Biochemical and Biophysical Research Communications 375 (2008) 372–377

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Syntaxin 1A is required for normal *in utero* development

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ARTICLE INFO

*Article history:*
Received 25 July 2008
Available online 17 August 2008

*Keywords:*
Syntaxin
Knockout mouse
Lethal
Development
PCR
Southern blot

ABSTRACT

We have generated a syntaxin 1A knockout mouse by deletion of exons 3 through 6 and a concomitant insertion of a stop codon in exon 2. Heterozygous knockout animals were viable with no apparent phenotype. In contrast, the vast majority of homozygous animals died *in utero*, with embryos examined at day E15 showing a drastic reduction in body size and development when compared to WT and heterozygous littermates. Surprisingly, out of a total of 204 offspring from heterozygous breeding pairs only four homozygous animals were born alive and viable. These animals exhibited reduced body weight, but showed only mild behavioral deficiencies. Taken together, our data indicate that syntaxin 1A is an important regulator of normal *in utero* development, but may not be essential for normal brain function later in life.



Syntaxin 1 is a critical element in calcium triggered exocytosis in both neuronal and non-neuronal tissues [1,2]. In vertebrate synapses, syntaxin 1 is known to interact tightly with both SNAP-25 and a synaptic protein interaction site found in both N-type and P/Q-type calcium channels [3–5]. This macromolecular protein complex allows for the docking of synaptic vesicles via interactions with synaptotagmin and synaptobrevin, thus localizing vesicles within calcium microdomains supported by channel activity—a key requirement for efficient calcium triggered neurotransmitter release (for review see [6,7]). In addition to its role in exocytosis, syntaxin 1 is known to directly inhibit calcium channel activity by reducing channel availability [8–13]. From a structural point of view, syntaxin 1 is organized as a four helix bundle that can undergo conformational changes, as well as a membrane insertion domain near the C-terminal which is located adjacent to the BotC cleavage site [14–16]. The mammalian nervous system expresses two syntaxin 1 isoforms, syntaxin 1A and 1B, which are about 85% homologous at the amino acid level, are both cleaved by BotC, and appear to be differentially distributed in the brain [12]. In addition, alternate splicing may generate additional syntaxin 1A variants [14–16].

A recent study reported on the functional knockout of syntaxin 1A in mice [17]. The authors reported that the mice were viable with apparent impairment in long term potentiation and conditioned fear memory, perhaps suggesting the possibility of compensation from syntaxin 1B. These authors targeted their gene deletion strategy towards exons 9 and 10, thus effectively removing the membrane insertion domain of syntaxin 1A, but sparing much of the syntaxin 1A molecule. The same group as well as others reported on the existence of a syntaxin variant (termed syntaxin 1C) that lacks the membrane insertion region as a result of alternate splicing and these truncated splice isoforms are also found in EST databases [18–20]. To determine if the lack of phenotype in the syntaxin 1A knockout animals may have been due to the fact that the authors effectively converted syntaxin 1A into a syntaxin 1C truncation protein, we created a syntaxin 1A null mouse in which we deleted exons three through six, and inserted an additional stop codon into exon 2. Our findings show that the deletion of the syntaxin 1A gene resulted in reduced embryonic growth leading to death *in utero*. Hence, syntaxin 1A appears to may be a key requirement for appropriate embryonic development.

## Materials and methods

To target and excise the endogenous syntaxin-1A gene a cDNA construct utilizing the pPNT vector was used. A segment encompassing a portion of intron 2 to exon 10 was isolated from mouse DNA using PCR two oligos (ccaaagagcctcactgagc, acctccgtggttgatccc were used according to the manufacturer's instructions, and the reaction product was analyzed on a 1.0% agarose gel; DNA bands were isolated, cloned into pGEM-T-easy (Stratagene), and sequenced to confirm identity. DNA from the in house embryonic cell line was used to optimize targeting of the vector as substantial nucleotide differences do occur between mouse lines. The confirmed DNA strand was cloned into the pPPNT vector for targeting

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doi:10.1016/j.bbrc.2008.08.031

the mouse embryonic stem cells. Using XhoI and BamHI (Fig. 1A) the region targeted for recombination included exon 3 until the end of exon 6 and was excised and replaced with the neomycin resistance cassette (see dotted line in Fig. 1). The in frame stop codon was introduced using standard site directed mutagenesis protocol from Stratagene. This targeting vector was electroporated into embryonic stem cells and subsequently grown on irradiated mouse fibroblasts as per the University of Calgary embryonic stem cell facility protocols. Colonies which survived the neomycin treatment were purified, grown to confluency and then screened with PCR and Southern analysis to observe if proper incorporation had occurred. For PCR, DNA and oligos 3 and 4 (oligo 3-ccaggagctgttccc and oligo 4-ggcaccctgggcaataccgc), were used according to the manufacturer's (Qiagen) instructions, the reaction product was analyzed on 2.0% agarose gel; DNA bands were isolated using a gel extraction kit (Qiagen), cloned into pGEM-T-easy (Stratagene), and sequenced to confirm identity. All further PCRs were cloned into pGEM-T-easy (Stratagene) and sequenced to confirm identity. Clones which showed a potential removal of the syntaxin 1A gene were screened with Southern blot analysis using 1 μg of ES cell DNA digested with KPN I (1 site in clone and other outside region) overnight at 37 °C. Digested DNA was electrophoresed through a 1% agarose gel, transferred to nylon membrane (Bio-Rad) overnight. The blot was rehydrated in 2XSSC (1Xs = 0.15 M sodium chloride and 0.015 M sodium citrate.) and prehybridized with 2× Denhardts solution in 2XSSC for 3 h. The random primed 33P probe corresponded to exon 2 was added to fresh solution (2XSSC and 2Xs Denharts) and incubated at 55 °C. The blot was washed in 0.2XSSC and the exposed to radiographic film overnight at −80 °C. Of 254 colonies screened, 6 had undergone homologous recombination and were subsequently grown and sent for blastocyte microinjection at the ESTM facility (University of Calgary). The microinjection process resulted in two females and five chimeric males of which the two females were outcrossed to produce heterozygote animals. To determine genetic identity of all offspring or embryos we used genomic PCR and oligos specific to the wt allele and vector or targeted (oligo 3-ccaggagctgttccc and oligo 4-ggcaccctgggcaataccgc) or wild type DNA/deleted regions of gene or wild type allele (oligo 3-ccaggagctgttccc and oligo 5-cccagtgcaaggaaccc) to determine genotype (Fig. 1A). DNA, oligos and10 mM dNTPs, and 3 U of Hot PROOF Start Taq (Qiagen) were used according to the manufacturer's instructions. Southern blot analysis was performed using 10 μg of mouse tail DNA digested with KPN I overnight at 37 °C. Digested DNA was electrophoresed through a 1% agarose gel, transferred to nylon membrane (Bio-Rad) overnight then the blot was allowed to dry. The blot was rehydrated in 2XSSC (1X's = 0.15 M sodium chloride and 0.015 M sodium citrate.) and prehybridized with 2× denhardts solution in 2XSSC for 3 h. The random primed $^{33}$P probe corresponded to exon 2 was added to fresh solution (2XSSC and 2Xs Denharts) and incubated overnight at 55 °C. The blot was washed in 0.2XSSC three times for 2 h each and the exposed to radiographic film overnight

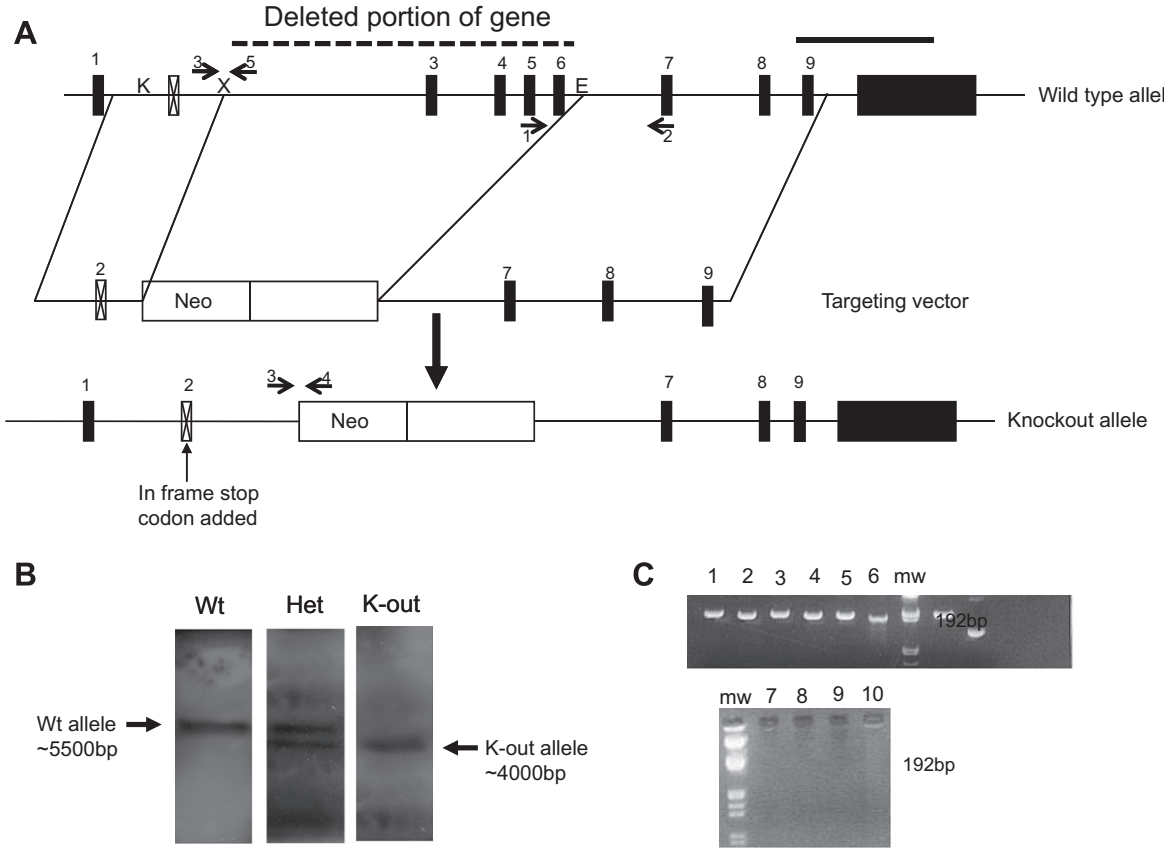


**Fig. 1.** Schematic of the targeting protocol for the construction of the knockout mouse. (A) The top line shows the normal wt gene intron/exon structure. Within this schematic is the indicated KpnI (K) site used in Southern blots and the XhoI (X) and EcoRI (E) used in synthesize of the knockout vector. The PCR oligos used in the genotyping of the mouse pups are indicated by the numbers 3 and 5. Oligos used in the screening of ES cells are shown by the arrows and numbers 1 and 2 under exons 5 and 7, respectively. The next line shows the targeting vector where exons 3–6 have been replaced by the neomycin portion of the cassette. In addition, to prevent further translation a stop codon was engineered into exon 2. The bottom line demonstrates the integration of the cassette into the allele and disruption of the syntaxin gene (knockout allele). In addition, more oligos used in the genotyping of the mouse pups are shown by the arrows and numbered 3 and 4. (B) Southern blot panel done with DNA isolated from wild type, heterozygous and KO mice. Shown is the decrease in size from ~5500 to 4000 bp of the targeted allele with the neomycin resistance gene incorporated in to the genome. (C) RT/PCR analysis of RNA extracted from wild type (lanes 1–3), heterozygous (lanes 4–6) or knockout mouse brains (lanes 7–10).