previously that PPF at 50 ms is enhanced in Tkneo mutant SC-CA1 synapses of adolescent (P21 - P23) when compared to WT mice, but that there is no similar enhancement of facilitation in HET slices (Bark et al., 2004). We have now confirmed that this enhanced facilitation is maintained in adult Tkneo slices, suggesting that this is not strictly a developmental phenotype. This indicates that alterations of SNAP-25 isoform and/or total level do play a role in regulating specific characteristics of synaptic plasticity.

Comparisons between these findings and those of other groups provide conflicting evidence about the effect of SNAP-25 expression levels on PPF (Corradini et al., 2009) and this may reflect a state-dependent regulation that is affected by the experimental paradigm. For example, in examination of HET mice, which have reduced expression of SNAP-25, Pozzi et al. (Pozzi et al., 2008) reported enhanced PPF at SC-CA1 synapses at intervals of 10 ms or less, but not at intervals greater than 20 ms. Figure 1A makes a comparison of PPF in Tkneo, HET, and WT slices at interpulse intervals between 10 and 100 ms. At each interval, PPF is greatest in the Tkneo mice, but there is not a significant difference in PPF between HET and WT mice except at the 10 ms interpulse interval. Consistent with the observations of Pozzi et al. mentioned above, there is greater PPF in slices from HET than from WT mice at the 10 ms interval. Moreover, Bark and colleagues have reported that mice with a targeted mutation of the *Snap25* gene that entirely eliminates the expression of the SNAP-25b, but not the SNAP-25a isoform (without affecting the level of total SNAP-25), exhibit reduced PPF (Johansson et al., 2008). These measurements of PPF, however, were performed at room temperature whereas both the experiments that we report here and have reported previously (Bark et al., 2004) were conducted at near physiological temperatures (32°C). Indeed, we have recently demonstrated that PPF is affected by age $\times$ temperature interactions and that this plasticity could be masked, or even expressed as depression, depending on the experimental conditions (Schiess et al., 2010). It is evident; therefore, that SNAP-25 must play an intimate role in mediating short-term plasticity, but how that plasticity is resolved can be dependent on the experimental protocol. This stresses the need to directly compare these properties under consistent experimental conditions.

3.3 SNAP-25 effects on Ca^{2+} regulation

We found previously that reduced expression or the complete absence of SNAP-25 did not significantly affect the amplitude of Ca^{2+} transients elicited by $[\text{K}^+]_o$ -dependent depolarization in the soma of cultured hippocampal neurons (Tafoya et al., 2008). In contrast, Pozzi and co-workers (Pozzi et al., 2008) showed that Ca^{2+} transients were enhanced largely in neurite processes of hippocampal neuron cultures from these mutants, which may be attributed to the loss of SNAP-25-mediated control of the inactivation kinetics of VGCCs. Interestingly, the regulation of Ca^{2+} transients was restored by expression of exogenous SNAP-25b, but not SNAP-25a, which is consistent with a maturation process that is not readily reflected by the rather immature state of neuronal cultures.

Our measurements of stimulus-driven presynaptic $[Ca^{2+}]_{res}$ transients show that mature SC-CA1 presynaptic terminals in acute slices from Tkneo and HET mice, which express comparably reduced levels of SNAP-25, exhibit similar alterations of $[Ca^{2+}]_{res}$ dynamics compared to WT synapses. For example, although there was no difference in the amplitude of the $[Ca^{2+}]_{res}$ signal among the three genotypes, both Tkneo and HET slices had a greater rate of decay ($\int v\Delta F/F_0$) of the presynaptic $[Ca^{2+}]_{res}$ transient than that of WT slices following a single stimulus (Figure 4D). This observation appears contrary to the findings of Matteoli and colleagues in cultured hippocampal neurons (Pozzi et al., 2008; Verderio et al., 2004) that decreased expression of SNAP-25 leads to increased Ca^{2+} accumulation due to a reduced effect of SNARE-mediated inactivation of VGCCs (Zhong et al., 1999). Several factors may contribute to these differences, but importantly the transient presynaptic $[Ca^{2+}]_{res}$ responses, which we measured with low affinity Mg Green (Figure 4A), are more likely to reflect different temporal and spatial processes than $[Ca^{2+}]_i$ responses measured with Fura-2 following K^+ depolarization. Our data from Tkneo and HET presynaptic terminals show a faster clearance of $[Ca^{2+}]_{res}$ than WT that could be dependent on either diffusion or buffering (Collin et al., 2005; Wasling et al., 2004). However, while the $[Ca^{2+}]_{res}$ decay kinetics in the adult rodent SC-CA1 synapse do not appear to be significantly affected by Ca^{2+} buffer saturation (Blatow et al., 2003), an increased role of buffer saturation has been reported in immature P6 – P12 synapses (Wasling et al., 2004). Interestingly, this coincides with the developmental period when the level of SNAP-25 expression increases (Bark et al., 1995). The increasing buffering capacity of the presynaptic terminal that normally occurs during this period might therefore correlate with the concomitant increase in SNAP-25b. Consistent with this interpretation, we found that adult WT presynaptic SC-CA1 terminals exhibit the expected non-saturating buffer characteristics, whereas both Tkneo and HET synaptic terminals exhibit some degree of buffer saturation that is reflected by the positive slopes of the relationship of PPR to $[Ca^{2+}]_o$ between 1 mM and 2 mM. In addition, the overall shape of this relationship for Tkneo and HET synaptic terminals is similar and differs significantly from that for WT terminals (Figure 5). These data indicate that the buffer saturation could be dependent upon the amounts of SNAP-25, but independent of the expressed isoform, or alternatively could result from compensatory mechanisms secondary to, or in addition to, the level of SNAP-25 expression. Nevertheless, this effect is apparently insufficient to lead to the enhancement of the PPR of HET synapses at 50 ms interpulse intervals that we observed in Tkneo slices. While it remains unclear how this difference in buffering might serve to diminish the effect of a VGCC-mediated increased influx of Ca^{2+} in SNAP-25-deficient presynaptic terminals of HET and Tkneo mice, it does suggest that such homeostatic regulation of $[Ca^{2+}]_{res}$ dynamics is likely to play a dominant role in regulating the availability of Ca^{2+} for synaptic plasticity.

The modification of $[Ca^{2+}]_{res}$ dynamics that results from altered expression of SNAP-25 isoforms is observed in both the postsynaptic response and presynaptic $[Ca^{2+}]_{res}$ transients following paired stimuli in Tkneo mice. Neither of these measures was correlated with the level of SNAP-25 expression (WT vs. HET) nor the isoform (WT vs. HET vs. Tkneo) (Figure 1D and 4C), but there was a significant difference in the second $[Ca^{2+}]_{res}$ transient in synapses from Tkneo mice (Figure 4E). This is consistent with a decrease in the rate of Ca^{2+} influx during repeated stimuli or a decrease in the clearance of $[Ca^{2+}]_{res}$ in Tkneo mutant synapses. The greater cumulative $\Delta R/R_0$ signal during the repeated pulse trains in Tkneo synapses, which suggests an increase of $[Ca^{2+}]_{res}$ (Figure 6C), is also consistent with additional mechanisms affected either directly or indirectly by the manner in which SNAP-25 isoforms differently effect $[Ca^{2+}]_{res}$ influx or removal processes following repetitive stimuli. The difference in the clearance of $[Ca^{2+}]_{res}$ between Tkneo and both WT and HET mice may thereby contribute to the genotype-dependent differences in plasticity among these synapses. This suggests that the effect of SNAP-25 levels in the regulation of

Ca^{2+} dynamics, particularly the change in Ca^{2+} saturation sensitivity, may be distinct from the role played by the SNAP-25 isoform. An important additional possibility is that these alterations in Ca^{2+} dynamics could be dependent on compensatory maturation within the synaptic circuitry.

3.4 Modeling synaptic vesicle pools

Differences in $[\text{Ca}^{2+}]_{\text{res}}$ clearance in the SC-CA1 terminal fields of Tkneo, HET, and WT mice do not fully describe the enhanced PPF at this synapse so additional mechanisms must be considered. One potential mechanism that could contribute to a greater PPF is the involvement of change in basal release probability (P_R) in Tkneo synapses. However, measurement of the rate of activity-dependent MK801 blockade of NMDA receptors indicates that neither the P_R nor the amplitude of R1 at half-maximum stimulation differs among Tkneo, WT, or HET synapses at P21 - P25 (Bark et al., 2004). Consistent with our modeling results (Bark et al., 2004; Schiess et al., 2006) (Supplemental material), this suggests that the primary mechanism underlying increased facilitation in Tkneo mice is an increased sensitivity of release mechanisms to Ca^{2+} during R2 that is fueled by a greater recruitment of vesicles to a facilitated state during the initial pulse. Interestingly, in chromaffin cells, SNAP-25b has been found to be responsible for an increase in the size of the RRP in rescued *Snap25* null mutants (Delgado-Martinez et al., 2007; Nagy et al., 2004; Sorensen et al., 2003). Furthermore, the transition between SNAP-25 isoforms appears to be temporally correlated with an increase in size of the RRP in cultured hippocampal neurons (Mozhayeva et al., 2002).

Our model predicts changes in the Hill equation parameters for Ca^{2+} binding to a facilitatory site that suggest a SNAP-25-dependent alteration in either $[\text{Ca}^{2+}]_{\text{res}}$ buffering or the affinity of this facilitatory site. Another potential mechanism for the influence of this SNARE protein on presynaptic $[\text{Ca}^{2+}]_{\text{res}}$ regulation is the association of SNAP-25 with VGCCs though the synprint site that provides a feedback mechanism to modulate the dynamics of Ca^{2+} influx and intracellular Ca^{2+} kinetics (Catterall, 1999; Pozzi et al., 2008). Furthermore, stimulation frequency-dependent alterations in PPF have been reported at these synapses in an exon 5b ablated mutant that expresses solely SNAP-25a (Johansson et al., 2008). This may reflect a change in the regulation of presynaptic $[\text{Ca}^{2+}]_i$ due to the exclusive expression of SNAP-25a, which is less effective in restricting Ca^{2+} influx through VGCCs (Pozzi et al., 2008). Thus, while $[\text{Ca}^{2+}]_{\text{res}}$ plays an important role in short-term plasticity, our data suggest that multiple converging factors, each of which may be influenced by SNAP-25, ultimately act to regulate $[\text{Ca}^{2+}]_{\text{res}}$ and thereby help to govern synaptic facilitation.

We report here that the level of SNAP-25 expression affects distinct properties of presynaptic $[\text{Ca}^{2+}]_{\text{res}}$ in adult SC-CA1 synapses and that differential expression of the two SNAP-25 isoforms is involved in determining the amount of facilitation at these synapses. This indicates that the regulated expression of SNAP-25 is likely to be essential in sculpting the properties of specific synapses as they contribute to nervous system circuits. The shift in expression between SNAP-25a and SNAP-25b isoforms may contribute to a refinement of the Ca^{2+} -dependent vesicular fusion processes underlying the mature phenotype of synaptic plasticity (Bark et al., 2004; Johansson et al., 2008; Pozzi et al., 2008). Indeed, detailed investigation of the kinetics of neuroexocytosis in reconstituted null mutant chromaffin cells and cultured neurons suggests that SNAP-25b is more effective than SNAP-25a or the constitutive cellular homologue SNAP-23 in maintaining vesicles in the primed state, which leads to a greater size of the RRP (Delgado-Martinez et al., 2007; Sorensen et al., 2003). In addition, SNAP-25 is phosphorylated by protein kinase C (Kataoka et al., 2006a), which correlates well with the developmentally-regulated expression of SNAP-25b mRNA (Bark et al., 1995) and protein (Prescott and Chamberlain, 2011; Yamamori et al., 2011) that increases until about 3 weeks of age. There is evidence that the level of this phosphorylation

in turn modulates Ca^{2+} flux through VGCCs (Pozzi et al., 2008). Moreover, Takahashi and colleagues have recently reported that a mouse mutant, designed to eliminate phosphorylation at Ser187, shows behavioral impairments as well as enhanced seizure activity (Kataoka et al., 2011). This suggests that SNAP-25b may be a preferential target for phosphorylation and a reduction in this phosphorylation may contribute to the differences seen in the presynaptic facilitation and Ca^{2+} dynamics observed in the Tkneo and HET synapses.

4. Experimental Procedure

4.1 Slice preparation

All experiments were conducted according to the relevant national and international guidelines and were approved by the Institutional Animal Care and Use Committee at the University of New Mexico Health Sciences Center, and the National Institutes of Health. Experiments were performed on coronal hippocampal slices prepared from adult mice (60 to 120 days, average 100 days). Strains bearing the *Snap25^{Tkneo}* (Mouse Genome Informatics allele designation *Snap25^{tm2Mcw}*) (Bark et al., 2004) and *Snap25* null (*Snap25^{-/-}*, MGI allele designation *Snap25^{tm1Mcw}*) (Washbourne et al., 2002) mutations are maintained by brother:sister heterozygote matings after 7 backcross generations to C57Bl/6 mice at the UNM HSC Animal Resource Facility. Homozygous *Tkneo* (Tkneo) mice, wild type (WT) mice, and heterozygote null (HET) mice were selected after genotyping from litters of the respective heterozygote matings. Genotypes were confirmed for all experimental animals from tail clips taken after removal of the brain for physiological studies.

Animals were deeply anaesthetized by i.p. injection of 250 mg kg^{-1} ketamine, brains were rapidly removed, and slices were cut at 300 μm with a vibroslicer (Pelco 101, St Louis, MO, USA) in an ice bath with a cutting solution containing (mM): 220 sucrose, 3 KCl, 1.2 NaH_2PO_4 , 26 NaHCO_3 , 12 MgSO_4 , 0.2 CaCl_2 , 10 glucose, and 0.01 mg ml^{-1} ketamine equilibrated with 95% O_2 –5% CO_2 . Slices were then transferred to a bath with artificial cerebrospinal fluid (ACSF) containing (mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4 , 1.3 MgSO_4 , 26 NaHCO_3 , 2.5 CaCl_2 , and 10 glucose equilibrated with 95% O_2 –5% CO_2 at 30°C for 1 h and then maintained at room temperature until transfer to a temperature-controlled recording chamber (Warner Instruments, Hamden, CT, USA or Scientific Systems Design, Mercerville, NJ, USA), which was maintained at 32°C and continuously perfused at 2 ml min^{-1} with ACSF saturated with 95% O_2 –5% CO_2 .

4.2 Presynaptic Ca^{2+} imaging

Presynaptic fibers were filled with the Ca^{2+} fluorophore, Mg Green AM or Fura-2 AM (Molecular Probes, Eugene, OR, USA) using an established technique (Atluri and Regehr, 1996; Kamiya and Ozawa, 1999; Regehr and Tank, 1991; Scullin et al., 2010; Sinha et al., 1997; Wu and Saggau, 1994). This technique allowed simultaneous measurements of presynaptic $[\text{Ca}^{2+}]_i$ and postsynaptic fEPSPs. In some instances, either the presynaptic or postsynaptic recording was not successful, but the data from the successful component were still included. To minimize the effect of exogenous buffers in experiments designed to measure the timecourse of $\Delta F/F_0$ decay, we used Mg-Green, with a Ca^{2+} binding $K_D = 6 \mu\text{M}$ (Atluri and Regehr, 1996; Regehr and Atluri, 1995). To control for bleaching in longer experiments with Fura-2, we measured the fluorescent ratio at the beginning and end of each stimulus train (40ms 350nm then 40ms 380nm).

Briefly, an ejection electrode (tip diameter 5 – 10 μm) containing the fluorophore (0.9 mM Mg Green AM or Fura-2 AM, 10% DMSO, 1% pluronic acid in ACSF) was lowered into the fiber pathway between the stimulating electrode and the presynaptic terminal field to be

investigated. While observing the emission image following excitation (490 nm for Mg-Green or 350 nm for Fura-2), with a 10 × water immersion lens on an Olympus BX51Wi microscope, an air pressure pulse was applied with a syringe to the ejection electrode until a small bright spot ($\approx 1 \mu\text{l}$) was observed in the fiber pathway. The slice was then maintained with a 2 ml min^{-1} flow of oxygenated ACSF at 32°C for 1 h to allow intracellular diffusion of the dye to the presynaptic imaging site $\approx 500 \mu\text{m}$ away from the ejection site. The excitation source was then reduced to a 100–200 μm diameter spot with a diaphragm in the epi-illumination path, and the emitted light was measured with a photomultiplier tube (PMT). By using standard wide field epifluorescent microscopy, the PMT signal summed the fluorescence response from an area of presynaptic terminals, which approximated the area that was electrically summed in the postsynaptic field potential recording. A single stimulus or pairs of stimuli were delivered orthodromically at 0.05 or 0.067 Hz by a Master 8 pulse generator and Iso-Flex constant current stimulator (AMPI Instruments, Jerusalem, Israel) under control of the imaging system (TILL Photonics, Pleasanton, CA, USA). Mg Green fluorescence responses are reported as the ratio of the change in fluorescence to the pre-stimulus fluorescence ($\Delta F/F_0$). The $\Delta F/F_0$ signals were corrected for bleaching by subtraction of a linearly sloping baseline and were inverted so that increasing presynaptic $[\text{Ca}^{2+}]_i$ produced an upward deflection. To diminish noise inherent with the low-affinity Ca^{2+} indicator, it was necessary to average five fluorescence responses and to filter the PMT signal at 1 kHz. For Fura-2 studies, we determined the 350 to 380 nm fluorescent ratio at each time point and normalized this to the pre-stimulus value ($\Delta R/R_0$). $[\text{Ca}^{2+}]_i$ responses to caffeine were measured using Fura-2 ratiometric imaging at 15 s intervals comparing $\Delta R/R_0$ in ACSF to $\Delta R/R_0$ after a minimum of 70 s in 10 mM caffeine. Only traces in which 40 mM KCl produced a measurable increase in $\Delta R/R_0$ were included in this analysis.

We used two tests to demonstrate that the measured $\Delta F/F_0$ signal was consistent with a $[\text{Ca}^{2+}]_i$ response predominately from the presynaptic Schaffer collateral axons and axon terminals (Atluri and Regehr, 1996; Kamiya and Ozawa, 1999; Scullin and Partridge, 2010; Sinha et al., 1997; Wu and Saggau, 1994). First, in the presence of 10 μM CNQX, 25 μM D-AP5, and 20 μM bicuculline, the postsynaptic response was blocked, while the presynaptic fiber volley and $\Delta F/F_0$ signal were left unchanged confirming that the $\Delta F/F_0$ signal was not a postsynaptic response. Second, subsequent addition of 600 nM TTX blocked both the presynaptic fiber volley and the $\Delta F/F_0$ signal arguing against direct stimulation of inadvertently filled postsynaptic dendrites.

4.3 Field potential recordings

We used standard electrophysiological techniques for extracellular population spike (PS) recordings in the SC-CA1 pyramidal neuron synapse in the *stratum pyramidale* in *in vitro* hippocampal slices (Schiess et al., 2006). In order to test for bias introduced by the measurement technique, we compared fEPSP slope measurements from *stratum radiatum* in figure 1A with PS measurements in figure 1D and found that these two measurements gave similar results. In addition, because of the presynaptic nature of PPF, which we have found to result from an increase in the reliability of quantal release (Schiess et al., 2010), and the observation that PS measurements are more resistant to contamination from perforant path (temporoammonic) stimulation than are fEPSP slope measurements (Colbert and Levy, 1992), we chose to use the more robust PS measurements in the remainder of the experiments.

Importantly, we restricted our PS measurements to the 50% point on the input-output curve where the relationship between synaptic efficacy and release probably is expected to be relatively linear. Briefly, PSs or fEPSPs were recorded with an Axoclamp 2B or Multiclamp 700B amplifier (both from Axon Instruments, Union City, CA) and a Digidata 1322A interface using pCLAMP 9.2 or 10 software (Axon Instruments) for experimental control

and data analysis. Potentials were digitized at 50 kHz and filtered at 2 kHz. Presynaptic constant current pulses (150 μ s duration) were applied to Schaffer collateral fibers with an Iso-Flex constant current stimulator (AMPI Instruments, Jerusalem, Israel) through a concentric bipolar electrode (FHC, Bowdoinham, ME) at a current, which was adjusted to produce 40–60% of the maximum PS amplitude. For simplicity, we define the PS response to the first of paired-pulses as R1 and that to the second as R2. The paired-pulse ratio (PPR) of the PS response was calculated as the ratio of R2 to R1 at a 50 ms interpulse interval and paired pulse facilitation (PPF) was calculated as the difference between R2 and R1 normalized to R1. In the series of experiments shown in figure 1A, the interpulse interval was varied between 10 ms and 100 ms and PPF was calculated from fEPSP slopes. In experiments to assess $[Ca^{2+}]_i$ accumulation, tetani at 10 Hz were delivered for 2 s once every 4 s..

4.4 Data analysis

The $\Delta F/F_0$ signal was digitally filtered with a five point center-weighted filter and numerically integrated after being normalized to the $\Delta F/F_0$ peak value ($\int v\Delta F/F_0$) yielding a value with units of time that was proportional to the timecourse of $[Ca^{2+}]_i$ decay. Fitting and statistical analysis were carried out with Matlab 7.0 (Mathworks, Natick, MA) or Prostat 4.0 (Polysoft International, Pearl River, NY). Coefficient of Determination (R^2) was used as a measure of goodness of fit for linear regressions. Average data are presented as means $\pm$ s.e.m., and statistical significance was determined at $P < 0.05$. Statistical significance indicated as: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data points that deviated from the mean by more than 2 standard deviations were considered outliers and were eliminated.

4.5 Drugs

Stock solutions of drugs were stored frozen in aliquots and diluted to the appropriate concentration in ACSF on the day of the experiment. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), d-(1)-2-amino-5-phosphonopentanoic acid (d-AP5), bicuculline, thapsigargin, and tetrodotoxin (TTX) were obtained from Tocris (Ellisville, MO, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Drugs were applied through a bath perfusion system for a minimum of 10 min (>2.5 bath exchanges) before recording commenced.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Facilitation and $[Ca^{2+}]_{res}$ were studied in SNAP-25a overexpressing mutant mice.
- SNAP-25 isoform or total level did not affect initial fEPSP amplitude.
- SNAP-25 isoform affected $[Ca^{2+}]_{res}$ during the first and second of paired pulses..
- Modeling studies suggest a role of SNAP-25 in $[Ca^{2+}]_i$ buffering.

Figure 1.

Influence of genotype on short-term facilitation. **A.** Effect of inter-pulse interval on PPF in the three genotypes. PPF was calculated from slopes of fEPSPs in slices in which the CA1 synaptic field had been surgically isolated from CA3. Significance determined by two-way ANOVA followed by a simple main effect analysis using one-way ANOVA. (Interpulse interval $F_{3,396} = 8.368$, $P < 0.0005$; Genotype $F_{2,396} = 15.404$, $P < 0.0005$; Interpulse interval * Genotype $F_{6,396} = 0.994$, $P = 0.429$) (WT, $n=17$; Tkneo, $n=13$; HET, $n=19$). **B.** Representative paired-pulse fEPSP traces at 50 ms inter-pulse interval (A1 WT, A2 Tkneo). **C.** Amplitude of the PS from the first of paired-pulses at half maximum stimulus intensity (mean $\pm$ S.E.M.). ANOVA of R1 amplitude vs. genotype shows no significant differences ($F_{2,77} = 1.24$, $P = 0.30$) (WT, $n=30$; Tkneo, $n=30$; HET, $n=20$). **D.** PPR at 50 ms inter-pulse

interval. One way ANOVA of PPR versus genotype shows significance ($F_{2,77} = 10.98$ $P < 0.0001$) horizontal bars show Fishers post-hoc test comparing PPR versus genotype, asterisks show Student's t-test against a theoretical mean of 1. (WT, n=30; Tkneo, n=30; HET, n=20).

Figure 2. Relationship of R1 to R2 amplitudes for paired pulses at 50 ms interpulse interval. Data were fit using our established model (see Supplemental material) that evaluates facilitation based on two states of release probability (representative PS data are shown in figure 1B). **A.** WT ($R^2 = 0.9332$, $n = 32$); **B.** Tkneo ($R^2 = 0.9636$, $n = 32$); **C.** HET ($R^2 = 0.9371$, $n = 18$). Dotted lines indicate equal responses for first and second pulses.

Figure 3.

Facilitation during paired-pulses. **A.** Linear regression fit to peak $\Delta F/F_0$ responses to the first (F1) and second (F2) stimulus at 50 ms interpulse interval (WT, slope = 1.259, $R^2 = 0.8576$ blue; Tkneo, slope = 1.2974, $R^2 = 0.8059$ red; HET, slope = 1.2951, $R^2 = 0.9461$, green; representative $\Delta F/F_0$ data are shown in figure 4A). Dotted line indicates unity slope. **B.** Effect of varying $[Ca^{2+}]_o$ on normalized R1 response. (WT, slope = 0.763, $R^2 = 0.9832$, n=10 animals, blue; Tkneo, slope = 1.1705, $R^2 = 0.9018$, n = 10 animals, red; HET, slope = 2.2607, $R^2 = 0.87163$, n = 7 animals, green).

Figure 4.

Presynaptic $[Ca^{2+}]_{res}$ kinetics during paired pulses. **A.** Representative $\Delta F/F_0$ traces for a single pulse (gray) and paired pulses at 50 ms interpulse interval (**A1.** WT; **A2.** Tkneo). **B.** Representative traces showing rate of $[Ca^{2+}]_{res}$ clearance as approximated by $\int v\Delta F/F_0$. (F1 gray and F2 black) (**B1.** WT; **B2.** Tkneo). **C.** Peak amplitude of $\Delta F/F_0$ following the first of paired pulses for the three genotypes. One way ANOVA comparison of F1 amplitude shows no significant difference among the three genotypes ($F_{2,39} = 0.97$, $P = 0.39$). **D.** Rate of $[Ca^{2+}]_{res}$ clearance as approximated by $\int v\Delta F/F_0$ for the three genotypes. One way ANOVA comparison of F1 $\int v\Delta F/F_0$ with Fishers post hoc shows significant differences among the three genotypes ($F_{2,39} = 4.88$, $P = 0.012$). Horizontal bars show results of post-hoc comparison. Importantly, following normalization of $\Delta F/F_0$ to F1, $\int v\Delta F/F_0$ of F2 does not differ significantly among the three genotypes (ANOVA $F_{2,39} = 1.63$, $P = 0.21$, not shown). **E.** Ratio of $\int \Delta F/F_0$ for F2/F1 following paired stimuli for the three genotypes (ANOVA of Ratio $\int \Delta F/F_0$ with Fishers posthoc shows significant differences among the three genotypes ($F_{2,39} = 5.61$, $P = 0.0072$) horizontal bars show result of post-hoc comparison. Asterisks show Student's t-test against a theoretical mean of 2. (For C, D, and E: WT, n=16; Tkneo, n=17; HET, n= 9 animals).

Figure 5.

Effect of Ca^{2+} influx on facilitation. **A.** Representative paired pulse traces for 1.5 mM $[Ca^{2+}]_o$ (light color) and 2.5 mM $[Ca^{2+}]_o$ (dark color) for the three genotypes. Traces have been displaced by 5 ms for clarity. (WT, blue; Tkneo, red; HET, green) **B.** Relationship of PPR at 50 ms interpulse interval to $[Ca^{2+}]_o$ (WT, n = 10 animals, blue; Tkneo, n = 10 animals, red; HET, n = 7 animals, green).

Figure 6.

A. Percent change of $\int \Delta F/F_0$ measured with MgGreen following a single pulse in 3 μM thapsigargin compared to the $\int \Delta F/F_0$ response in ACSF in the same slice ($n = 4$). (Thapsigargin was dissolved in DMSO and an equivalent concentration of DMSO (0.1%) was included in the ACSF control.) **B.** The relative change in $\Delta R/R_0$ in response to bath application of 10 mM caffeine. Timecourse of $\Delta R/R_0$ using Fura-2 in WT (blue) and Tkneo (red). Bath application of 10 mM caffeine at 450–800 sec and 40 mM KCl at 800–1200 sec (WT $n = 10$; Tkneo, $n = 6$ animals). **C.** Accumulation of background Ca^{2+} during stimulus trains. Background Fura-2 AM ratio responses (350nm to 380 nm) between repeated 2 s 10 Hz stimulus trains repeated every 2 s normalized to the initial value. The $\Delta R/R_0$ signal is significantly different among the three genotypes (ANOVA, $F_{32,99} = 3.04$, $P < 0.0001$, Fishers post-hoc) (WT blue $n = 5$, Tkneo red $n = 4$, HET green $n = 4$). Significance between the WT and Tkneo indicated with blue asterisks and between Tkneo and HET with green asterisks.

Table 1

Data from fits to following equation

$$R2 = a \times P2 \times (S - R1) + R1 \times \left(1 - \frac{R1}{S}\right) \times (1 - a)$$

$$a = \left(\frac{YM}{1 + \left(\frac{EC \times S}{R1}\right)^{HN}} \right)$$

where $P2$ is the release probability of the second pulse, S is a scaling factor relating P_R1 and $R1$, and EC , HN and a_{max} are variables in the Hill equation, such that EC reflects the half maximal effective concentration or EC_{50} , HN is the Hill coefficient, and a_{max} = maximum y value for the Hill equation.

	WT	Tkneo	HET
HN	1.539±0.8124	1.969±0.5031	1.515±0.3519
S	8.207±1.536	10.26±1.05	10.26±1.381
EC	0.1318±0.0631	0.0679±0.0081	0.08916±0.0199
a_{max}	1		
$P2$	0.1768		
C.O.D.	0.933	0.967	0.993