Deficits in memory and motor performance in synaptotagmin IV mutant mice

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Synaptotagmin (Syt) IV is a synaptic vesicle protein. Syt IV expression is induced in the rat hippocampus after systemic kainic acid treatment. To examine the functional role of this protein *in vivo*, we derived Syt IV null [Syt IV(−/−)] mutant mice. Studies with the rotorod revealed that the Syt IV mutants have impaired motor coordination, a result consistent with constitutive Syt IV expression in the cerebellum. Because Syt IV is thought to modulate synaptic function, we also have examined Syt IV mutant mice in learning and memory tests. Our studies show that the Syt IV mutation disrupts contextual fear conditioning, a learning task sensitive to hippocampal and amygdala lesions. In contrast, cued fear conditioning is normal in the Syt IV mutants, suggesting that this mutation did not disrupt amygdala function. Conditioned taste aversion, which also depends on the amygdala, is normal in the Syt IV mutants. Consistent with the idea that the Syt IV mutation preferentially affects hippocampal function, Syt IV mutant mice also display impaired social transmission of food preference. These studies demonstrate that Syt IV is critical for brain function and suggest that the Syt IV mutation affects hippocampal-dependent learning and memory, as well as motor coordination.

Synaptic neurotransmission is one of the most highly regulated of all vesicle trafficking events. Although many of the molecular components of synaptic vesicles, the presynaptic cytosol, and presynaptic plasma membranes have been identified, the mechanisms by which these components regulate stimulus-evoked vesicle fusion and recycling remain unclear (1).

Synaptotagmins (Syts) are a large family (2–4) of synaptic vesicle proteins (5–7) characterized by a pair of cytosolic C2 domains, homologous to those originally described in protein kinase C (8). Through their C2 domains, Syts bind calcium and phospholipids (9) and interact with a variety of other presynaptic molecules (reviewed in ref. 2). Disruption of the murine Syt I gene (10), or the *Drosophila* and *Caenorhabditis elegans* Syt genes (11, 12), severely impairs stimulus-evoked, calcium-dependent synaptic release. In addition, *C. elegans* Syt mutants display reduced retrieval of synaptic vesicles from the plasma membrane after depolarization (13). These observations suggest that Syts function as calcium regulators in the exocytotic fusion reaction and as part of the endocytotic vesicle retrieval apparatus.

We isolated a member of the Syt family, Syt IV, as a depolarization-inducible immediate-early gene in the rat pheochromocytoma PC12 cell line (14). Syt IV mRNA also is induced predominantly in the rat hippocampus and piriform cortex after kainic acid-elicited seizures (14). In contrast, substantial basal expression of Syt IV is observed in the cerebellum (15). Both basal and induced Syt IV expressions are relatively low in other areas of the brain, e.g., the amygdala (14). Syt IV protein, after its stimulation-induced synthesis, colocalizes with Syt I in secretory vesicles of PC12 cells (7). Unlike other Syts, the Syt IV C2A domain has a single amino acid change, a serine for aspartate substitution at residue 244, that inactivates the calcium-binding pocket and renders this domain incapable of binding calcium or phospholipid (4, 16). This mutation is conserved in the mouse (17), *Drosophila* (18), and human (G.D.F. and H.R.H., unpublished observations) Syt IV orthologues. Evolution has, with a single amino acid substitution, selected against calcium-dependent binding activity in the C2A domain of Syt IV, but retained the residues required for other effector functions. Syt IV can hetero-oligomerize with Syt I (19, 20) and reduce the ability of Syt I to penetrate and bind phospholipid membranes (18), in effect functioning as a "dominant negative."

The data suggest Syt IV may modulate the calcium sensitivity of the synaptic vesicle fusion reaction *in vivo*. Indeed, overexpression of Syt IV in neurons at the *Drosophila* neuromuscular junction attenuates synaptic release, as reflected in a reduced postsynaptic response (18). Thus, the relative levels of Syt IV protein in vesicles, modulated by depolarization, may alter the probability of vesicle fusion and lead to changes in synaptic output.

Specific patterns of electrical activity can, by inducing changes in gene expression, promote long-lasting, stable changes in synaptic function. Neurons of the hippocampus, a major site of information storage in the brain (21), can display increases in synaptic efficacy *in vivo* during learning (22) and in brain slice preparations after tetanic stimulation (23). Such activity-dependent changes in synaptic strength are thought to be a basis for memory storage in the brain (24, 25). Although the locus of expression of long-term synaptic plasticity remains controversial (25), evidence suggests it may be mediated, at least in part, through presynaptic mechanisms that alter the probability of vesicle fusion and neurotransmitter release (26, 27). To further examine the function of Syt IV and its potential role in synaptic plasticity and memory, we have developed a Syt IV knockout mouse. Mice homozygous for the Syt IV mutation are viable and appear normal. However, the Syt IV mutant mice exhibit deficits in motor control and memory tasks associated with hippocampal function.

Materials and Methods

Generation of Syt IV Homozygous Mutant Mice. The murine Syt IV gene, partially contained within a 7.0-kb *Bam*HI fragment, was disrupted through the insertion of a neomycin resistance cassette (neo^R) inserted in an exon in the first C2 domain of Syt IV. A homologous recombination-targeting construct was created by inserting the Syt IV/neo^R fragment into a vector with flanking thymidine kinase (28) genes. RW-4 embryonic stem cells (Genome Systems, St. Louis) were grown, electroporated with *Pvu*I-linearized targeting vector, and selected as described by supplier. Double-resistant colonies were picked on days 7–9 of selection, expanded, and analyzed by Southern blot with a 1.3-kb

Abbreviations: Syt, synaptotagmin; STFP, social transmission of food preference; CTA, conditioned taste aversion; MANOVA, multivariate analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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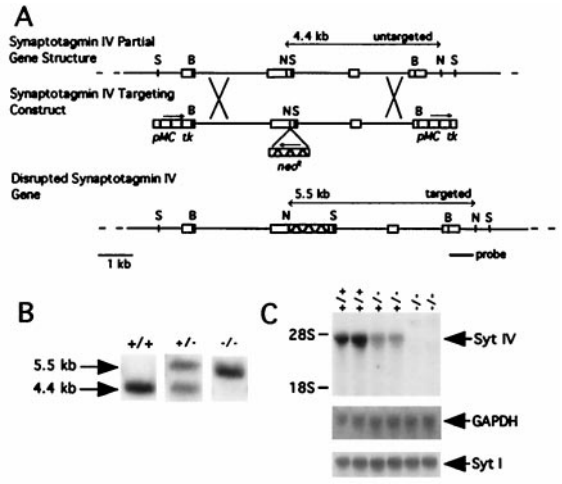

Fig. 1. Mutation of the Syt IV gene by homologous recombination. (*A*) Partial structure of the wild-type Syt IV gene (*Top*), the targeting construct (*Middle*), and the mutated Syt IV gene after homologous recombination (*Bottom*). In the targeting construct, a neomycin resistance (neo^R) gene was inserted into the exonic sequence, and the genomic fragment was flanked by herpes simplex virus thymidine kinase (tk) genes. Expected sizes of wild type and disrupted Syt IV gene after digestion with *Nco*I and hybridization with a 3′ flanking probe (solid bar) are indicated. Targeted clones were verified by PCR (data not shown). Relevant restriction enzymes are *Sac*I (S), *Bam*HI (B), and *Nco*I (N). (*B*) Southern blot analysis of offspring from a mating of Syt IV heterozygotes. Tail DNAs were digested with *Nco*I and hybridized with the 3′ flanking probe. Syt IV genotypes are indicated above each lane. (*C*) Northern blot analysis. Total RNA (10 μg) from the brains of two mice of each genotype were hybridized with Syt IV (*Top*), GAPDH (*Middle*), and Syt I (*Bottom*) cDNA probes. Relative positions of 18S and 28S ribosomal RNAs are indicated in *Top*.

3′ external probe (shown in Fig. 1) or by confirmatory PCR analysis (data not shown). Mice heterozygous for the mutation, obtained by mating male chimeras to C57BL/6 females, were then intercrossed to obtain Syt IV homozygous mutants. Genotypes of all animals were determined by Southern blot analysis of tail DNA. Wild type and mutant C57BL/6 × 129SvJ F_2 hybrids were used in all experiments.

RNA Analysis. Total RNA was extracted from the brains of two wild-type (+/+), heterozygous (+/−), and homozygous (−/−) Syt IV mutant animals by using TRIzol reagent (GIBCO/BRL) according to the manufacturer's instructions. Total RNA (10 μg) from each brain was denatured, subjected to electrophoresis, and transferred, by capillary blotting, to Hybond-N (Amersham Pharmacia) membranes. Rat-derived Syt IV (14), Syt I (5), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (29) probes were hybridized to filters in 43% formamide/5× standard saline phosphate/EDTA (0.15 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/10× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% BSA)/2% SDS/100 μg/ml salmon sperm DNA for 18 h at 42°C. The filters were washed three times for 30 min in 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.5% SDS at 55°C and then exposed to autoradiographic film overnight at −80°C.

Histology. Histology on wild-type and Syt IV mutant mouse brains was performed as described previously (30), with slight modification. Coronal and sagittal sections were cut at 20 μm and stained with 0.5% thionin/1%acetic acid.

Behavioral Analyses. Wild-type, heterozygous, and homozygous Syt IV mutants were examined systematically for general health and gross neurologic and motor function, including testing on an accelerating rotorod, as described by Crawley and Paylor (31). In addition, vertical and horizontal rod and coat hanger tests were performed as described previously (32), with only slight modifications.

Pavlovian Fear Conditioning. Separate context and cued conditioning chambers, constructed as described previously (33), were used in these experiments. Wild-type mice exhibit minimal generalization between these contexts (see Fig. 4*C*).

Twenty-four-hour contextual fear. Mice were placed into the conditioning chamber and, after a 4-min baseline period, received a 2-s, 0.75-mA foot shock. At 60 s later, the mice were returned to their home cages. One day later, the mice were placed back in the conditioning chamber for a 4-min contextual freezing test.

Overtraining. The same mice were returned to the conditioning chambers and, after 4 min, received three 2-s, 0.75-mA foot shocks separated by 60 s. The mice were returned to their home cages 1 min after the final foot shock. On the next day, they were returned to the conditioning chambers for another 4-min contextual freezing test.

Twenty-four-hour tone fear. A separate group of mice was subjected to tone conditioning. On the training day, the mice were placed into the training-context and, after 150 s, received a 10-s, 90-dB (A-scale), 2.8-kHz tone that coterminated with a 2-s, 0.75-mA foot shock. At 30 min after training, the mice were returned to the same context for a contextual fear memory test that lasted 4 min. On the next day, they were brought to novel chambers (in a different room), and after a 2-minute baseline, the original training tone was played for 3 min. The mice were returned to the training context 30 days later for a delayed 4-min contextual memory test to confirm this group of Syt IV mutant mice also exhibited a long-term contextual memory deficit.

Computer scoring. Freezing was scored by a computerized technique using NIH IMAGE (National Institutes of Health, Bethesda, MD) on a Macintosh computer. We recently have validated this measure elsewhere (33). The program generates freezing scores comparable to established norms (34) by comparing digitized video frames at 1 Hz. We also assessed baseline activity (at 1 Hz) by using this program, as well as shock reactivity (at 10 Hz) in cm/s (33, 35).

Social Transmission of Food Preference (STFP). STFP was performed as described previously (37), with slight modification. A single demonstrator mouse was allowed to eat scented food and then was placed immediately in its home cage with observers for a 20-min interaction period. The observers were then tested for food preference either immediately or 24 h later.

Conditioned Taste Aversion (CTA). Mice were water-deprived for 20 h and then given, in a separate cage, free access to water from two bottles for 1 h. On day 2, water access was limited to 45 min, and on days 3–6, access was limited to 30 min. The mice were weighed every other day. If more than 30–40% weight loss had occurred, they were removed from the analysis. A single Syt IV mutant mouse was removed based on this criterion. On day 7, the mice were presented with 0.1% saccharin sodium salt in water from one bottle for 15 min. Half the mice were injected i.p. with 0.02 M LiCl in saline (2% of body weight), and half were injected with saline. Two hours after injection, the mice were given access to drinking water for 30 min to prevent dehydration. CTA was tested on day 8. In a 40-min test trial, the mice were presented with saccharin-flavored and normal tap water in separate bottles, with the bottle positions counterbalanced. The weights of these bottles were determined before and after testing and used to determine water consumed. The preference score was calculated as (saccharin water consumed)/[(saccharin water con-

sumed) + (normal water consumed)]. The higher the preference score, the higher the preference for the saccharin water.

Statistics. Genotype and dependent measures were entered into a general multivariate analysis of variance (MANOVA). Multiple planned post hoc comparisons were made with the Wald test (38). For between-groups comparisons, this test is equivalent to the unpaired two-tailed *t* test. For within-subjects comparisons, it is equivalent to the paired two-tailed *t* test. Significance was set at $\alpha < 0.05$.

For more detailed descriptions of the methods used in this paper, see supplementary *Materials and Methods* published on our web site at http://www.gregorydferguson.homepage.com.

Results

Generation of Syt IV Homozygous Mutant Mice. We isolated a murine Syt IV genomic clone by screening a genomic phage library with a rat cDNA probe at low stringency. A 7.0-kb *Bam*HI fragment, which includes the translation start site and both C2 domains, was subcloned from this phage and used to construct the targeting vector (Fig. 1*A*). To inactivate the Syt IV gene, a neomycin resistance gene was inserted into an exon that lies within the first C2 domain. This targeting construct was linearized and electroporated into embryonic stem cells. Embryonic stem colonies surviving drug selection were analyzed by Southern blot and PCR (data not shown) for homologous recombination The frequency of positive clones was 1 in 150. Embryonic stem cells harboring a mutant Syt IV allele injected into blastocysts gave rise to chimeric progeny that transmitted the mutation through the germ line. The resultant mice were genotyped by Southern blotting of tail biopsy samples (Fig. 1*B*). Analysis of total brain RNA by Northern blotting confirmed that mice homozygous for the Syt IV mutation (hereafter referred to as "mutants") produced no detectable Syt IV mRNA, and that heterozygous mice produced approximately half the wild-type levels of Syt IV mRNA (Fig. 1*C*). GAPDH levels indicated equal amounts of RNA were loaded in each lane. In addition, there was no compensatory up-regulation of Syt I mRNA levels in the Syt IV mutants.

Syt IV Mutant Mice Are Viable and Appear Normal. Mating of mice heterozygous for the Syt IV mutation yielded a near Mendelian distribution of genotypes in the offspring: +/+ (n = 34, 23%); +/− (n = 84, 56%); and −/− (n = 31, 21%). Syt IV mutant mice are viable and appear normal to at least 12 months of age, indicating that the Syt IV gene is not essential for survival. In contrast, Syt I homozygous knockout mice die within 48 h after birth (10). In general, the Syt IV mutant mice appear healthy and well groomed, and show no overt abnormalities. Consistent with these observations, light microscopic analysis of thionin-stained coronal (Fig. 2) and sagittal (data not shown) brain sections revealed no gross neuroanatomical abnormalities in the Syt IV mutant mice.

Behavioral Assessment of Syt IV Mutants. A detailed and systematic analysis of the gross neurologic and motor function (31) of the Syt IV mutants revealed no significant differences between wild-type and Syt IV mutant mice in a variety of tests of strength, balance, reflex, locomotion, and coordination that included a hanging wire test, vertical and horizontal beam tests, and gait measurements (data not shown).

We did observe a performance deficit, however, in the Syt IV mutant mice on the accelerating rotorod. In this task, animals must make continuous adjustments in balance and posture to remain upright on a rod that increases in rotational velocity during each 5-min trial. The Syt IV mutant mice (n = 15) exhibited an immediate and significant performance deficit when compared with wild-type mice (n = 10) [Fig. 3*A*; +/+ = 176.6 ± 21 s; −/− = 94.5 ± 20 s; F (1, 23) = 7.5; P = 0.01]. In addition, wild-type mice were able to stay on the accelerating rotorod for significantly more time than were the Syt IV mutants over the five trials (Fig. 3*A*). There was significant improvement in both genotypes across days of training (Fig. 3*A*), but no genotype by trial interaction (Fig. 3*A*). Wild-type and Syt IV mutant mice were also tested at constant speeds of 18 rpm and 6 rpm. When compared with wild-type mice, Syt IV mutant mice performed normally at the low speed (data not shown; F (1, 20) = 1.3; $P > 0.25$) but performed poorly when compared with wild-type animals at the higher speed (Fig. 3*B*). These data suggest the basis for the performance deficit on the accelerating rotorod in the Syt IV mutants is in motor coordination rather than in motor learning.

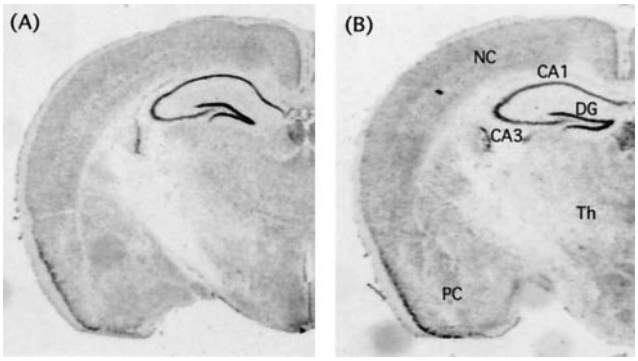

Fig. 2. Histological analysis. Histological sections containing hippocampus from Syt IV mutant (*A*) and wild-type (B) mice after staining with 0.5% thionin. Indicated are dentate gyrus (DG), pyramidal cells of the Schaffer collateral fiber tract (CA1 and CA3), thalamus (Th), neocortex (NC), and piriform cortex (PC).

Fear Conditioning in Syt IV Mutants. In previous studies, we postulated that Syt IV may modulate synaptic function and, therefore, have a role in learning and memory (7). To test this hypothesis, we first examined the Syt IV mutant mice in Pavlovian contextual and cued conditioned freezing (34, 39). In this paradigm, mice learn to fear an otherwise innocuous stimulus [the conditional stimulus (CS)], a context or tone, by pairing the CS with an aversive stimulus [(the unconditional stimulus (US)],

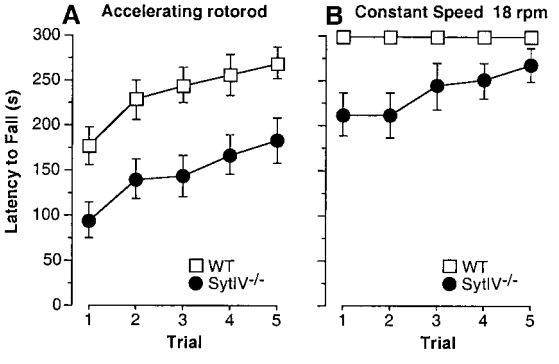

Fig. 3. Impaired rotorod performance in Syt iv mutant mice. (*A*) Fall latencies of wild-type (n = 10) and Syt IV mutant (n = 15) mice on an accelerating rotorod over five trials. The rotorod increased from 3 rpm to 30 rpm during each 5-min trial. Data points and bars represent mean ± SEM, respectively. MANOVA revealed a significant main effect of genotype [F (1, 23) = 9.6; $P <$ 0.01] and significant main effect of trial [F (1, 4) = 16.5; $P < 0.0001$], but no genotype by trial interaction [F (4, 92) = 0.2; $P > 0.9$]. (*B*) Fall latency at a constant 18 rpm as above. MANOVA revealed a significant main effect of genotype [F (1, 23) = 9; $P < 0.01$].

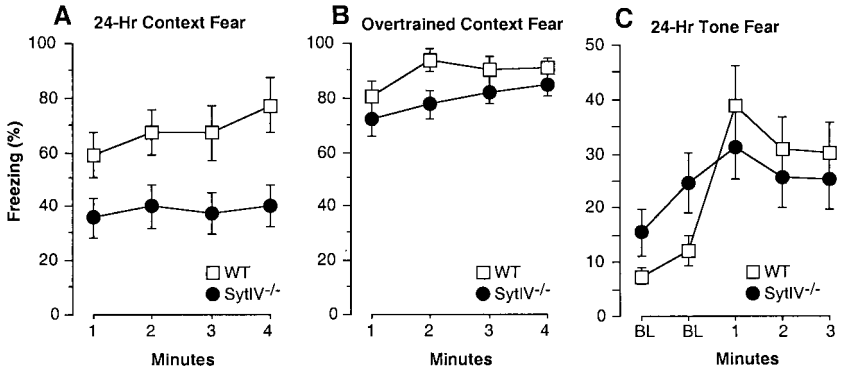

Fig. 4. Fear conditioning in Syt IV mutant mice. (*A*) Twenty-four-hour context test in wild type ($n = 10$) and Syt IV mutants ($n = 15$) after a single foot shock (0.75 mA for 2 s). Mean percentage of freezing during each minute of the 4-min context test is shown. Data points and bars represent mean ± SEM, respectively. MANOVA indicated a significant main effect of genotype [$F(1, 23) = 7.6$; $P = 0.01$]. (*B*) The trained and tested mice from *A* were given three additional foot shocks, separated by 60 s, and then tested 24 h later as above. MANOVA revealed no significant effect of genotype [$F(1, 22) = 2.6$; $P > 0.1$]. (*C*) Twenty-four-hour cued conditioning in another group of wild-type ($n = 15$) and Syt IV mutant ($n = 14$) mice. Mice were trained as above with a 10-s tone (90 dB; 2.8 kHz) coterminated with a single foot shock and subsequently tone tested in a novel context. Mean percentage of freezing during a 2-min baseline period before tone and for 3 min immediately after tone are shown. Data points and bars represent mean ± SEM, respectively. MANOVA revealed no significant effect of genotype [$F(1, 27) = 0.01$; $P = 0.9$] and a significant genotype-by-time interaction [$F(4, 108) = 3.2$; $P < 0.02$]. There was a significant effect of genotype during the second minute of baseline period [$F(1, 27) = 4.2$; $P < 0.05$]. There were no significant differences at any other minute.

such as a foot shock. When reexposed to the CS, either the context or the tone, mice exhibit freezing, an adaptive fear response in rodents in which all movements, except those required to breathe, cease (40). Both cued and contextual conditionings are sensitive to amygdala lesions (41). In contrast, contextual conditioning is thought to be more sensitive to hippocampal lesions (34, 41, 42, 43).

Baseline activity, baseline freezing, and shock reactivity. Initially, we tested the Syt IV mutant and wild-type mice for differences in shock reactivity and spontaneous activity. Baseline activity was assessed in a subset of animals during the 4-min period before the first shock in the conditioning chambers. The baseline activity of the Syt IV mutants and wild-type mice did not differ [$F(1, 23) = 2.3$; $P > 0.1$; data not shown]. Baseline freezing during the first minute was characteristically low and did not differ across genotypes [$+/+ = 9.3 \pm 5\%$; $-/- = 7.1 \pm 2.3\%$; $F(1, 23) = 0.2$; $P > 0.6$; data not shown]. Shock reactivity was assessed by measuring the movement velocity (cm/s) of the mice in the 2 s before and during the 2-s foot shock. Previous studies have shown movement velocity (cm/s) is a good measure of nociception (33, 35, 36). Both genotypes exhibited the characteristic increase in velocity when exposed to the shock [$F(1, 23) = 195$; $P < 0.0001$; data not shown] and did not differ in this respect [$F(1, 23) = 1.6$; $P > 0.2$; data not shown]. Thus, any differences in fear conditioning could not be attributable to increased activity (which might interfere with freezing), alterations in baseline freezing, or decreased sensitivity to the shock US.

Twenty-four-hour contextual fear test. For context training, Syt IV mutant ($n = 15$) and wild-type ($n = 10$) mice were placed in the conditioning chambers for 4 min, given a single foot shock, and returned to their home cage after an additional minute. At 24 h later, the mice were tested in the same conditioning chambers for 4 min. Syt IV mutants exhibited a significant deficit in freezing relative to wild-type mice in contextual memory when tested 1 day after training (Fig. 4*A*).

Overtraining. To determine whether Syt IV mutants *can* exhibit high levels of freezing (that is, that the Syt IV mutation does not produce a deficit in freezing, but rather in memory), mice were given three additional shocks and then tested 24 h later. After overtraining, Syt IV mutant mice exhibit robust freezing and are not significantly different from wild-type mice (Fig. 4*B*), indicating the Syt IV mutants are capable of the high levels of freezing achieved by wild-type mice shown in Fig. 4*A*.

Twenty-Four-Hour Cued Conditioning. Twenty-four-hour tone memory was tested in a separate group of wild-type ($n = 15$) and Syt IV mutant ($n = 14$) mice. These animals received a single tone-shock pairing and were then given a tone test, in a novel context, 24 h later. After a 2-min baseline period to assess generalization from the training context, the training tone was played for 3 min. Although there are no significant differences in the overall levels of freezing between the Syt IV mutant and wild-type mice (Fig. 4*C*), there is a significant genotype by time interaction (Fig. 4*C*). This interaction seems to be because Syt IV mutants exhibit higher pretone baseline freezing than do wild-type mice (Fig. 4*C*), perhaps caused by an overgeneralization from the training context. The levels of freezing are significantly different during the second pretone baseline minute, but not during any other minute of the freezing test (Fig. 4*C*).

Heterozygous Mice. Syt IV $+/-$ mice were examined in all tests except the STFP and CTA, and did not differ from wild-type controls on any test (data not shown).

STFP in Syt IV Mutant Mice. To examine the Syt IV mutants in another hippocampus-dependent memory test, we used the STFP test (44). This task exploits the tendency of mice to prefer foods that they have recently smelled on the breath of other mice. This task, which does not use aversive stimuli, tests the ability to learn and remember information transmitted in olfactory cues during social interactions (37).

We first tested Syt IV mutant ($n = 16$) and wild-type ($n = 15$) mice for food preference 24 h after interaction with demonstrators. Planned comparisons of the data in Fig. 5 for each genotype revealed a deficit in the Syt IV mutants. Whereas wild-type mice eat significantly more cued food than uncued food (Fig. 5*A*), the primary indicator of learning on this task, Syt IV mutant mice do not (Fig. 5*A*). Wild-type and Syt IV mutant mice ate similar amounts of total food (cued and uncued) during this test (wild-type, 0.992 ± 0.085 g; Syt IV mutants, 1.081 ± 0.059 g; $F(1, 29) = 0.74$; $P = 0.4$).

It is possible that Syt IV mutant mice are deficient in olfaction, social interaction, or other aspects of the STFP test. To examine

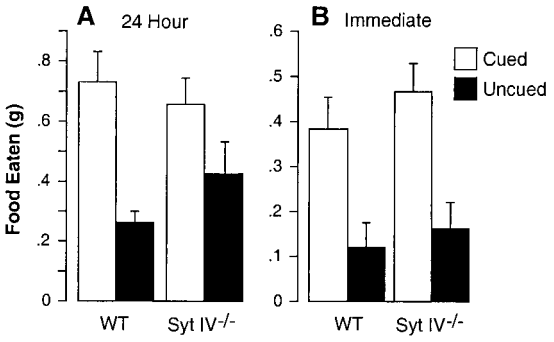

Fig. 5. STFP. (*A*) Wild-type ($n = 15$) and Syt IV mutant ($n = 16$) mice were tested for food preference 24 h after a single interaction with a demonstrator mouse that had just eaten cued food. Columns and error bars represent mean ± SEM, respectively. Planned comparisons reveal that wild-type mice exhibit a strong preference for cued food. [F (1, 14) = 13.7; $P < 0.01$]. In contrast, Syt IV mutant mice did not demonstrate a statistically different preference for cued food [F (1, 15) = 1.5; $P > 0.2$]. (*B*) Wild-type ($n = 9$) and Syt IV mutant ($n = 10$) mice were tested for food preference immediately after a single interaction with a demonstrator mouse that had just eaten cued food. Wild-type and Syt IV mutant mice both exhibit a strong preference for cued food. Wild-type [F (1, 8) = 5.8; $P < 0.05$]; Syt IV mutant mice [F (1, 9) = 12.8; $P < 0.01$].

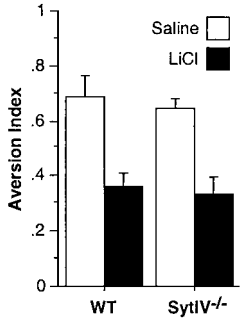

Fig. 6. CTA. Saccharin-flavored water was paired with an i.p. injection of saline (wild type, $n = 5$; Syt IV mutant, $n = 6$) or LiCl (wild type, $n = 5$; Syt IV mutant, $n = 6$). At 24 h later, all mice were tested for saccharin aversion. Aversion index is calculated as [total saccharin water consumed, in ml]/[total saccharin water, in ml]+ [total unflavored water consumed, in ml]. Columns and error bars represent mean ± SEM, respectively. MANOVA revealed significant main effect of treatment [F (1, 18) = 28; $P < 0.0001$] in wild-type and Syt IV mutant mice, but no difference in genotype [F (1, 18) = 0.3; $P > 0.5$] or genotype-by-treatment interaction [F (1, 18) = 0.1; $P > 0.9$].

these possibilities, we tested an additional group of wild-type and Syt IV mutant mice for food preference immediately after interaction with demonstrators. Both wild-type and Syt IV mutant animals exhibited a robust preference for cued food when tested immediately after training (Fig. 5*B*), indicating that the Syt IV mutants can perform all aspects of the STFP test normally.

CTA in Syt IV Mutant Mice. Because cued conditioning is normal in the Syt IV mutants, we examined the Syt IV mutants in another amygdala-dependent task, CTA (45, 46). By pairing a taste stimulus with an agent that causes mild nausea and malaise, normal mice learn and subsequently remember to avoid this taste stimulus. A strong CTA response can be elicited in mice by pairing saccharin flavor with i.p. injection of LiCl, an agent that causes a transient visceral malaise.

Syt IV mutant ($n = 6$) and wild-type ($n = 5$) mice injected with LiCl exhibit a robust CTA when compared with Syt IV mutant ($n = 6$) and wild-type ($n = 5$) mice injected with vehicle saline (Fig. 6). Syt IV mutants do not differ from wild-type mice in this respect (Fig. 6). Normal CTA suggests, again, that amygdala function is unaffected by the Syt IV mutation.

Discussion

To characterize the functional role of Syt IV *in vivo*, we developed a Syt IV knockout mouse. Because the Syt IV mutants are viable, we are able to examine them in a variety of behavioral paradigms. By using behavioral tasks with known neural substrates, we have identified specific brain regions in which Syt IV function appears critical. This approach allows us to identify candidate neuronal populations in which to examine the physiological effect of the Syt IV mutation.

Contextual fear conditioning (Fig. 4*A*), which relies on hippocampus and amygdala function (41, 43), and the hippocampus-dependent (44) STFPs task (Fig. 5*A*), when tested 24 h after training, are deficient in Syt IV mutants. In contrast, Syt IV mutants are normal in CTA (Fig. 6) and cued fear conditioning (Fig. 4*C*), which require amygdala function (41, 45). Additionally, these mutants perform normally in the immediate STFP task, a hippocampal-independent task. Because the mechanisms for production of the freezing response for contextual and cued fear conditioning are similar (43, 47, 48), it is unlikely that Syt IV mutants exhibited deficits in freezing *per se*. For example, after context overtraining, Syt IV mutants exhibited freezing comparable to that of wild-type animals (Fig. 4*B*). These data suggest that the Syt IV mutation leads to selective functional deficits in the hippocampus, although sparing amygdala function.

Previous studies demonstrated that mice lesioned in the hippocampus *before* training can, in some cases, be conditioned to context, suggesting that, in the absence of hippocampal function, other brain regions can support contextual conditioning under certain conditions (34, 41). In contrast to pretraining lesions, hippocampal lesions immediately *after* training severely impair contextual conditioning, suggesting that—when contextual information is acquired with a functional hippocampus—this structure also is initially critical for its storage (34, 42, 43). Thus, *postacquisition* hippocampal function may be disrupted by the Syt IV mutation, and Syt IV may play a role in stabilizing memories after they initially are established. That is, like kainic acid-induced seizures (14), learning may trigger changes in Syt IV expression that are critical for memory consolidation. This suggestion is supported by evidence of intact performance in the STFP task when tested immediately after training. Nonetheless, it remains to be determined more precisely when and where Syt IV is involved in stabilizing memory.

The cAMP-responsive element binding protein (CREB) pathway, from upstream activating proteins to transcription factor, have been strongly implicated in learning and memory (49). Disruption of the α and δ isoforms of CREB in mice lead to memory deficits (39). Similarly, adenylyl cyclase type I knockout mice exhibit deficits in long term memory (50) and perform poorly on the accelerating rotorod (32). Much like Syt IV mutant mice, transgenic mice overexpressing a dominant negative protein kinase A regulatory subunit exhibit deficits in contextual, but not cued, conditioning and have normal CTA response (51). CREB can be phosphorylated by adenylyl cyclase-dependent protein kinase A (pK_a) (52). Forskolin or depolarization, potent activators of the pK_a pathway, induce Syt IV mRNA levels in PC12 cells (14), suggesting that the Syt IV gene may be regulated by CREB. Indeed, we have identified a consensus cAMP-response element (CRE) at position −486 in the Syt IV promoter (our unpublished observations). Thus, Syt IV may reside downstream of CREB and function as a so-called "CREB

effector'' (53) to modulate stimulation-induced neuronal plasticity.

Although sensorimotor learning and, therefore, performance on the rotorod cannot easily be attributed to a single brain region, it is widely accepted that the cerebellum is a major component in this circuit (54). Mice with structural abnormalities in the cerebellum (36) or with disruptions in genes enriched in cerebellum (32) exhibit performance deficits on the rotorod. A performance deficit on the rotorod is consistent with the elevated basal expression of Syt IV observed in cerebellum (14, 15). This result suggests that the basal, uninduced levels of Syt IV have, in at least some neurons, an important role in normal physiology. However, the Syt IV mutant mice improve at approximately the same rate as do wild-type mice during rotorod performance (see Fig. 3*A*), indicating some form of motor learning is preserved in Syt IV mutant mice.

In summary, these studies indicate that Syt IV, although not essential for survival, is required for normal motor function and selected aspects of learning and memory. Behavioral analysis suggests that neuronal function is perturbed in hippocampus and cerebellum. Because the gross neuroanatomy of these brain regions appears unaltered, we propose that the basis for these functional deficits lies in faulty regulation of synaptic transmission. Future studies examining the electrophysiological properties of neurons from Syt IV mutant mice will be required to test this possibility.

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