An Ancient Duplication of Exon 5 in the Snap25 Gene Is Required for Complex Neuronal Development/Function

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Abstract

Alternative splicing is an evolutionary innovation to create functionally diverse proteins from a limited number of genes. SNAP-25 plays a central role in neuroexocytosis by bridging synaptic vesicles to the plasma membrane during regulated exocytosis. The SNAP-25 polypeptide is encoded by a single copy gene, but in higher vertebrates a duplication of exon 5 has resulted in two mutually exclusive splice variants, SNAP-25a and SNAP-25b. To address a potential physiological difference between the two SNAP-25 proteins, we generated gene targeted SNAP-25b deficient mouse mutants by replacing the SNAP-25b specific exon with a second SNAP-25a equivalent. Elimination of SNAP-25b expression resulted in developmental defects, spontaneous seizures, and impaired short-term synaptic plasticity. In adult mutants, morphological changes in hippocampus and drastically altered neuropeptide expression were accompanied by severe impairment of spatial learning. We conclude that the ancient exon duplication in the Snap25 gene provides additional SNAP-25-function required for complex neuronal processes in higher eukaryotes.

Introduction

The evolution of more advanced organisms has required adaptation of genomes to be able to generate new gene functions. The original genome sequence analyses of different organisms surprisingly revealed that the number of genes in the human genome is only around 30,000, versus 20,000 in much simpler organisms such as the nematode Caenorhabditis elegans [1,2]. However, a closer examination of these 30,000 identified human genes suggested that as many as one third of them might be false and the number of protein-coding genes in humans are close to the 19,000 found in the domestic dog [3,4]. Instead the increased protein complexity of higher eukaryotes appears to be the consequence of the same gene encoding several functional proteins. This has been accomplished by duplication of genes or gene segments and transcriptional and post-transcriptional regulation. The major contribution to protein diversity is alternative splicing and some 40–60% of all mammalian genes generate more than one protein [5,6]. Interestingly, as many as 10% of all genes in mammals contain tandemly duplicated exons, suggesting that exon duplication followed by functionally diverging mutations have been a fast and successful evolutionary mechanism to increase protein variety [7,8]. Duplicated exons are often subjected to mutually exclusive alternative splicing, incorporating only one of the two exons in the resulting polypeptide [8].

Regulated membrane fusion forms the basis for synaptic transmission but is also fundamental for appropriate release of hormones and modulatory neuropeptides [9–13]. One of the final steps prior to vesicle fusion with the plasma membrane is the formation of a trans-membrane soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) complex [14,15]. This specialized SNARE complex is bridging vesicles to the plasma membrane during regulated exocytosis and consists of three compartmentally defined proteins: The vesicle-associated VAMP2/synaptobrevin, a protein with a plasma membrane anchor syntaxin 1a, and the cytoplasmic synaptosomal-associated protein of 25 kDa, SNAP-25, that associates with membranes through palmitoylation. The three SNARE proteins are held together by strong protein-protein interactions, whereby the cytoplasmic domains form a four α-helix coiled-coiled bundle [16]. The detailed mechanisms mediating regulated exocytosis are still not fully elucidated but the current hypothesis is that SNARE proteins operate at the actual fusion event and have intrinsic capabilities to perform membrane fusion [17,18]. A possibility is that the SNARE proteins are candidates for adjusting thresholds for synaptic plasticity in more advanced neuronal systems. They form the central fusogenic core at the plasma membrane. It is notable that the number of SNARE proteins, and alternative isoform variants, have increased through evolution and their expression is strictly regulated both anatomically and temporally [19–23].
In higher vertebrates SNAP-25 is expressed as two developmentally regulated and complementary distributed splice variants termed SNAP-25a and SNAP-25b [23]. The alternative splicing is an obligate choice between two closely spaced tandemly arranged exon 5 sequences, and nine of 206 amino acids in the two polypeptides differ. The alternative splicing modifies a domain of the SNAP-25 protein spanning a quartet of cysteine residues that are substrates for post-translational palmitoylation and required for membrane targeting [24,25]. In mouse brain, SNAP-25a precedes SNAP-25b expression during development, but by the second postnatal (PN) week SNAP-25b becomes the major splice variant, concomitantly with a dramatic increase in SNAP-25 expression [26]. In fact, in adult mouse brain the SNAP-25b transcript represents more than 90% of total SNAP-25 mRNA [26]. Targeted disruption of the mouse Snap25 gene has demonstrated that complete removal of SNAP-25 results in total absence of evoked neuroexocytosis and embryonic lethality [27]. Separate overexpression of the two SNAP-25 isoforms in embryonic adrenal chromaffin cells from these SNAP-25 knock-out (KO) mice showed that the SNAP-25b isoform had a higher capability to stabilize the pool of primed vesicles than SNAP-25a, since the burst of Ca$^{2+}$-evoked catecholamine release differed [28]. Recently, genome-wide scans and linkage analysis have indicated an association between polymorphisms in the human SNAP-25 gene and vulnerability to develop attention deficit hyperactivity disorder, ADHD [29,30]. In humans, different SNAP-25 alleles also demonstrate inheritance correlated to intelligence [31,32].

To specifically explore the physiological importance of the exon 5 duplication in the Snap25 gene we used a novel approach by developing SNAP-25b KO/SNAP-25a knock-in mouse mutants. The exon 5b was genetically eliminated and replaced with a second supplementary exon 5a, to maintain alternative splicing and normal expression levels, but allowing only SNAP-25a to be expressed. The SNAP-25b deficient mouse mutants exist in two versions, neo-containing with a Tkneo marker retained and neo-excised with the selection gene removed. Based on results from electrophysiological, behavioral and immunohistochemical experiments we conclude, that under physiological conditions, mice deficient in SNAP-25b have developmental defects, impaired short-term synaptic plasticity, a seizure-prone phenotype and malfunctioning cognitive performance. We suggest that the ancient duplication in the Snap25 gene followed by regulated alternative splicing between two similar but distinct exon 5 sequences is required for accurate synaptic function during development. Furthermore, a balanced expression of the two isoforms is a prerequisite for maintaining an operational neuronal network also during adulthood in advanced organisms.

### Results

**Mouse Mutants Not Expressing SNAP-25b**

In the Snap25 gene, exon 5a and 5b are closely spaced and differ in only nine of 39 amino acids [23,26]. To develop mouse mutants that only express SNAP-25a, a gene targeting vector with an additional exon 5a sequence, replacing the mouse exon 5b sequence, was generated (Figure 1A). The vector construct spanning two exon 5a sequences arranged in tandem and exon 6 from the mouse Snap25 gene also contained a Tkneo selection cassette flanked by loxP recombination sites (Figure 1A). Only the exon 5b sequence was changed to encode the exon 5a amino acids, and the original splicing signals for expression of the downstream exon 5 were kept intact. Three independent mouse lines were backcrossed on C57BL/6N Crl (B6) mice for at least ten generations, thus establishing fully congenic strains. After intercrossing of heterozygous SNAP-25a and SNAP-25b deficient mice exhibited neurological defects and were sacrificed. Thus, we have developed a SNAP-25b deficient mouse mutant by replacing exon 5b with an additional exon 5a equivalent, thereby preventing expression of SNAP-25b but not the alternative splicing.

**SNAP-25 in Neo-Containing SNAP-25b Deficient Mice**

We previously demonstrated that targeted insertion of a Tkneo selection cassette in the Snap25 gene impaired alternative splicing and repressed total gene expression [33]. Initially, we therefore investigated the level of SNAP-25 mRNA and protein expression in brain of neo-containing SNAP-25-deficient mutants and wild-type (WT) littermates at PN14. Semi-quantitative RT-PCR analysis demonstrated that neo-containing SNAP-25b deficient mice expressed approximately 50% of the SNAP-25 mRNA levels present in WT littermates (Figure 1B). In order to determine the relative ratio of SNAP-25a and SNAP-25b mRNA expression in brain at PN14, isolated RNA from homozygous and heterozygous SNAP-25b deficient mutants and WT littermates was subjected to an RT-PCR assay based on the presence of exclusive restriction enzyme sites in exons 5a and 5b. At PN14, SNAP-25b levels were five times higher than SNAP-25a in WT mice, heterozygous neo-containing mutants had equal amounts of both SNAP-25 mRNA isoforms, while homozygous neo-containing SNAP-25b deficient mutants only expressed SNAP-25a (Figure 1C).

Western blotting of protein homogenates from PN14-15 homozygous neo-containing SNAP-25b deficient mice brains revealed that SNAP-25 protein levels were also lower in mutants when compared to WT littermates (Figure 1D). SNAP-25b deficient mice expressed approximately 80% of normal SNAP-25 protein levels. Thus, there was no direct correlation between SNAP-25 mRNA and protein levels (compare Figures 1B and 1D). The protein levels of the cellular SNAP-25 homolog, SNAP-23, and the binding partner to SNAP-25, syntaxin 1, were not
Figure 1. Generation of SNAP-25b Deficient Mouse Mutants. (A) Schematic diagram demonstrating the targeting construct and the development of a modified Snap25 allele with eliminated SNAP-25b expression. The mouse genomic sequence was derived from the WT Snap25 gene and addition of an additional exon 5a was performed using PCR. A Tkneo selection gene, surrounded by loxP repeats, was inserted at the EcoRI (E) site located downstream of the two tandemly arranged exons 5a and upstream of exon 6. Letters denote restriction sites: E, EcoRI; H, HindIII; P, PstI; S, SacI; X, XbaI. (B) Total SNAP-25 mRNA level in mouse brain at PN14 was investigated by semi-quantitative RT-PCR. Levels of SNAP-25 mRNA, quantified relative to GAPDH mRNA levels, were determined in neo-containing SNAP-25b deficient mutants (KO) and compared to WT littermates. SNAP-25 mRNA in neo-containing SNAP-25b deficient mutant brain was significantly reduced to 55.5 ± 2.7% compared to WT (mean ± S.E.M., n = 6 mice for each genotype, each sample repeated three times, *p = 0.0312, Wilcoxon’s signed-rank test). (C) A semi-quantitative RT-PCR restriction enzyme assay was used to determine relative levels of SNAP-25a and SNAP-25b isoform mRNAs in WT (+/+), neo-containing heterozygous (+/-) and neo-containing homozygous (−/−) mutant brains at PN14. A PvuI restriction site exclusive for exon 5a and a StyI site only present in exon 5b was used to determine relative levels of SNAP-25a and SNAP-25b. WT mice had 16.7 ± 1.9% SNAP-25a mRNA, neo-containing heterozygous (+/-) mice 51.6 ± 1.7% SNAP-25a mRNA, and homozygous SNAP-25b deficient mutants only, the SNAP-25a mRNA isoform (n = 5 mice of each genotype, run in two replicates, mean ± S.E.M., ***p < 0.0001, Student’s t-test). (D) Western blotting was used to analyze synaptic protein levels in brain at PN14-15, standardized against α-tubulin. The level of SNAP-25 protein in neo-containing SNAP-25b deficient mutants was 80.7 ± 3.6% compared to WT (mean ± S.E.M., *p = 0.0312, Wilcoxon’s signed-rank test), whereas expression of SNAP-23 protein was 98.9 ± 3.5% (p = 1, n.s.) and that of syntaxin 1 protein 107.6 ± 4.9% (p = 0.4375, n.s.), compared to WT animals. The levels of SNAP-25 protein differed significantly in neo-containing SNAP-25b...
deficient mutants compared to WT littermates, but not the levels of SNAP-23 and syntaxin 1 proteins (n = 6 mice of each genotype, run in three replicates). (E) Representative images of SNAP-25 immunoreactivity in coronal sections at low magnification (i, iii) and in the hippocampus at higher magnification (ii, iv) and WT (i, ii) mice at PN14 (n = 2 mice of each genotype, and three levels were analyzed from each animal). Identical microscope settings were used for WT and KO (neo-containing SNAP-25b deficient mutants) images. Scale bar = 1 mm for the low magnification and 200 μm for the high magnification figures. (F) Weight curves of homozygous neo-containing SNAP-25b deficient mutants compared to WT littermates between PN5 to PN15. Body weight gain was significantly reduced in young neo-containing SNAP-25b deficient mutants when compared to WT (**p<0.0001, two-way repeated measures ANOVA, n = 7 mice of each genotype). (G) Bone growth is affected in neo-containing SNAP-25b deficient mice. Mean ± S.E.M. thickness of the hypertrophic, proliferative, and reserve zones in femur and tibia in WT (black bars) and neo-containing SNAP-25b deficient (white bars) PN14 mice. *p<0.05 (n=4). Data was analyzed with unpaired Student’s t-test. doi:10.1371/journal.pgen.1000278.g001

significantly different in mutants compared to control animals (Figure 1D). Immunohistochemical analysis of SNAP-25 showed a similar appearance in homozygous neo-containing SNAP-25b deficient mutant and WT mouse brain at PN14, and no obvious pathological changes were observed (Figure 1E).

Together, our results demonstrate that homozygous neo-containing SNAP-25b deficient mouse mutants only express SNAP-25a and that total SNAP-25 mRNA and protein levels are reduced. Unexpectedly, despite that neo-containing SNAP-25b deficient mutants express only 50% of normal SNAP-25 mRNA levels, protein levels of SNAP-25 are only reduced to 80% of WT littermates.

Neo-Containing SNAP-25b Deficient Mutants Die Young

Neo-containing SNAP-25b deficient mutants exhibited a severe phenotype that included a reduced gain in body weight after the first PN week (Figure 1F). The growth-deficiency was not due to an inability of mutants to feed properly, as dissection revealed stomachs filled with milk (data not shown). To further investigate developmental defects, bone growth was analyzed in PN14 mutants and WT littermates. Bone development was determined by measuring the hypertrophic, proliferative and reserve zones in hind limb sections (Figure 1G). In both the femur and tibia, the hypertrophic zones were significantly reduced in homozygous neo-containing SNAP-25b deficient mutants when compared to WT littermates. The proliferative and reserve zones were not significantly different (Figure 1G). Around PN10 homozygous SNAP-25b deficient mice were easily identified by their smaller size and extreme activity with hypertensive episodes. From PN11, neo-containing SNAP-25b deficient mutants exhibited frequent episodes with tremors and seizure activity and were therefore sacrificed at PN14-17. The early PN development also appeared postponed, indicated by that eye opening occurred approximately one day later compared to WT littermates and that the SNAP-25b deficient mutants also demonstrated inability to sound concurrently in time when WT littermates acquired that ability. Heterozygous neo-containing SNAP-25b mutants were indistinguishable from WT littermates during early PN development.

Our results show that homozygous neo-containing SNAP-25b deficient mice, lacking SNAP-25b expression and having a moderate reduction in total levels of SNAP-25 protein, exhibit severe developmental and behavioral defects.

Removal of the Selection Gene, Tkneo, Reduces the Severity of the Phenotype

The reduced SNAP-25 expression in neo-containing SNAP-25b deficient mutants could be due to the presence of the Tkneo selection gene. Therefore, neo-containing heterozygous SNAP-25b deficient mutants were crossbred with a global Cre transgene [34]. The Tkneo gene was excised and the Cre transgene was thereafter crossed out from the neo-excised SNAP-25b deficient mutants (Figure 2A).

Neo-excised SNAP-25b mutants were backcrossed onto a B6 background for more than ten generations. Intercrosses using heterozygous breeding pairs indicated the expected mendelian distribution (28% homozygous mutants born out of 222 mice). Unlike neo-containing homozygous SNAP-25b deficient mutants, the growth of neo-excised homozygous mutants between PN5 and 15 was not severely affected. No difference in body weight was observed between mutants and WT littermates when genders were mixed. However, when females were analyzed separately they demonstrated a small but significant reduction in body weight (Text S1, Figure S1A and S1B). After the first PN weeks the body weights were normalized and no significant differences were observed in adult females (data not shown). Neo-excised SNAP-25b deficient mutants also demonstrated spontaneously occurring seizures although less frequent and not prior to young adulthood.

In conclusion, in vivo excising of Tkneo from the targeted Snap25 gene rescues the most severe developmental defects observed in homozygous neo-containing SNAP-25b deficient mice.

SNAP-25b Deficient Mutants Exhibit No Reduction in SNAP-25 Expression

Contrary to homozygous neo-containing animals, neo-excised SNAP-25b deficient mutants showed SNAP-25 mRNA levels similar to WT littermates at PN14-15 (Figure 2B). SNAP-25b mRNA in WT mice was, as expected, five times higher than SNAP-25a (Figure 2C). In heterozygous neo-excised mutants the relative SNAP-25a/SNAP-25b mRNA ratio was 2:1, while SNAP-25b was absent in homozygous neo-excised mutants. In neo-excised mutants SNAP-25 protein expression was moderately but significantly increased to 111% compared to WT (Figure 2D). SNAP-23 and syntaxin 1 protein levels were not altered from levels observed in control littermates.

Our results demonstrate that removal of Tkneo restores SNAP-25 expression and moderately increases total SNAP-25 protein levels in brain of homozygous neo-excised SNAP-25b deficient mutants.

SNARE Complexes from Neo-Excised SNAP-25b Deficient Mutants Are Less Stable Than Those from WT Mouse Brain

In adult brain of WT mice, both splice variants of SNAP-25 are expressed but SNAP-25b is the predominant isoform comprising 90-95% of total SNAP-25 mRNA [26]. To investigate if the stability of SNARE complexes was altered in the brain of our neo-excised SNAP-25b deficient mutants compared to WT mice, we analyzed SDS-resistant SNARE complexes at different temperatures (Figure 2E). Adult brain tissue homogenates only containing SNAP-25a (neo-excised SNAP-25b deficient mutants, KO) or predominantly SNAP-25b (WT) were incubated at different temperatures, either 4°C or heated, prior to gel electrophoresis and Western blotting. In non-heated samples, several immunoreactive bands were observed in SNAP-25b deficient and in WT brain homogenates (Figure 2E). Except for a SDS resistant
complex observed at ~97 kD, identified as the ternary SNARE complex, additional intermediate complexes were detected. At 4°C and stepwise increasing temperatures (70°C–90°C) the ratio of the quantified protein band migrating at ~28 kDa (free SNAP-25) to higher molecular weight SNAP-25 containing complexes indicated that the stability of SNARE complexes was reduced in SNAP-25b deficient mouse brain. Quantification of immunoreactive bands from WT and neo-excised SNAP-25b deficient mutants mice showed that the percentage of total SNAP-25 still present in the ternary complex differed significantly at 80°C, 85°C and 90°C.

**Figure 2. In vivo Excision of the Tkneo Gene Results in Altered Gene Expression.** (A) A schematic diagram demonstrating the in vivo excision of the Tkneo gene. Heterozygous neo-containing SNAP-25b deficient females were mated with heterozygous Prm1Cre males. Male offspring carrying both the mutation in the Snap25 gene and the Cre transgene were mated with 86 females. In vivo excision was demonstrated by PCR, and the Cre transgene was crossed out from the confirmed neo-excised SNAP-25b deficient mice. (B) To investigate total SNAP-25 mRNA levels in mouse brain of neo-excised SNAP-25b deficient mutants at PN14-15, the same radioactive semi-quantitative RT-PCR assay as for neo-containing mouse mutants (see Figure 1B) was used. Total SNAP-25 mRNA levels (SNAP-25a + SNAP-25b) in neo-excised SNAP-25b deficient mice brain at PN14-15 were 117.3 ± 6.8% of WT (n = 6 mice for each genotype, repeated three times each, mean ± S.E.M., *p = 0.0625, Wilcoxon’s signed-rank test). (C) SNAP-25a/SNAP-25b mRNA ratio at PN14 were for WT littermates 17.5 ± 1.2% and 82.5 ± 1.2%, respectively (mean ± S.E.M., ***p < 0.0001 Student’s t-test), in heterozygous neo-excised SNAP-25b mutants 68.9 ± 2.1% SNAP-25a and 31.1 ± 2.1% SNAP-25b (***p < 0.0001), and in homozygous neo-excised SNAP-25b deficient mice 100% SNAP-25a (n = 5 mice for each genotype, repeated twice). (D) Protein levels in brain at PN14-15. In neo-excised SNAP-25b deficient mice, there was 110.9 ± 2.3% (*p = 0.0312) SNAP-25 protein, 106.4 ± 4.2% (p = 0.3125, n.s.) SNAP-23, and 99.4 ± 3.5% (p = 0.8438, n.s.) syntaxin 1 relative to WT expression levels (n = 6 mice for each genotype, run in three replicates, mean ± S.E.M., Wilcoxon’s signed-rank test). (E) Immunoblot demonstrating temperature dependence and stability of the ternary SDS resistant SNARE complex in neo-excised SNAP-25b deficient (KO) and WT mouse brain cortex. The samples separated in lanes 1–5 (from left to right) are from adult WT brain cortex, and the preparations in lanes 6–10 are from neo-excised SNAP-25b deficient mutants (KO only expressing SNAP-25a). The high molecular weight ~97 kD complex was identified as SNAP-25 in ternary SNARE complex, while the band migrating as ~28 kDa is free SNAP-25 protein. KO brain homogenates heated to 80°C showed 44 ± 8% SNAP-25 still present in ternary complex compared to 4°C samples, significantly lower than for SNAP-25 in WT brain (71.2 ± 7.2, *p = 0.01). At 85°C and 90°C, the difference in percentage of SNAP-25 remaining in complex between WT and KO was as following: 59.8 ± 7 and 36.9 ± 3.4 (*p = 0.018), and 48.3 ± 12.3 and 4.5 ± 2.2 (***p = 0.0081), respectively. All experiments were repeated at least three times. Results were analyzed with unpaired Student’s t-test and summarized as mean ± S.E.M. doi:10.1371/journal.pgen.1000278.g002
**p<0.01]. The ternary complex almost disappeared in SNAP-25b deficient brain preparations treated at 90°C while the WT samples still showed a strong immunoreactive signal containing 48.3% of total SNAP-25 in non-disassociated complex (Figure 2E).** Boiling of the samples resulted in the loss of all immunoreactive bands except the monomeric SNAP-25 in both neo-excised SNAP-25b deficient and WT mice.

Our results demonstrate that SNARE complexes isolated from brain of adult neo-excised SNAP-25b deficient mutants are less stable than SNARE complexes from adult WT mice.

**Neo-Excised SNAP-25b Deficient Mutants Demonstrate a Reduction of Facilitated Release**

SNAP-25 is essential for evoked synaptic transmission [27] and its activation is dependent on cytoplasmic free Ca$^{2+}$-concentrations [35]. The different stability of SNARE complexes found in neo-excised SNAP-25b deficient mutants compared to WT mice (Figure 2E) and that SNAP-25b demonstrates an increased association with plasma membrane fractions compared to SNAP-25a (see Text S1, Figure S2) suggest that SNAP-25a and SNAP-25b are differently associated with SNARE complexes close to, or immediately upstream of fusion. Therefore, we compared paired-pulse facilitation (PPF, Figure 3E) of AMPA receptor-

**Pathological Changes Develop in Hippocampus by Age**

In young neo-containing and neo-excised SNAP-25b deficient mouse mutants no obvious difference in structural and morphological appearance of the brain was observed by histological and immunohistochemical analysis (Figure 1E, and data not shown). However, in the stratum lucidum (SLu) of the CA3 region of 4-month-old neo-excised SNAP-25b deficient mutants, SNAP-25-positive (+) mossy fibers had expanded and formed large bundles (compare Figure 5E and 5F), separating the synaptophysin’ nerve endings into island-like structures (Figure 5G and 5H). These morphological differences observed with immunohistochemistry were also evident in neo-excised SNAP-25b deficient mouse mutants at 2 months of age and appeared to progress with age. The CA1 and cerebellum were unaffected at all ages analyzed (data not shown). Thus, the absence of SNAP-25b results in progressing pathological changes in brain areas important for cognitive functions.

**Change in Expression of Neuropeptides, BDNF, and Doublecortin**

Epileptic activity causes dramatic changes in peptide expression in the hippocampal formation (HF), in particular affecting the granule cell mossy fiber system, termed ‘epilepsia peptides profile’ [39–42]. Since we observed seizure activity in our mouse mutants, we examined with immunohistochemistry the expression of cholecystokinin (CCK), neuropeptide Y (NPY) and brain-derived neurotrophic factor (BDNF) in the HF of 8-week-old neo-excised SNAP-25b deficient mutants and WT littermates.
Numbers of doublecortin (DCx)+ migrating neuronal precursor cells were also inspected, as seizures may increase neurogenesis. WT mice displayed a wide expression of CCK immunoreactivity (ir) in the HF, being strongest in SLu and supragranular layer of the dentate gyrus (DG) (Figure 6A and 6E). Double-labeling experiments demonstrated that CCK+ terminals partially overlapped with SNAP-25-ir in SLu (Figure 6I–6K); however, SNAP-25-ir had a wider distribution in this layer. CCK-ir in SLu was virtually absent in homozygous neo-excision SNAP-25b deficient mutants (Figure 6B and 6F, c.f. Figure 6A and 6E), but was increased in cortex (Figure 6B). NPY+ fibers and interneurons were fairly evenly distributed in the WT mouse HF (Figure 6C), but NPY-ir was strongly increased in SLu and the polymorph layer of neo-excision SNAP-25b deficient mutants (Figure 6D and 6H, c.f. Figure 6C and 6G). NPY+ terminals/fibers in SLu of mutant mice overlapped with SNAP-25, although SNAP-25-ir had a more extensive distribution (Figure 6I). Diffuse NPY-ir was increased in the molecular layer of the DG, with lower levels in cortex of neo-excision SNAP-25b deficient mice (Figure 6C, c.f. Figure 6D). BDNF-ir was weak in SLu and polymorph layer of the WT.
In WT mice, DCx
+ cell bodies were distributed as a single layer in the DG subgranular zone (SGZ), while DCx
+ processes had an even localization in the molecular and granular cell layers (Figure 6Q and 6S). The number of DCx
+ cell bodies was increased by 140% in the SGZ of neo-excised SNAP-25b deficient mice, including cells migrating through the granular cell layer, with a large increase in DCx
+ fibers in the molecular layer (Figure 6R and 6T, c.f. Figure 6Q and 6S and Text S1, Figure S3).

In conclusion, deletion of SNAP-25b results in dramatic alterations in neuropeptide levels in the HF, which together with increased levels of BDNF and DCx may provide an environment that promotes neuroprotection/neuroproliferation in response to disrupted synaptic transmission and seizure activity in neo-excised SNAP-25b deficient mutants.

Discussion

The protein sequences of the two splice variants of SNAP-25, SNAP-25a and SNAP-25b, are remarkably well conserved in higher eukaryotes and demonstrate 100% amino acid identity in human, mouse and chicken [23,26,43]. Duplicated exon 5
sequences have also been described in bony fish [44] but not in cartilaginous fishes as the ray *Torpedo marmorata* or in the fruit fly *Drosophila melanogaster* [45]. The *Torpedo* SNAP-25 protein shares 81% and the *Drosophila* protein 61% amino acid identity with the mouse SNAP-25b protein, respectively [45]. Thus, the duplication of exon 5 in the *Snap25* gene must have occurred during early bony fish development more than 400 million years ago, when cartilaginous and bony fish divided and arose from the Placoderms, the first primitive jawed fishes [46]. Although bony fish appear to have rather immature brain structures, the sensory and motor coordinating functions are well developed. After the original duplication event, subsequent accumulation of mutations, presumably in both exon 5a and 5b, have resulted in the two similar but distinct SNAP-25 proteins present in all higher vertebrates.

We asked ourselves if the ancient duplication of exon 5, followed by mutually exclusive splicing, and resulting in increased protein diversity of the SNAP-25 protein was a prerequisite for the development of more intricate neuronal functions found in higher vertebrates. We now show that expression of SNAP-25b is important for early postnatal development and synaptic plasticity. Moreover, genetic elimination of SNAP-25b results in progressive morphological and neurochemical changes in CNS and impairment of cognitive function in adult animals. However, in brains of homozygous *neo*-containing SNAP-25b deficient mutants SNAP-25...
25 protein levels were reduced by 20%, indicating that the Tkneo gene suppressed gene expression. Reduced SNAP-25 protein levels in absence of SNAP-25b are lethal, as demonstrated in the homozygous neo-containing SNAP-25b deficient mutants. The phenotype becomes evident already in the second PN week with characteristic tremors and increased activity, including hyperactive episodes and reduced growth; all signs of uncontrolled neurotransmitter and hormonal release. The neo-excisied SNAP-25b deficient mutants, with restored levels of SNAP-25 expression, escaped the critical early PN period with minor defects. Thus, the increased vulnerability in neo-excisied SNAP-25b deficient mutants is the result of both lack of the SNAP-25b isoform and reduced levels of protein, underlining the importance of a balanced expression of SNAP-25 isoforms.

Figure 6. SNAP-25, CCK, NPY, BDNF, and DCx Immunoreactivity in the Hippocampal Formation of Adult Neo-excisied SNAP-25b Deficient and WT Mice. (A) CCK-ir in HF of WT mice, shown at higher magnification in (E). In SNAP-25b deficient mice (B), CCK-ir disappears in PoDG and SLu, higher magnification in (F). (C) NPY-ir in WT HF, shown at higher magnification in (G). (D) Increased NPY-ir in PoDG, Mol, and SLu of SNAP-25b deficient (KO) mice, the latter at higher magnification in (H). Confocal micrographs of double-staining experiments taken from WT (I-K), and neo-excisied SNAP-25b deficient (L) mice. CCK- (I) and SNAP-25-ir (J) in fibers/terminals of SLu in WT mice. Arrowheads in (J) point to strong SNAP-25 fibers. (K) Double-staining of CCK- (green) and SNAP-25-ir (red) in WT SLu. (L) NPY-ir, lacking in fibers/terminals of WT SLu, is highly expressed in neo-excisied SNAP-25b deficient mutants. In homozygous neo-excisied SNAP-25b deficient mice, SNAP-25-ir fibers in SLu are often thicker (arrowheads in (L) compared to WT mice [(L)] cf. (J,K)). Insets in (K,L) show respective photomicrographs at higher magnification. (M) BDNF-ir in WT HF, the PoDG, and SLu shown at higher magnification in (O). (N) Increased BDNF-ir in PoDG and SLu of mutant mice, the DG shown at higher magnification in (P). (Q) Strong DCx+ cell bodies in SGZ of WT DG, shown at higher magnification in (S). (R) Increased number of cell bodies randomly distributed in SGZ and granular cell layer of neo-excisied SNAP-25b deficient mutants, with higher levels of DCx+ fibers in Mol, shown at higher magnification in (T). Arrowheads in (T) point to DCx+ cell bodies in the granular cell layer. Cx, cortex; DG, dentate gyrus; LMol, lacunosum Mol; Mol, molecular layer of DG; Or, oriens layer of HF; PoDG, polymorph layer of DG; Py, pyramidal layer of HF; Rad, stratum radiatum of HF; SGrDG, supragranular layer of DG; SGZ, subgranular zone of DG; SLu, stratum lucidum of HF. Scale bars: (D = A–C = 500 μm), (H = E–G = 200 μm), (I = J–L = 50 μm), (K, inset = L inset = 10 μm), (M = N = 500 μm), (O = P = 200 μm), (Q = R = 100 μm), (S = T = 50 μm). WT (n = 2), SNAP-25b mutant mice (n = 2).

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SNARE Complexes in Adult SNAP-25b Deficient Mouse Mutants Are Less Stable and Demonstrate a Different Cellular Localization Than Those in WT Mouse Brain

In the SNARE complex with syntaxin and VAMP, SNAP-25 contributes with two amphiphatic α-helices to the four-helix-coiled-coiled SNARE structure [16]. The nine amino acids that distinguish SNAP-25b from SNAP-25a encompass a region in the SNAP-25 protein that spans the last part of the N-terminal SNARE motif and the first part of the linker that separate the N- and C-terminal α-helices. Substitutions of two non-conservative amino acid differences in the SNAP-25a protein to resemble the SNAP-25b sequence followed by transient overexpression in mouse chromaffin SNAP-25 deficient cells changed the secretion capability of SNAP-25a to become similar to that of SNAP-25b [47]. In addition, it has been established that SNAP-25b containing SNARE complexes are more stable than those containing SNAP-25a [16], and that SNAP-25b when overexpressed, is more efficient in driving fusion of catecholamine-containing vesicles from chromaffin cells [28]. We took advantage of the fact that homozygous neo-excised SNAP-25b deficient mutants only express SNAP-25a instead of predominantly SNAP-25b in an adult WT mouse brain. We have now been able to show for the first time that ex vivo SNAP-25b complexes in brain homogenates from neo-excised SNAP-25b deficient mutants dissociated at lower temperatures than WT SNARE complexes (mostly holding SNAP-25b). In this respect our attention was drawn to a SNAP-25b mouse mutant named the “blind-drunk mouse” (Bdr) with retinal degeneration (caused by choice of the brain strain) and a mild phenotype with ataxia and impaired sensorimotor gating due to a dominant mutation in SNAP-25b [48]. The Bdr mouse expresses a SNAP-25 protein with even higher affinity for syntaxin than WT SNAP-25b. The SNAP-25b/Bdr SNAP-25 complexes, apparently more stable than complexes with WT SNAP-25b, result in impairment of both spontaneous and evoked release from cortical neurons and lack of facilitation during trains of high-frequency stimulation [48]. The outcome from studies of SNAP-25b/Bdr, SNAP-25b and SNAP-25a containing complexes suggest that the difference in secretory phenotype might be dependent on the difference in complexity-stability; and possibly on the interaction with accessory factors due to the presence of different amino acids exposed on the surface of the N-terminal amphipathic α-helix.

SNAP-25a and SNAP-25b also exhibit differences in the linker region between the N- and C-terminal SNARE motifs, where a quartet of cysteines, implicated in membrane anchoring of the protein, exists in two different contexts [23-25]. We hypothesized that a difference in plasma membrane association between SNAP-25a and SNAP-25b also could add to the phenotype observed in our mutants. Therefore we performed sucrose density gradient fractionation of brain homogenates from adult neo-excised SNAP-25b deficient mutants (only expressing SNAP-25a) and WT littermates (predominantly expressing SNAP-25b). In agreement with our assumption the mutants only had around 80% of the SNAP-25 protein levels found in WT plasma membrane fractions. Instead, in mutant brain more SNAP-25 was detected in low-density fractions, representing soluble protein and/or protein associated with small intracellular organelles. This difference in subcellular localization of the two SNAP-25 isoforms might contribute to the phenotype of the mutants. Whether that depends on the ability of the two isoforms to associate with membranes due to the altered organization of the cysteine residues that are substrates for palmitoylation, or, whether SNAP-25a and SNAP-25b reside in complex with additional secretory factors that guide the subcellular localizations needs to be further investigated.

Short-Term Synaptic Plasticity Is Impaired in SNAP-25b Deficient Mice

To explore a possible effect on presynaptic mechanisms we studied PPF at Schaffer collateral-CA1 pyramidal synapses. We observed a reduction of PPF in young neo-containing and neo-excised SNAP-25b deficient mutants during low-frequency stimulation (0.2 Hz), suggesting a specific effect of SNAP-25b-deficiency. During higher frequency stimulation (0.5 Hz), when Ca2+ may be expected to be elevated during longer time periods in the stimulated presynaptic terminals, a reduction of the PPF-ratio was observed in neo-containing experiments while there was only a non-significant tendency to a reduction in neo-excised SNAP-25b deficient mice. Whether this is a result of the presumed higher average Ca2+-concentration or due to other factors will need to be investigated in future studies.

Normally, only few synaptic vesicles are “fusion-competent” and require no further modifications prior to exocytosis. Remaining vesicles close to the site of fusion need to be “primed”, that is undergoing ATP- and Ca2+-dependent maturation steps in order to be mobilized to the fusion-competent state [49]. It has been demonstrated that there is a 2-3 fold higher ability of SNAP-25b to keep vesicles in the primed state than SNAP-25a, a feature not due to facilitated priming but rather dependent on a lower de-priming rate [28,50,51]. Furthermore, SNAP-25b has been suggested to sustain the exocytotic bursts in a more efficient way than SNAP-25a in flash photolysis experiments with caged Ca2+ in chromaffin cells [28]. The results from the above mentioned investigations indicate that SNAP-25 is not only involved in the membrane fusion reaction but is also likely to play regulatory roles prior to exocytosis, such as in mobilization, docking and priming of vesicles [28,38,47]. Thus, the results from our PPF studies on SNAP-25b deficient mutants may represent an effect of reduced stability and availability of primed vesicles and are in line with the suggested weaker exocytotic bursts by synapses with SNAP-25a-containing SNARE complexes. Interestingly Bronik et al. [30] showed that there is no synaptic facilitation in the few cultured SNAP-25 KO hippocampal neurons that respond to extracellular + dependent types of facilitation including PPF. At physiological levels of SNAP-25 expression and elevated Ca2+-concentrations the replacement of SNAP-25b by SNAP-25a does not affect synaptic release enough to result in a statistically significant effect. However, there was a tendency to a reduction that needs to be investigated further.

Elimination of SNAP-25b Expression Impairs Cognitive Function, Triggers Morphological Changes, and Induces Neuroprotective Mechanisms

SNAP-25b is normally highly expressed in brain areas involved in cognitive function, such as the hippocampus. Therefore we examined mutants in behavioral tasks dependent on hippocampal mechanisms. Neo-excised SNAP-25b deficient mutants demonstrated a higher anxiety index compared to WT mice, however, without alterations in overall locomotor activity in the elevated plus maze. This finding is consistent with the profound thigmotaxis observed in the computerized measurements obtained in the water maze task. In the Morris water maze task, homozygous neo-excised SNAP-25b deficient mutants were severely impaired in their ability to acquire the spatial learning task. This impairment cannot simply be explained by visual or motor disturbances although there was a trend for female neo-excised SNAP-25b deficient mutants to perform less well in the visible platform test. The failure of the mutants to consistently improve their performance in the
visible platform test is probably related to the same mechanisms as those underlying the deficiency in spatial learning. It is important to stress that the mutants did not display impaired swim performance since they did not differ from WT in their swim speed. However, unlike the WT mice, the mutants displayed a high level of thigmotaxis in the water maze task. This suggests that the deficiency in spatial learning in the mutants could partly be related to their anxious phenotype evidenced by their profound thigmotaxis and/or related to attention deficits observed in the visible platform test.

Both neo-containing and neo-excised SNAP-25b deficient mutants demonstrate spontaneously occurring convulsive seizures and freezing behavior. The neo-containing SNAP-25b mutants are most severely affected with frequent attacks starting around PN12-13, whereas in neo-excised SNAP-25b deficient mutants seizure activity is rare prior to young adulthood. These behavioral impairments were paralleled by increasingly pronounced anatomical and immunohistochemical changes, which are only observed after the debut of seizure activity. In neo-excised SNAP-25b deficient mice, mossy fibers appear notably enlarged and swollen with certain areas almost devoid of synaptophysin immunoreactivity, suggesting a locally decreased density of functional nerve endings. Increased NPY expression in mossy fibers after seizures is believed to be neuroprotective by dampening excitatory activity, while BDNF can regulate NPY expression [52,53]. Both NPY and BDNF have been suggested to promote dentate SGZ neurogenesis [54,55], and their elevated expression may contribute to the increase in DCX+ migrating neuronal precursor cells observed in our mutants. It has been reported that cells generated in the adult dentate gyrus mature into functional neurons that integrate into hippocampal circuitry [55,56], and it was recently shown that newly generated neurons after epilepsy exhibit dampening characteristics [57]. Taken together, it is conceivable that pathology develops in the SNAP-25-expressing mossy fiber area in response to aberrant presynaptic plasticity and synaptic contacts. However, a detailed ultrastructural study will be required to determine any alterations in synaptic morphology.

Conclusions

We here demonstrate the physiological importance of the ancient exon 5 duplication in the Snap25 gene. The functions of SNAP-25a and SNAP-25b appear to complement each other in tuning the presynaptic exocytotic machinery towards different modes of release. For complex neuronal circuitries this modulation of release is necessary, and is also instrumental in protecting highly plastic brain areas from accumulating degenerative morphological changes with age. The development of higher brain functions during evolution has been complemented with expanding number of gene products, sometimes by gene duplications but primarily by increasing protein diversity and complexity via alternative splicing. The importance of alternatively spliced isoforms from other genes has previously been extensively analyzed in vivo, by hindering expression of selected exons using gene targeted mouse models [58]. This is the first time an exon knock-out/knock-in of tandem duplicated exons has been performed. We specifically investigated the physiological importance of removing one isoform while preserving total expression levels of the protein by a simultaneous knock-in of an extra copy of the remaining exon variant.

Interestingly, a growing amount of reports are connecting SNAP-25 function with a wide variety of behavioral and neuropsychiatric disorders, as well as linking it with cognitive capability in humans [29–32]. Our present findings in the SNAP-25b deficient mouse mutants suggest that even small alterations in the strictly regulated temporal and anatomical expression of SNAP-25 isoforms could add to these variations.

Methods

Generation of SNAP-25b Mutant Knock-Out Mice

To generate SNAP-25b deficient mouse mutants a targeting vector was generated where the exon 5b, located downstream of exon 5a in the Snap25 gene, was substituted with an additional exon 5a. A Tkneo gene surrounded by loxP repeats was introduced into the vector [34] (Figure 1A). Homologous DNA recombination in embryonic stem cells (ES cells) was performed using standard procedures [59]. Chimeras that demonstrated germline transmission were chosen for establishing SNAP-25b KO mouse lines (see Text S1). Three mouse lines, bred independently from each other for at least ten generations on C57BL/6N Crl (B6) mice, were termed “neo-containing” as the Tkneo gene inserted into the targeting vector was still present in the modified Snap25 gene. Two of the neo-containing lines were crossed with the Protamine-Cre recombinase transgene Prm1Cre [34], resulting in in vivo excision of the Tkneo gene. The neo-excised lines were bred independently onto B6 background until congenic, and the Prm1Cre transgene crossed out. Genotyping was routinely performed by PCR and by Southern blotting when necessary. In vivo expression of the introduced chicken exon 5a was demonstrated by RT-PCR (Text S1). All animal breeding and studies were done in accordance with the guidelines from local ethical committees.

RNA Analysis

Mouse brains, minus the cerebellum, were isolated after terminal CO2 anesthesia and frozen in liquid N2. Total RNA was isolated from brain (PN14-15) using the GenElute Mammalian Total RNA kit (Sigma). For quantification of total SNAP-25 mRNA; 1 μg RNA, 10 μl of each primer and a trace of [32P]dCTP (3000 μCi/μmol, PerkinElmer Life Sciences) were used in 25 μl reactions with the SuperScript III RT-PCR System (Invitrogen). Semi-quantitative RT-PCR was performed with 20 cycles of amplification and SNAP-25 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers in the same reaction (see Text S1 for primer sequences and PCR programs). The RT-PCR products were separated on an 8% polyacrylamide Tris-borate EDTA (TBE) gel that was dried, and detected using a phosphoimager (BAS-1500, Fujifilm). Signal intensities were quantified in Image Gauge V3.45 (Fujifilm). Determination of SNAP-25a/b mRNA ratio expression was essentially performed as described [26] (and Text S1). Statistical analyses were made using Wilcoxon’s signed-rank test.

Western Blotting

Mouse brains were homogenized in (in mM): 20 HEPES, 1 MgCl2, 250 D-sucrose, 2 EDTA and protease inhibitor cocktail (Roche Diagnostics GmbH), pH 7.4. For whole cell homogenates cells were lysed with 1% NP-40 (Sigma) and 5 μg protein was run on 10% Tris-glycine/NUI-PAGE gels (Novex, Invitrogen) followed by Western blotting. Primary antibodies used were a rabbit polyclonal antibody against SNAP-25 from Synaptic Systems (1:20,000 dilution), a rabbit polyclonal anti-SNAP-25 (1:1000, Synaptic Systems), mouse monoclonals anti-syntaxin 1, HPC-1 (1:50,000 and 1:100,000) and anti-α-tubulin, clone DM 1A (1:45,000), both from Sigma. Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulins (IgGs) from Dako Corporation and Rockland. Statistical analyses were made using Wilcoxon’s signed-rank test for paired data.

Determination of Stability of SNAP-25a and SNAP-25b Containing SNARE Complexes

Pulverized adult WT and homozygous neo-excised SNAP-25 deficient (KO) mouse brain tissues were homogenized in buffer
described for whole cell homogenates. SDS sample buffer [0.5M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% 2-mercaptoethanol and 0.05% Bromophenol Blue] was added to equal amounts (20–40 μg) of WT and KO homogenate before heating treatment at 70, 75, 80, 85, 90 and 100°C (boiling), or kept at 4°C, for 20 min. Treated samples were immediately loaded and separated on 10% Bis-Tris NU-PAGE gels (Invitrogen). SMII1 antibody (1,500,000) was used to detect immunoreactivity of bands migrating as ternary SNARE complex or solitaire SNAP-25 protein. To measure the grade of disassembly of heat-resistant SNARE complex in WT and SNAP-25b KO tissue, percentage monomeric SNAP-25 protein of total SNAP-25 in ternary complex at 4°C was calculated and compared with the value calculated at a certain temperature. All experiments were performed at least three times. Results were analyzed with unpaired Student’s t-test.

Histology and Immunohistochemistry
For histological analysis, mice were anesthetized with isoflurane and transcardially perfused with PBS followed by freshly prepared 4% paraformaldehyde in PBS. Organs were post-fixed overnight, dehydrated in graded ethanol and embedded in paraffin according to standard procedures. 4 μm sections were stained with hematoxylin-eosin or cresyl violet.

For immunohistochemistry of paraffin embedded tissue, sections were collected on Superfrost Plus slides, deparaffinized (xylene), dehydrated (ethanol) and boiled by microwaving for antigen unmasking (see also Text S1). Antibodies used were mouse anti-SNAP-25 (1:750, SMI 81, Sternberger Monoclonals), rabbit anti-SNAP-25 (1:100, Synaptic Systems), mouse anti-synaptophysin (1:200, SVP38, Sigma), and anti-mouse IgG-Alexa 488 or anti rabbit IgG-Alexa 546 (1:250, Molecular Probes).

Neuropilin peptide immunohistochemistry was performed as described [60] (see also Text S1). Antibodies used were mouse anti-SNAP-25 (1:750, 1:2,000 SMI 81, Sternberger Monoclonals), rabbit anti-SNAP-25 (1:100, Synaptic Systems), mouse anti-synaptophysin (1:200, SVP38, Sigma), chicken anti-BDNF (1:200, Promega) and goat anti-DCx (1:100, Santa Cruz Biotechnology). For tyramide signal amplification (TSA+, NEN Life Science Products) antibodies used were rabbit anti-CCK (1:8,000), or rabbit anti-NPY (1:3,000) (for details see Text S1). Corresponding secondary antibodies were HRP-swine anti-rabbit IgG (1:200, Dako), FITC-donkey anti-chicken, Cy3-donkey anti-goat and Cy5-donkey anti-mouse (all 1:100, and from Jackson ImmunoResearch Laboratories).

Determination of Growth Plate Zones in Hind Limb
Hind limb specimens were fixed in 4% formaldehyde in phosphate buffer and embedded in paraffin. Sections, 4–5 μm thick, were stained with hematoxylin and eosin. Growth plates of tibia and femur were scanned using a Zeiss Axiovert 35M microscope fitted with a LSR Astro Cam type TE3/A/S digital camera. Images were analyzed using Concord software from Life Science Resources Ltd. The width of the different zones was determined at least in triplicate per section. Per sample, at least two cross sections were measured. Data was analyzed with unpaired Students t-test.

Electrophysiology
PN12-16 mice were terminally anesthetized with 100% CO2 and sacrificed by decapitation. Brains were quickly removed and placed in ice-cold low Ca2+/ high Mg2+ artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 1.24 NaH2PO4, 0.5 CaCl2, 10 MgSO4, 26 NaHCO3, and 10 glucose, and oxygenated with 95% O2/5% CO2 (pH 7.4). Coronal hippocampal slices (350 μm thick) were cut on a vibratome (Leica VT1000S, Leica Microsystems) and transferred to regular aCSF (same composition as above but with 2.4 mM CaCl2 and 1.3 mM MgSO4) at RT. Patch-clamp electrodes (6–10 MΩ) were filled with [in mM]: 135 Cs-methane sulphonate, 10 HEPES, 1 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 2–5 QX-314, and 8 NaCl (pH 7.25; osmolarity 270–280 mOsm). Whole-cell voltage clamp (~70 mV) recordings of evoked excitatory postsynaptic currents (EPSCs) were obtained at RT from CA1 pyramidal neurons of the hippocampus, visualized with differential interference microscopy (DIC) using an Olympus BX50WI microscope. 50 μM picrotoxin (Sigma-Aldrich) was added to the aCSF to block GABA_A receptor-mediated synaptic transmission. EPSCs were evoked by stimulation of Schaffer collaterals with fine concentric Pt/Ir bipolar stimulation electrodes (Fredrik Haer and Co.) placed in the stratum radiatum. Paired stimuli were delivered at IPs from 40 to 300 ms at 0.2 and 0.5 Hz and data was collected after at least 5 minutes of baseline recording to make sure responses were stable. Statistical comparisons of PPF ratios between WT and SNAP-25b deficient mice were made with two-way repeated measurements analysis of variance (ANOVA). For additional information, see Text S1.

Mouse Behavioral Analysis
The elevated plus maze analysis was performed as described previously [61]. For the water maze task, the animals were handled by the operator for a period of five days prior to the test and spatial learning and memory were examined as described before [62]. Locomotor activity was recorded by means of a multicage red- and infrared-sensitive motion detection system as described earlier for mice [61].

Data from the behavioral testing was analyzed by non-parametric statistics using Kruskal-Wallis ANOVA followed by Mann-Whitney U as the Post-hoc test.

Supporting Information

Figure S1 Weight curves of neo-excised SNAP-25b deficient mouse mutants from PN5 to PN15. (A) Weight gain was reduced for neo-excised male mutants compared to male WT littermates (n = 6 animals of each genotype). (B) Neo-excised female SNAP-25b mouse mutants at the second PN week weigh less than their WT littermates (**p<0.01, n = 6 females of each genotype). Data was analyzed with two-way repeated measures ANOVA. Found at: doi:10.1371/journal.pgen.1000278.s001 (1.30 MB TIF)

Figure S2 Sucrose density gradient fractionation of brain homogenates from 2–5 months old neo-excised SNAP-25b deficient and WT mice for comparison of subcellular localization of SNAP-25a (KO) and SNAP-25b (WT) proteins. More SNAP-25a than SNAP-25b protein could be found in low-density fractions, as shown in the representative immunoblots. Neo-excised SNAP-25b deficient mice (KO) had 80.4±3.4% (*p = 0.05) SNAP-25 in plasma membrane (PM)-associated fractions using a polyclonal antibody, whereas monoclonal antibody resulted in 86.5±5.5% PM-associated protein (p = 0.1, n.s.). Mean±S.E.M., n = 3 for each genotype of animals, and each gradient was run in triplicate for each antibody, data was analyzed using Mann-Whitney U test. Found at: doi:10.1371/journal.pgen.1000278.s002 (1.21 MB TIF)

Figure S3 Doublecortin-ir cells in the hippocampal dentate gyrus. Number of DCx+ migrating neuronal precursor cells was increased by 140% in the neo-excised SNAP-25b deficient (KO) mutants. Number of cells was 298 ± 28 in the homozygous neo-excised SNAP-25b deficient KO mice compared to 125 ± 20 in WT (two levels were counted from each animal and in both hemispheres, n = 2 animals of each genotype, Students t-test, mean±SD). Found at: doi:10.1371/journal.pgen.1000278.s003 (1.39 MB TIF)
**Text S1** Supplementary information.

Found at: doi:10.1371/journal.pgen.1000278.s004 (2.98 MB DOC)

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**Author Contributions**

Conceived and designed the experiments: JUJ JJ SO CB. Performed the experiments: JUJ JJ SB DS SAM BR CB. Analyzed the data: JUJ JE JJ SB DS SAM MAW TH SO BR POB CB. Contributed reagents/materials/analysis tools: MAW TH SO BR POB CB. Wrote the paper: JUJ TH CB.
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