Point Mutation in Syntaxin-1A Causes Abnormal Vesicle Recycling, Behaviors, and Short Term Plasticity*

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Background: Roles of the syntaxin-1A-CaMKII interaction are not physiologically understood in vivo.

Results: A point mutation in syntaxin-1A caused abnormal plasticity, recycling, and behaviors in mice.

Conclusion: The CaMKII/syntaxin-1A interaction is essential for maintenance of neuronal plasticity.

Significance: Syntaxin-1A is involved in regulatory pathways in higher brain functions.

Syntaxin-1A is a t-SNARE that is involved in vesicle docking and vesicle fusion; it is important in presynaptic exocytosis in neurons because it interacts with many regulatory proteins. Previously, we found the following: 1) that autophosphorylated Ca2+/calmodulin-dependent protein kinase II (CaMKII), an important modulator of neural plasticity, interacts with syntaxin-1A to regulate exocytosis, and 2) that a syntaxin missense mutation (R151G) attenuated this interaction. To determine more precisely the physiological importance of this interaction between CaMKII and syntaxin, we generated mice with a knock-in (KI) syntaxin-1A (R151G) mutation. Complexin is a molecular clamp involved in exocytosis, and in the KI mice, recruitment of complexin to the SNARE complex was reduced because of an abnormal CaMKII/syntaxin interaction. Nevertheless, SNARE complex formation was not inhibited, and consequently, basal neurotransmission was normal. However, the KI mice did exhibit more enhanced presynaptic plasticity than wild-type littermates; this enhanced plasticity could be associated with synaptic response than did wild-type littermates; this pronounced response included several behavioral abnormalities. Notably, the R151G phenotypes were generally similar to previously reported CaMKII mutant phenotypes. Additionally, synaptic recycling in these KI mice was delayed, and the density of synaptic vesicles was reduced. Taken together, our results indicated that this single point mutation in syntaxin-1A causes abnormal regulation of neuronal plasticity and vesicle recycling and that the affected syntaxin-1A/CaMKII interaction is essential for normal brain and synaptic functions in vivo.

Syntaxin-1A is a t-SNARE, and in neurons, it is a main component of the presynaptic SNARE complex that executes exocytosis (1). Neuronal SNARE complexes include syntaxin-1A, SNAP-25 (another t-SNARE), and VAMP-2 (a v-SNARE) and are essential for both vesicle docking and fusion during the presynaptic exocytosis that leads to transmitter release. The neuronal isoform of syntaxin-1A, designated syntaxin-1B, can also be incorporated into SNARE complexes; however, the specific function of syntaxin-1B is not clear. Syntaxin-1A interacts with many kinds of proteins, and most of these interactions require the syntaxin-1A H3 domain, which is also crucial for SNARE complex formation. However, the interaction between syntaxin-1A and autophosphorylated Ca2+/calmodulin-dependent protein kinase II (CaMKII) requires a linker domain in syntaxin-1A that connects its N-terminal helices (Ha-Hc) and its H3 domain but not the H3 domain itself (2, 3). The interaction between syntaxin-1A and autophosphorylated CaMKII is important for normal brain and synaptic functions in vivo.

*This work was supported in part by Grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT)/Japan Society for the Promotion of Sciences KAKENHI 17023019, 22040040, and 24111515 (to M.I.), 11J40157 (to Y. W.), 25220008, 25640005, and 26540005 (to T. Manabe), the Comprehensive Center of Education and Research for Chemical Biology of Diseases (Global COE Program) (to T. Manabe), the Strategic Research Program for Brain Sciences from the MEXT (to T. Manabe), and a grant from the Uehara Memorial Foundation (to T. Manabe).

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2 The abbreviations used are: CaMKII, Ca2+-dependent protein kinase II; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; KI, knock-in; PPF, paired-pulse facilitation; PTP, post-tetanotic potentiation; RRP, readily releasable pool; STP, short term plasticity; SV, synaptic vesicle; D-APV, D-2-amino-5-phosphonovaleric acid; Cplx, complexin; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid.
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CaMKII is Ca$^{2+}$-dependent (2, 3). At the cellular level, an intermediate complex is necessary for exocytosis because CaMKII binds to syntaxin-1A; importantly, a missense mutation in syntaxin-1A (R151G) can inhibit this binding and thereby prevent syntaxin-1A from regulating exocytosis (2, 4).

CaMKII is an oligomeric protein kinase and is particularly abundant in neurons that have highly plastic properties, such as neurons in the hippocampus, cerebral cortex, or cerebellum (5, 6). CaMKII plays a central role in expression of postsynaptic long term plasticity. Whereas the role of CaMKII in long term plasticity is well documented, its role in presynaptic short term plasticity (STP) is not well understood. Notably, CaMKII is a major component of the presynaptic termini and the surface of synaptic vesicles (7, 8), and it is also a major component of postsynaptic densities.

We reasoned that interactions between syntaxin-1A and CaMKII may play an important role in the regulation of STP; therefore, we generated syntaxin-1A (R151G) knock-in (KI) mice that carry a single point mutation in syntaxin-1A. Based on the biochemical findings described here, we concluded that interactions between syntaxin-1A and CaMKII and the consequent recruitment of complexin to the SNARE complex were attenuated in the R151G KI mice. Based on our electrophysiological data, we concluded that the paired-pulse facilitation (PPF), an STP form, and post-tetanic potentiation (PTP), another STP form, were enhanced in the KI mice. In contrast, SNARE complex formation itself and basal transmission were normal in these KI mice. These phenotypes, together with abnormal behaviors indicative of excessive brain activity, may have resulted from insufficient CaMKII activity in presynaptic termini.

Additionally, the recycling of synaptic vesicles (SVs) was delayed in these KI mice, and the SV density was reduced. Therefore, knock-in of this single point mutation (syntaxin-1A (R151G)) attenuated the interaction between syntaxin-1A and CaMKII and resulted in abnormal regulation of exocytosis, STP, SV recycling, and higher brain functions that regulate behaviors. We conclude that the action of CaMKII in the presynaptic terminal is closely related to the regulation of STP because of an interaction between CaMKII and syntaxin-1A and the consequent action of complexin.

EXPERIMENTAL PROCEDURES

Animals

This research was approved by the respective Animal Care and Experimentation Committee of each institution to which the collaborators belong, and all experiments were performed according to guidelines of these committees.

Generation and Genetic Analysis of Syntaxin-1A (R151G) KI Mice

The ES cell line RENKA derived from C57BL/6N and the mouse strain C57BL/6N were used to produce, via homologous recombination, the syntaxin-1A (R151G) KI mice (B6N-CrlCrlJ-Stx1Aimm1). Syntaxin-1A genomic fragments carrying exons 3–5 (fragment-1, 4,478 bp) and exons 8–9 (fragment-2, 5,870 bp) were amplified via polymerase chain reaction (PCR) from genomic DNA prepared from C57BL/6N mice. Separately, two PCR fragments (232 bp (exons 5 and 6) and 960 bp (exons 6 and 7)), each with the point mutation (Gly-151, GGG) engineered into one primer, were connected via PCR; these sequential reactions resulted in a 1,170-bp fragment carrying exons 5–7 of the syntaxin-1A gene with the R151G point mutation in exon 6 (fragment-1b, 1,170 bp). Simultaneously, this mutation generated a new BstNI site, which facilitates detection of the R151G allele. Fragments-1a and -1b were connected at the BamHI site in exon 5 (fragment-1, 5,629 bp). The 1.8-kb DNA fragment, which carried the Pgk-1 promoter-driven neomycin phosphotransferase gene (neo) flanked by two 34-bp loxP sequences and two FLP recognition targets (ftr), was interposed between fragments-1 and -2, and inserted into pMC1DTPa (9).

The point mutation was confirmed by direct sequencing of the resultant PCR product, which included exon 6. The resulting chimeric mice were mated to C57BL/6N mice to yield heterozygous mice (9). Homozygous mutant mice and control mice were obtained by crossing heterozygous pairs. Genotypes were confirmed by a Southern blot analysis that is described below and, routinely, determined by PCR using the following primers: 5'-GAGTGGGAAACCTGCTCATC-3', 5'-ATAGAC-AACTTGCAGTCTCTCTC-3', and 5'-CCAGACTGCTTG-GGAAAAAAG-3'. A 539-bp product resulted from the wild-type (WT) locus, and a 695-bp product resulted from the KI locus.

To confirm homologous recombination via Southern blot analyses of genomic DNA, 5' and 3' external probes (400 and 549 bp, respectively) and an internal probe designed within the neo cassette (neo probe, 423 bp) were used. Each probe was PCR-amplified from genomic DNA. The PCR primer pairs used were as follows: 5'-GGCAAGAGCAGCGATGC-3' and 5'-CCTCACGTTCGGCAATC-3' (5' probe), 5'-ATTCTGGGCAATC3' and 5'-AGACTAATGTCGAGC-TATC-3' (3' probe), and 5'-GGGATGGCCATTTAGGAC-GAAC-3' and 5'-GATTTGCGTCTGGTGTCG-3' (neo probe). Genomic DNA was digested with NheI for the 3' and neo probes and with SpeI and XbaI for the 5' probe. The sizes of the resulting genomic fragment for the wild-type and KI alleles were as follows: XbaI, 10.6 kb (WT) and 8.3 kb (KI); SpeI, 20.2 kb (WT) and 15.1 kb (KI); NheI, 13.8 kb (WT) and 14.5 kb (KI) (Fig. 1, A and B).

The engineered R151G mutation generated a new restriction site for BstNI. The point mutation was confirmed by restriction fragment length polymorphism-PCR. Exon 6 was amplified with the oligonucleotide primers 5'-AGCTCCTGGGTTCG-3' and 5'-CTCAAACCACACCCACAG-3'. The 234-bp PCR product was digested with BstNI to monitor the new restriction site generated by the mutation. The resulting products were WT 218 bp and KI 163 and 55 bp.

Immunoblotting and Semiquantitative Analysis of Synaptic Proteins

Antibodies used are listed in Table 1. Synaptosomes samples were prepared from the brains of male mice that were 12–16 weeks old (10). Each synaptosome pellet was solubilized as follows (in mM): HEPES 20 (pH 7.4), NaCl 120, DTT 0.1, EGTA 0.1 with 1% Triton X-100, 0.2% CHAPS, phosphatase inhibitors, and protease inhibitors overnight at 4 °C.
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**TABLE 1**
List of antibodies used

| Antibody               | Source                                      | Type        |
|------------------------|---------------------------------------------|-------------|
| Amphiphysin            | Synaptic Systems                            | Rabbit pAb  |
| AP180                  | BD Transduction Laboratories, 610469         | mAb         |
| β-Actin                | Sigma, AC-15                                | mAb         |
| CaMII                  | Santa Cruz Biotechnology, M-176             | Rabbit pAb  |
| CaMII (pT286)          | Affinity BioReagents, clone 22B1            | mAb         |
| CaMKIIr                | Millipore, clone 6G9                       | mAb         |
| CaMKIIB                | Zymed Laboratories Inc.                     | mAb         |
| CASK                   | Upstate Biotechnology                        | mAb         |
| Clathrin heavy chain   | BD Transduction Laboratories, 610499        | mAb         |
| Complexin-1 and -2     | Synaptic Systems, 122 002                   | Rabbit pAb  |
| Complexin-2/Synaphin-1 | Gift from Dr. T. Abe, clone 12C5            | mAb         |
| DOC-2                  | BD Transduction Laboratories, clone 43      | mAb         |
| Dynamin                | Sigma, clone D5                             | mAb         |
| GST                    | GE Healthcare                               | Goat pAb    |
| Munc-13                | Synaptic Systems, clone 266B1               | mAb         |
| Munc-18                | BD Transduction Laboratories, clone 31      | mAb         |
| Myosin Va              | Sigma, LF-18                                | Rabbit pAb  |
| NMDAR1                 | M. Watanabe (co-author)                     | Rabbit pAb  |
| NMDAR2                 | M. Watanabe (co-author)                     | Rabbit pAb  |
| Piccolo                | Synaptic Systems, 140 002                   | Rabbit pAb  |
| Rho-GDI                | Santa Cruz Biotechnology, A-20             | Rabbit pAb  |
| SNAP-25                | Gift from Dr. M. Takahashi, clone BR05      | mAb         |
| Synapsin-la/ib         | Synaptic Systems, clone 46.1               | mAb         |
| Synapsin-lII           | BD Transduction Laboratories                | mAb         |
| Synaptophyalin         | Synaptic Systems, clone 7.2                | mAb         |
| Synaptotagmin-1.2      | Synaptic Systems, 105 002                   | Rabbit pAb  |
| Syntaxin-1A            | Synaptic Systems, 110 302                   | Rabbit pAb  |
| Syntaxin-1A            | Synaptic Systems, clone 78.3               | mAb         |
| Syntaxin-1B            | Synaptic Systems, 110 403                   | mAb         |
| Syntaxin-1A/B          | Gift from Dr. M. Takahashi, clone 10H5      | mAb         |
| α-Tubulin              | Medical & Biological Laboratories, PM054-7  | pAb         |
| VCP                    | Affinity BioReagents, clone 5               | Rabbit pAb  |

* These antibodies were used for both Western blot and immunoprecipitation. pAb is polyclonal antibody.

**GST-Pulldown Assay**

A cDNA fragment encoding mouse syntaxin-1A (residues 1–262), the syntaxin-1A point mutant (R151G), full-length mouse SNAP-25, the cytoplasmic region of rat VAMP-2 (residues 1–96), full-length bovine complexin-2/synaphin-1 (Cplx2) (a gift from Dr. T. Abe), or full-length rat CaMKIIα was introduced into an insect cell expression vector, pDEST8, pDEST10, or pDEST20, using LR Clonase II (Invitrogen). Purified bacmids were introduced to the Sf21 insect cell line with Cellfectin II transfection reagent (Invitrogen). Within 72–120 h after transfection, cells were collected into a lysis buffer (20 mM HEPES (pH 7.2), 150 mM NaCl, 0.1% Triton X-100), and proteins were extracted for 30 min at 4°C. Lysates from Sf21 cells expressing CaMKIIα, SNAP-25, VAMP-2, complexin, or some combination thereof were mixed in the lysis buffer with 0.8 μM human calmodulin and preincubated with glutathione-Sepharose 4B-agarose beads (GE Healthcare) at 4°C for 1 h. After centrifugation, each supernatant was divided equally into three tubes as follows: GST was added to one tube, GST-syntaxin-1A (WT) to the second tube, and GST-syntaxin-1A (R151G) to the third tube. A Ca\(^{2+}\)-containing buffer (0.5 mM Ca\(_{2+}\), 2 mM Mg\(_{2+}\), 0.5 mM ATP in final) was then added to each tube. Each mixture was incubated at 4°C for 2 h before addition of the GST-Sepharose 4B-agarose beads. The beads were washed four times with Ca\(^{2+}\)-containing lysis buffer and heated with SDS-sample buffer.

**Assay of SDS-resistant Complexes**

To study SNARE complex formation, synaptosomes from KI or from WT mice homogenized in 20 mM HEPES (pH 7.2), 150

Pairs of synaptosome samples included one KI sample and one WT sample (1–20 μg of protein) that were prepared at the same time and were then applied to the same SDS-polyacrylamide gel in triplicate. The obtained values were normalized to the values of valosin-containing protein or of Rho-guanine nucleotide dissociation inhibitor protein detected on the same blot. The normalized values were further standardized relative to the average of those of WT. The ECL prime chemiluminescence kit (GE Healthcare) was used to detect protein bands on immunoblots, and images were captured with a cooled CCD camera system (Light Capture-II, Atto Corp., Tokyo, Japan).

**Immunoprecipitation Studies**

For co-immunoprecipitation assays and analysis of syntaxin-1A-CaMII complexes, synaptosomes dissolved in Triton X-100 and CHAPS (1.5 mg/assay) were preincubated with protein G-Sepharose 4Fast Flow (GE Healthcare) at 4°C for 2 h. The supernatant was collected and divided into identical samples that were then incubated overnight either with anti-syntaxin-1A mAb and normal mouse IgG or with anti-CaMII polyclonal antibody and normal rabbit IgG at 4°C. For co-immunoprecipitation assays with complexin and syntaxin-1A, synaptosomes dissolved in Targitol, CHAPS, and n-dodecyl-β-D-maltoside (600 μg) were used. Next, anti-complexin polyclonal antibody was added to each sample, and the mixtures were incubated at 4°C for 2 h and then incubated with protein G-Sepharose beads for another 1 h. The one-sample t test was used for statistical comparisons of densitometric values.
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mm NaCl were directly lysed in 1% SDS. The protein concentration of each lysate was determined, and each lysate was then mixed with SDS sample buffer, and the sample from each genotype was then divided into two tubes as follows: for each genotype, one tube was boiled, and the other was kept at room temperature. The same blot was sequentially probed with anti-syntaxin-1A, syntaxin-1B, syntaxin-1A/B, and α-tubulin antibodies. The densitometric value of the monomeric form of each SNARE protein was analyzed.

Electrophysiological Analysis

Transverse hippocampal slices (400 μm thick) were cut from mice (8–15 weeks old) with a Vibratome tissue slicer and placed in an interface-type holding chamber for at least 1 h; the chamber contained Krebs-Ringer solution, which included the following (in mM): NaCl 119, NaHCO3 26.2, NaH2PO4 1, KCl 2.5, glucose 11, CaCl2 2.5, and MgSO4 1.3.

Synaptic responses were recorded using an Axopatch 1D amplifier (Molecular Devices, Sunnyvale, CA); signals were filtered at 1 kHz and digitized at 10 kHz with Digidata 1322A (Molecular Devices). Extracellular field-potential recordings were made in the stratum radiatum of the CA1 region using a glass electrode filled with 3 M NaCl. Whole-cell patch clamp recordings were performed using a patch pipette filled with the internal solution containing the following (in mM): cesium gluconate 122.5, CsCl 17.5, NaCl 8, HEPES 10, Mg-ATP 2, and Na3-GTP 0.3 (pH 7.2; 290–310 mosM). To evoke synaptic responses, Schaffer collateral/commissural fibers were stimulated at 0.1 Hz. For extracellular recordings, stimulus strength was adjusted to obtain α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated excitatory postsynaptic potentials (EPSPs) with a slope value between 0.10 and 0.15 mV/ms. For whole-cell patch clamp recordings, stimulus strength was adjusted to evoke excitatory postsynaptic currents (EPSCs) with an average amplitude of about 150 pA.

To examine input-output relationships, a low concentration of 6-cyano-7-nitroquinolinoxide-2,3-dione (1 μM), a non-N-methyl-D-aspartate (NMDA) receptor antagonist, was included to partially block AMPA receptor-mediated synaptic responses; this blocker reduced the nonlinear summation of EPSPs and enabled more accurate measurements of EPSP slopes. These experiments were performed in the presence of 25 μM d-2-amino-5-phosphonovaleric acid (d-APV) to block NMDA receptor-mediated synaptic responses. PPF was induced by two consecutive stimuli delivered at inter-pulse intervals of 30, 50, 100, 200, or 300 ms in the presence of 25 μM d-APV. The paired-pulse ratio was calculated by dividing the slope value of the second EPSP by that of the first EPSP. PTP was elicited by tetanic stimulation (100 Hz, 1 s) of afferent fibers in the presence of 50 μM d-APV.

For estimating the size of the readily releasable pool (RRP) of SVs and the rate of SV supply from the reserve pool, EPSCs were evoked at 20 Hz, and the cumulative EPSC amplitude (normalized to the base-line EPSC amplitude) was plotted against the stimulus number. The regression line was then drawn by fitting a line to the last 10 points (from the 91st to the 100th pulse), and the slopes of these regression lines and their intercepts with the y axis (cumulative EPSC) were determined (11).

All values are expressed as the mean ± S.E. The Student’s t test was used to determine whether there was a statistically significant difference (p < 0.05) between the means of groups (*, p < 0.05; **, p < 0.01).

Recycling Experiments

Neurons of DIV21–28, were washed with normal saline, and then stimulated with high-K+ saline ((in mm) NaCl 31.5, KCl 90, MgCl2 2, CaCl2 2, HEPES 25 (pH 7.4), and glucose 30) for 2 min at 37 °C in the presence of 10 μM FM4-64fx.

Scanning fluorescence images were acquired using a laser scanning microscope (LSM 510 META, Carl Zeiss Inc.) through a Plan-Apochromat ×100/1.4 objective at a sampling of 106 nm/pixel (912 × 912 pixels). ImageJ software (National Institutes of Health) was used to perform all measurements. Individual puncta of 9 × 9 pixels were manually selected to mark individual synapses. Larger puncta were rejected to avoid clusters of smaller synapses (11). For each experiment, more than 150 puncta were counted from five images per coverslip. For each set of samples in an experiment, each fluorescent value was normalized to the median of the fluorescent values from wild-type samples.

Electron Microscopy

While under deep pentobarbital anesthesia (100 mg/kg of body weight), each of three wild-type and each of three mutant mice were perfused transcardially with 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Hippocampal slices were further post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h, stained in block with 2% uranyl acetate for 1 h, dehydrated in graded alcohols, and embedded in Epon 812 resin. Ultrathin sections were cut with Ultracut™ (Leica). For quantitative analyses of morphological features at axo-spinous asymmetrical synapses in the distal stratum radiatum of the hippocampal CA1 region, electron micrographs were taken with an H7100 electron microscope (Hitachi, Tokyo, Japan). The length of each active zone and the densities of synapses, of SVs, and of docked SVs were measured using IPLab software (Scanalytics, Rockville, MD). Docked synaptic vesicles were defined as vesicles that were separated by less than 10 nm from the presynaptic membrane.

Behavioral Analysis

All behavioral tests were carried out with male mice of C57BL/N strain, including the KI mice that were at least 10 weeks old at the start of testing. Raw data from the behavioral tests, the date on which each experiment was performed, and the age of each mouse at the time of each respective experiment are shown in the mouse phenotype database. Mice were housed in groups (3–4 mice per cage) in a room with a 12-h light/dark cycle (lights on at 7:00 a.m.) with ad libitum access to food and water. All behavioral testing procedures were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University, and the National Institute for Physiological Sciences (12, 13). The software applications used for the behav-
ioral studies (Image HC, Image FZ, Image TS, and Image RM) were based on the public domain ImageJ program (rsb.info.nih.gov), which was modified for each test by authors (available through O’Hara & Co., Tokyo, Japan). Statistical analysis was conducted with StatView (SAS Institute, Cary, NC). Data were analyzed by two-way analysis of variance or two-way analysis of variance with repeated measures, unless noted otherwise. Values in graphs are expressed as the mean ± S.E.

Open Field Test—Locomotor activity was measured via an open field test as described previously (13). In each trial, data were collected for 120 min.

Social Interaction Test in Home Cage—Social interaction monitoring in the home cage was conducted as described previously (14). Social interaction was measured by counting the number of particles in each frame as follows: two particles indicated the mice were not in contact with each other, and one particle indicated contact between the two mice.

Contextual and Cued Fear Conditioning—The contextual and cued fear conditioning test was performed as described previously (12, 13). White noise (60 db), which served as the conditioned stimulus, was presented for 30 s, followed by a mild (2 s, 0.35 mA) foot shock, which served as the unconditioned
stimulus. For each mouse, context testing was conducted for 180 s 1 day after conditioning in the same chamber. Data acquisition, control of stimuli (i.e., tones and shocks), and data analysis of stimuli and outcomes were similar to that in WT mice.

FIGURE 2. R151G mutation leads to inefficient binding of syntaxin-1A to CaMKII and of complexin to syntaxin-1A. A and B, interaction between syntaxin-1A (Stx-1A) and CaMKII requires Arg-151 of Syx-1A in vivo. Representative images of (A) and graphs (B) depicting the quantifications of immunoblots (IB) resulting from the co-immunoprecipitation of Stx-1A (left) and CaMKII (right) from brain synaptosomes isolated from WT and Stx-1A (R151G) KI mice. IP, immunoprecipitation. The amounts of co-immunoprecipitated proteins were normalized to immunoprecipitated Stx-1A or CaMKII, and a percentage calculated relative to the amount in WT mice is shown. The amount of Stx-1A that co-immunoprecipitated with CaMKII was significantly reduced in KI mice compared with that in WT mice (mean ± S.D., 27.00 ± 4.583%; n = 3; *, p < 0.05 in one sample t test). C and D, interaction between Stx-1A and complexin (Cplx) was attenuated in the samples from KI mice compared with that in WT mice (mean ± S.D., 41.50 ± 22.03%; result of three independent experiments, †, p < 0.05 in one sample t test). E and F, reduced association between Stx-1A (R151G) and Cplx in an in vitro GST pulldown assay. Lysates from Sf21 cells that expressed CaMKII, SNAP-25, VAMP-2(1–96), and/or Cplx2 were incubated with GST-syntaxin-1A (1–262; WT), GST-Stx-1A (1–262; R151G), or GST in the presence of Ca2+ and ATP. Representative immunoblot of three duplicate experiments (E) and the respective quantification (F) are shown. The densitometric amounts of pulled down Cplx and SNAP-25 were normalized to the amount of pulled down GST-syntaxin-1A (GST-Stx-1A). Results were obtained from at least three sets of synaptosomes sampled from different preparations. Densitometric values of each protein were normalized to the level of valosin-containing protein or of Rho-guanine nucleotide dissociation inhibitor. The relative ratios were calculated using average values from the WT as standards. Median and interquartile ranges are shown. GST-Syx-1A-associated Cplx was significantly reduced in KI mice relative to that in WT mice (†, p < 0.05 in Mann Whitney test, Median, 25% percentiles, 75% percentiles: 1.00, 0.5904, 1.410; 0.4515, 0.1134, 0.5665; WT and KI, respectively).

FIGURE 3. SNARE complex formation in the synaptosomes from KI mice was similar to those from WT mice. A, representative immunoblot of boiled (+) or non-boiled synaptosomes from KI or WT mice. B, quantification of the analysis depicted in A showed fold change of monomeric syntaxins after boiling. No significant differences were observed between KI and WT samples (paired t test). The result of immunoprecipitation and GST pulldown assays indicated that binding of complexin to the SNARE complex was reduced; therefore, we examined whether the formation of the SNARE complex was affected by the R151G mutation introduced into the KI mice. The assembled SNARE complexes were resistant to SDS sample buffer at RT (20) but dissociated into monomeric SNARE proteins at 100 °C. To evaluate the amount of assembled SNARE complex, we compared WT and KI samples with regard to the increase in monomeric syntaxins induced by the boiling of SDS-resistant SNARE complex. Statistically significant analyses were performed with data from two duplicate experiments using three synaptosome pairs from different preparations. No significant difference was observed between WT and KI samples in paired t tests. C and D, ternary SNARE complex was assembled in the mixture of recombinant SNARE proteins. Recombinant SNAP-25 and VAMP-2 were mixed with GST-syntaxin-1A (WT) or GST-syntaxin-1A (R151G) (R). The SDS-containing sample buffer was added to the mixture but was kept at room temperature. The same PVDF membrane was sequentially immunoblotted using anti-VAMP-2, syntaxin-1, and SNAP-25 antibodies. Images obtained using the CCD camera were pseudo-colored and merged (D) using ImageJ software. The arrows indicated the band recognized by all those three antibodies. The white asterisks in C indicated the monomers recognized by each of three antibodies, respectively.
ysis were performed automatically with a program based on ImageJ. When this stimulated area was below a certain threshold (i.e. 20 pixels), the behavior was categorized as “freezing.”

Porsolt Forced Swim Test—Depression-related behavior was assessed via the Porsolt forced swim test as described previously (13). Mice were placed in the cylinders, and immobility and distance traveled were recorded over a 10-min test period. When the area equaled or exceeded the threshold, the mouse was considered to be “moving.” Immobility lasting less than 2 s was not included in the analysis.

Eight-arm Radial Maze—Working memory was measured in fully automated eight-arm radial maze apparatuses (O’Hara & Co., Tokyo, Japan) as described previously (15). Data acquisition and data analysis were performed using Image RM software. The software was based on the public domain ImageJ program (http://rsb.info.nih.gov/ij/), which was modified for the test by authors (available through O’Hara & Co., Tokyo, Japan).

RESULTS

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FIGURE 4. Basal synaptic transmission and presynaptic short term plasticity. A, input-output relationships of AMPA receptor-mediated EPSPs at the Schaffer collateral-CA1 pyramidal cell synapse in acute hippocampal slices were indistinguishable (n = 21 (WT) and n = 22 (KI); p > 0.05) between KI mice (●) and their WT littermates (○). The maximum initial slope of the AMPA receptor-mediated EPSPs evoked with stimulus intensities ranging from 1.80 to 6.48 V is plotted as a function of the fiber volley amplitude. The inset shows sample traces of EPSPs (averages of 10 consecutive sweeps) evoked with various stimulus strengths. B, paired-pulse ratio at the inter-pulse intervals of 100 and 200 ms was higher in KI mice (●) than in WT mice (○), whereas it was indistinguishable between the KI and WT mice at inter-pulse intervals of 30, 50, or 300 ms (n = 32 (WT) and n = 38 (KI); *, p < 0.05; **, p < 0.01). C, post-tetanic potentiation of 1–6 min (except for 3 min) after tetanic stimulation was larger in KI (●) than in WT (○) (n = 22 (WT) and n = 23 (KI); *, p < 0.05; **, p < 0.01). D and E, after obtaining a stable baseline at 0.1 Hz at least for 5 min (D), a 5-Hz stimulation was applied for 3 min (E), and then the stimulation frequency was returned to 0.1 Hz (D). Synaptic responses during the 5-Hz stimulation, except for those during the first 18 s, were significantly larger in KI mice than in WT mice (E) (n = 13 (WT) and n = 13 (KI); *, p < 0.05). The synaptic depression that occurred soon after 5-Hz stimulation was significantly smaller in KI mice than in WT mice (D) (*, p < 0.05).
open (active) form (16), and the linker domain in syntaxin is believed to play an important role in this conversion. Currently, the only known protein that binds to the linker domain is the autophosphorylated form of CaMKII (2). To examine the functional role of the structural conversion of syntaxin-1A, we used the C57BL/6N strain of mice to generate KI mice that carry a syntaxin-1A (R151G) mutation (Fig. 1A). This missense mutation within the syntaxin-1A linker domain severely reduced the CaMKII/syntaxin-1A interaction (4), and in KI mice, the syntaxin-1A (R151G) mutation was confirmed via both Southern blot analysis and Western blot analysis (Fig. 1B and C). The KI mice developed normally and were fertile; moreover, they did not show any marked macroscopic abnormalities or any abnormal phenotypes when assayed under a dissecting microscope.

**Reduction of the CaMKII/Syntaxin Interaction in KI Mice Inhibits Recruitment of Complexin to SNARE Complexes**—First, we analyzed the interaction between CaMKII and syntaxin-1A in KI and control mice. Based on immunoprecipitation data from synaptosomal fractions, we confirmed that samples from KI mice had much less signal from CaMKII/syntaxin-1A complexes than did samples from WT mice (Fig. 1D and E). Synaptosome-1A and an isoform, syntaxin-1B, are among many varied synapse-associated proteins for which the amount of protein in these (R151G) KI mice did not differ relative to that of the respective protein in wild-type (WT) mice (Fig. 1F and G).

**FIGURE 5. Estimation of the size of SV pools and of release probability during repetitive synaptic activation.** A, EPSCs were evoked at 20 Hz in CA1 pyramidal cells to estimate the size of SV pools and release probability during repetitive synaptic activation. B, during 20-Hz stimulation, EPSCs were initially transiently facilitated and were then depressed in the later phase. At the majority of the points in the later phase, the depression was significantly smaller in KI mice than in WT mice (A and B). (*, p < 0.05; **, p < 0.01). C, cumulative plot of EPSC amplitudes, which were normalized to those of the base-line EPSC, is shown. The broken lines represent the regression lines drawn fitted to the last 10 points (from the 91st to the 100th pulse) (n = 23 (WT) and n = 24 (KI); *, p < 0.05). D and E, slopes of the regression lines (D) and the intercept of the regression lines with the y axis (cumulative EPSC) (E). F, time course of EPSC amplitudes before and after 20-Hz stimulation. Short term potentiation soon after the 20-Hz stimulation was significantly larger in KI mice than in WT mice (#, p < 0.05). The 1st point, p = 0.0006; the 2nd point, p = 0.0003; the 3rd point, p = 0.0043; the 4th point, p = 0.0218.
a small presynaptic cytosolic protein, binds to the SNARE complex (1, 17–19); as a result, complexin can inhibit or stimulate exocytosis. In KI mice, the binding of syntaxin-1A to complexin I/II in the synaptosomal fraction was reduced to 40% of that in WT mice (Fig. 2, C and D). To confirm that the R151G mutation inhibited the interaction between syntaxin-1A and complexin, we performed an in vitro reconstitution study. In the presence of the autophosphorylated CaMKII, the amount of complexin bound to R151G syntaxin-1A was reduced to less than 50% of the WT syntaxin-1A-bound complexin (Fig. 2, E and F; for complexin, \( p = 0.0411 \)). The amount of SNAP-25 bound to mutated syntaxin-1A did not significantly differ from that bound to the WT. These results indicated that the R151G mutation resulted not only in an attenuated CaMKII/syntaxin interaction but also in attenuated recruitment of complexin to the SNARE complex.

We examined whether the SNARE complex formation was perturbed by the R151G mutation. At room temperature, formation of the assembled SNARE complex, i.e. the SDS-resistant complex (20), was not changed (Fig. 3A), and there were no statistically significant differences between WT and mutant samples (Fig. 3B). These results indicate that this point mutation does affect formation of the SNARE complex, which is involved in docking and fusion directly. With samples from either WT or mutant mice, the amount of syntaxin-1B in the SNARE complex did not change (Fig. 3, B–D).

**KI Mice Exhibited Abnormal STP**—Next, we used electrophysiological techniques and acutely dissected hippocampal slices to examine basal synaptic transmission and presynaptic STP at Schaffer collateral-CA1 synapses from KI mice and their WT littermates. The input-output relationships of EPSPs that are mediated by AMPA receptors in KI mice were not significantly different from those in WT mice (Fig. 4A), indicating that basal synaptic transmission was normal in mutants and that the initial SV release probability was similar between the KI and WT mice. In contrast, at inter-pulse intervals of 100 ms
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To examine the involvement of SV dynamics in STP, we analyzed synaptic responses to prolonged low frequency (5 Hz) stimulation; these responses are dependent upon the release probability, the size of SV pools, and the rate of SV supply to the pools. Once a stable base line had been maintained for more than 5 min (Fig. 4D), 5-Hz stimulation was applied for 3 min, and recordings were taken (Fig. 4E). Just after the start of 5-Hz stimulation, EPSPs exhibited transient facilitation in WT mice; this facilitation was followed by synaptic depression that persisted throughout the remaining stimulation (Fig. 4E). KI mice also showed similar changes, but throughout the stimulations, the synaptic responses were consistently larger in KI mice than in WT mice (Fig. 4D and E). Furthermore, the synaptic depression that became evident soon after stimulation was significantly smaller in KI mice than in WT mice (WT, 69.6 ± 1.8%; KI, 76.7 ± 2.1%; p = 0.0127). In contrast, the peaks of the depression (WT, 61.3 ± 1.4%; KI, 62.1 ± 2.4%; p = 0.7917) and the recovery time constants after the 5-Hz stimulation (WT, 2.98 ± 0.36 min; KI, 2.86 ± 0.32 min; p = 0.8043) were indistinguishable between the genotypes (Fig. 4D). These results indicated that the rate of SV supply was faster and/or the release probability during repetitive stimulation was higher in KI mice than in WT mice and that the initial release probability did not differ between KI and WT mice; these findings suggested that only plastic changes in synaptic responses were affected, but basal synaptic transmission was intact in KI mice.

Estimation of the Size of SV Pools and of Release Probability during Repetitive Synaptic Activation—Measurements of cumulative synaptic responses during high levels of synaptic activation are often used to accurately estimate the size of an average RRP of SVs and the rate of SV supply to an average RRP (23). Therefore, we used the whole-cell patch clamp recording technique to analyze synaptic responses at the Schaffer collateral-CA1 synapse; we used 20-Hz stimulation because it is sufficient to deplete SVs in the RRP (23). We found that the responses that were induced by high frequency stimulation (e.g., 20-Hz) in KI mice had larger amplitudes than those in WT mice (Fig. 5A). EPSCs were transiently facilitated during the early phase 20-Hz stimulation and then depressed during the later
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phase 20-Hz stimulation (Fig. 5B). As observed in field-potential recordings at 5 Hz (Fig. 4D), the late phase depression was significantly smaller in KI mice than in WT mice (Fig. 5B). Furthermore, transient potentiation after 20-Hz stimulation was also larger in KI mice than in WT mice (Fig. 5F), as is evident from the field-potential recordings at 100 Hz (Fig. 4C).

The intercept of the regression line of the cumulative plot of EPSCs with the axis of the cumulative EPSC amplitude typically represents the RRP size, and the slope of the regression line represents the rate of SV supply when the release probability is constant (24, 25). The slope of the regression line in KI mice (51.5 ± 3.4 (n = 23)) was significantly larger (p = 0.0374) than that in WT mice (39.8 ± 3.4 (n = 24)) (Fig. 5, C and D), but the intercepts did not differ between KI and WT mice (Fig. 4, C and E), indicating that the pool size was similar between the genotypes (WT, 4,794.1 ± 377.0; KI, 4645.2 ± 291.3; p = 0.7574).

FIGURE 8. Behavioral abnormalities in KI mice. A, relative to WT mice, R151G KI mice displayed hyper-locomotor activity and decreased anxiety-like behavior in the open field tests. The KI mice showed increased distance traveled (panel a) (F1,38 = 12.970, p = 0.0009), increased vertical activity (panel b) (F1,38 = 16.781, p = 0.0002), and increased stereotypic behaviors (panel c) (F1,38 = 9.921, p = 0.0032) relative to the WT mice. KI mice stayed for a significantly longer time in the center area (panel d) (F1,38 = 11.835, p = 0.0014) than did WT mice. **, p < 0.01. B, R151G KI mice showed hyper-locomotor activity in the home cage analyses. Social interactions in the home cage did not differ significantly between genotypes (top) (F1,17 = 0.649, p = 0.4316). However, the activity levels of the KI mice were higher than WT controls (bottom) (F1,17 = 4.905, p = 0.0407). The open bars indicate the light periods, and the solid bar indicates the dark period. Analyses were performed automatically with Image HC software. *, p < 0.05. C, contextual memory in KI mice. Panel a, KI mice showed less freezing following the conditioning than did WT mice (F1,37 = 11.802, p = 0.0015). Panel b, reduced freezing was observed in KI mice during the contextual test (left) (F1,37 = 10.879, p = 0.0022) and during the cued testing in the altered context (right) (pretone period, F1,37 = 12.279, p = 0.0012; tone period, F1,37 = 3.406, p = 0.0730), which was conducted 24 h after conditioning. Panel c, KI mice also showed decreased freezing during the contextual test (left) (F1,37 = 9.633, p = 0.0037), and during the cued testing in the altered context (right) (pretone period, F1,37 = 14.062, p = 0.0006; tone period, F1,37 = 10.515, p = 0.0025), which was conducted 12 days after conditioning. **, p < 0.01. D, altered performance of KI mice in the Porsolt forced swim test. For the tests conducted on the 1st day, there were no significant differences in immobility between KI and WT mice (F1,38 = 0.669, p = 0.4185), but the KI mice traveled longer distances than did WT mice (F1,38 = 6.630, p = 0.0140; *, p < 0.05). On the following day, KI mice traveled longer distances than did WT mice (p = 0.0091; ***, p < 0.01). Notably, the KI mice displayed decreased immobility (F1,38 = 8.278, p = 0.0065; ††, p < 0.01) and increased distance traveled (F1,38 = 10.962, p = 0.0020; †††, p < 0.01) relative to the WT mice during first 1 min of the test. E, KI mice showed a performance deficit in the initial phase of training of the eight-arm radial maze test (1–18 session, F1,38 = 7.512, p = 0.0095), which assesses working memory. After sufficient training sessions, the performance of the KI mice was comparable with that of WT (19–30 session, F1,36 = 0.133, p = 0.7171). ***, p < 0.01.
However, the SV supply rate and/or the release probability during higher frequency stimulation were higher in KI mice than in WT mice, suggesting that SV turnover after frequent stimulation is specifically overactivated in KI mice relative to that in WT mice.

Delayed Recycling of SVs in Syntaxin (R151G) KI Mice—Next, we examined whether endocytosis, like exocytosis, was affected in these KI mice. Using the cultured hippocampal neurons, we confirmed that KI cells showed normal synaptogenesis in vitro (Fig. 6A). The distribution of synaptic proteins in KI cells was quantitatively similar to that in WT (Fig. 6B). FM4-64 is a hydrophilic fluorescent dye that only becomes incorporated into the cell membrane during endocytosis. Using this dye and high K+/H11001 stimulation, we examined SV recycling and observed a significant delay of SV recycling (Fig. 6, C and D) in KI cells.

If SV recycling is continuously disturbed for long periods, KI mice may exhibit an abnormal distribution of SVs. Thus, we performed a quantitative electron microscopic analysis of hippocampal neurons. Although the number or the size of the synapses was not changed in KI mice, the SV density was reduced (Fig. 7A) to approximately two-thirds of that in WT mice (Fig. 7B). These results indicated that the (R151G) mutation affected normal SV recycling, and as a result, mature KI mice had a lower SV density than mature WT mice.

Behavioral Analyses Indicate that KI Mice Exhibit Hyperactive Locomotion—Generalized characteristics did not differ significantly between KI and WT mice. Under these conditions, behavioral tests are very useful for quantitatively evaluating the higher brain functions of transgenic mice (12). The KI mice exhibited locomotor hyperactivity in the open field and fewer anxiety-like behaviors in the open field (Fig. 8A). The KI mice were also hyperactive in their home cages (Fig. 8B). Contextual memory tests revealed that the KI mice showed less freezing following conditioning (Fig. 8C). In the Porsolt forced swim test, the KI mice traveled longer distances than WT mice on the 1st day as well as the following day. In particular, the KI mice exhibited significantly longer distances traveled and significantly less immobility at the first 1 min of the 2nd days’ test (Fig. 8D, see ††). The KI mice showed a performance deficit in the initial phase of training of the eight-arm radial maze test, which is a test that assesses working memory (Fig. 8E).

Based on all these findings, the KI mice, which have only a single point mutation (syntaxin-1A R151G), showed disorders in several higher brain functions. In particular, like CaMKII+/− mice, the KI mice showed hyperactivity, decreased anxiety-like behaviors, decreased depression-like behavior, and impairment of working memory (Fig. 8, A, B, D, and E) (13, 15). These findings indicate that the R151G mutation causes insufficient CaMKII action in the presynaptic terminal.

DISCUSSION

Syntaxin-1A is a key player in presynaptic exocytosis as a SNARE complex component. We generated and examined syntaxin-1A (R151G) KI mice, which have a single point mutation that attenuated the interaction between CaMKII and syntaxin-1A. A key biochemical abnormality caused by this KI mutation was reduced recruitment of complexin, which normally functions as a molecular clamp, to SNARE complexes (Fig. 2). The mutation affected neither normal SNARE complex formation (Fig. 3) nor the associated basal neurotransmitter release (Fig. 4). However, the KI mutation was associated with abnormal short term plasticity, excess SV release (Fig. 5), delayed SV recycling (Fig. 6), and reduced SV density in mature mice (Fig. 7); additionally, the mutation caused abnormal behaviors, including hyperactivity (Fig. 8). These results clearly indicated that syntaxin-1A is essential not only for exocytosis but also for the regulation of STP, SV recycling, and higher brain function through regulation of transmitter release. All of these were disturbed by a single R151G point mutation.

Role of Complexin in Regulating STP—Although the RRP size in syntaxin-1A-KI was similar to that in WT, the SV supply rate and/or the release probability during higher frequency stimulation was higher in KI than in WT (Fig. 4). Therefore, we asked the following two questions. 1) How does the CaMKII/syntaxin-1A interaction regulate exocytosis and play a role in STP? 2) How does impairment of this interaction lead to enhanced PPF? To answer these questions, we focused on complexin. Complexin is bound to the SNARE complex and thereby regu-
lates exocytosis after SV docking. However, the role of complexin remains unclear. In particular, the effect of complexin on exocytosis appears to be bidirectional. Initially, complexin adopts a conformation that inhibits vesicle fusion; it then undergoes a conformational change, thereby adopting a conformation that stimulates exocytosis (29–33). Because CaMKII-syntaxin-1A was necessary for recruitment of complexin to the SNARE complex and because the mutant syntaxin-1A (R151G) inhibited this recruitment both in in vitro reconstitution (Fig. 2, A and B) and in vivo immunoprecipitation studies (Fig. 2, A–D), the “clamping” action of complexin on the SNARE complex is probably insufficient and incomplete. Repetitive synaptic stimulation probably induces an accumulation and therefore a rise of [Ca$^{2+}$]i in the presynaptic terminal that includes the residual Ca$^{2+}$ that remained after the first pulse in the PPF experiments (34). These processes result in elevated autophosphorylation of CaMKII and enhance complexin recruitment to the SNARE complex. Data from many reconstitution studies and some electrophysiological studies have shown that complexin itself has an inhibitory effect on vesicular fusion and that it acts as a “clamp” (30, 32, 35); thus, it is very likely that, in the CNS including hippocampal neurons, recruitment of complexin via the CaMKII-syntaxin-1A interaction inhibits transmitter release during repetitive stimulation and that impairment of this recruitment leads to the increase of the second response in PPF. This hypothesis could also completely account for the enhanced PTP as well (Fig. 4B).

**Similarity of R151G Phenotypes to CaMKII Mutations**—We demonstrated that syntaxin-1A (R151G) KI mice exhibited enhanced STP (Figs. 4 and 5) and abnormal behaviors (Fig. 8) and that both phenotypes were associated with and may be caused by an attenuated CaMKII-syntaxin-1A interaction and the consequent reduction in the recruitment of complexin to SNARE complexes (Fig. 2). However, the basal release probability in KI mice was not significantly different from that in WT mice (Fig. 4A), indicating that the CaMKII-dependent mechanisms acted only during the plastic stage, which occurs after stimulation. These results suggested that presynaptic CaMKII regulated STP and exocytosis via interactions with the proteins involved in the SNARE mechanism.

Our results seemed generally consistent with the results from previous experiments conducted by other groups that used CaMKIIα-KO homozygous or heterozygous mice (36, 37) or presynaptic cell-specific CaMKIIα-KO mice (Fig. 9) (38). In addition, the R151G KI mice exhibited abnormalities in higher brain functions that were very similar to those of CaMKIIα−/− mice (Fig. 8) (13, 15, 38). These findings appear also to be consistent with the hypothesis that presynaptic CaMKII activity was reduced because of the R151G mutation. Further studies are required to resolve these issues.

**Abnormal SV Recycling and Syntaxin-1A**—We also showed that the mutation caused a delay in SV recycling in the KI mice, and probably as a consequence of this delay, SV density was reduced in the brains of these mice. We do not currently know the exact reason for these abnormalities caused by a single point mutation of syntaxin-1A. One possible interpretation is that excess SV release caused by the stimulation also caused excess SV consumption, and endocytosis was not sufficiently rapid to prevent a delay in SV recycling. We could not compare the effects of the R151G mutation in syntaxin-1A directly to those of CaMKII mutations because the designs of the previous studies differed too greatly from our study design. Further investigation into whether the endocytic abnormality is dependent upon CaMKII or complexin should be a next step.

**Acknowledgments**—We thank Drs. Teruo Abe and Masami Taka-hashi for generously providing us with antibodies. The production of the gene-targeted mice and their behavioral analysis were supported by TOGONO and HOKATSU-NOU (Comprehensive Brain Research Network from Ministry of Education, Culture, Sports, Science and Technology).

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