Construction of $Ca_v3.2$ N-glycosylation mutants

$Ca_v3.2$ N-glycosylation mutants (N192Q, N271Q, and N1466Q) were generated by mutating Asn192, Asn271, and Asn1466 residues of human $Ca_v3.2$ (GenBank accession no AF051946) into Gln residues using two-step PCR methods. The specific methods are as follows.

N192Q: The forward and reverse primers to amplify the upper fragments covering 215 to 585 (nucleotide number of $Ca_v3.2$) are 5'-ACCCGGCCTTGGCGGCCA-3' and 5'-GAGGCTCACCTGGTGTCCGTCCAACGAGTA-3'. The forward and reverse primers to amplify the lower fragments covering 565 to 1147 are 5'-GACGGACACCAGGTGAGCCTCTCGGCTATC-3' and 5'-TGATGTCGACCCAGCCTTCCAGCGT-3'. The upper and lower fragments were overlapped and extended by second-step PCR. N192Q pcDNA3 was constructed by ligating *Sfi*I (227)-*BspE*I (2560) PCR fragment and *BspE*I (2560, $Ca_v3.2$)-*Age*I (3844, $Ca_v3.2$) fragment into $Ca_v3.2$ pcDNA3 opened with *Sfi*I (227, $Ca_v3.2$) and *Age*I (3844, $Ca_v3.2$).

N271Q: The forward and reverse primers to amplify the upper fragments covering 215 to 823 (nucleotide no of $Ca_v3.2$) were 5'- GCGGCCACGGTCTTCTTCTG-3' and 5'-GAAGGTCAGCTGGTTGTTCCTGACAAAGGC-3'. The forward and reverse primers to amplify the lower fragments covering 806 to 1147 were 5'-AACAACCAGCTGACCTTCCTGCGGCCG-3' and 5'-TGATGTCGACCCAGCCTTCCAGCGT-3'. The upper and lower fragments were overlapped and extended by second-step PCR. N271Q pcDNA3 was constructed by ligating *Sfi*I (227)-*BspE*I (2560) PCR fragment and *BspE*I (2560, $Ca_v3.2$)-*Age*I (3844, $Ca_v3.2$) fragment into $Ca_v3.2$ pcDNA3 opened with *Sfi*I (227, $Ca_v3.2$) and *Age*I (3844, $Ca_v3.2$).

N1466Q: The forward and reverse primers to amplify the upper fragments covering 3840 to 4407 are 5'-AGAACCGGTTCCGCGTCT-3' and 5'-GGTGGAGATCTGCCTGGTGTCGGGGCCCT-3'. The forward and reverse primers to amplify the lower fragments covering 4390 to 6537 are 5'- ACCAGGCAGATCTCCACCAAGGCACAG-3' and 5'-AGAGCGGGCTCGGCCTCA-3'. The upper and lower fragments were overlapped and extended by second-step PCR. N1466Q pcDNA3 was constructed by ligating *Age*I (3844)-*Bsu36*I (6520) PCR fragment into $Ca_v3.2$ pcDNA3 opened with *Age*I (3844, $Ca_v3.2$) and *Bsu36*I (6520, $Ca_v3.2$).

EGFP-$Ca_v3.2$, EGFP-N192Q, EGFP-N271Q, and EGFP-N1466Q: For the green fluorescence experiments, we tagged EGFP (enhanced green fluorescence protein) cDNA to the amino termini of wild-type $Ca_v3.2$ and N-glycosylation mutants by ligating EGFP (*Cla*I-*Not*I) and *Not*I (263, $Ca_v3.2$)-*BspE*I (2560, $Ca_v3.2$) fragment into plasmids ($Ca_v3.2$, N192Q, N271Q, and N1466Q in pcDNA3) opened with *Cla*I (5'-polylinker) and *BspE*I (2560, $Ca_v3.2$).

Purification of Cav3.2 Channel- Stable HEK-293 cells expressing the $_{6HIS/FLAG}$Cav3.2 channel were harvested by washing with 10 mls of 37^{o}C PBS, followed by incubation with PBS containing 5 mM EDTA for several minutes. Cells were collected by centrifugation at 900 rpm for 10 minutes, washed with ice cold PBS, resuspended in buffer containing 20 mM HEPES, pH 7.4, 1 mM EGTA, and protease inhibitors, and flash frozen. Cells were lysed with a 21 g needle, and membranes collected by centrifugation at 35,000 RPM for 45 minutes in a 45 Ti rotor at 4^{o}C. Membranes were resuspended in 4 mls of buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% CHAPS and protease inhibitors using an 18 g needle, and allowed to rotate end over end for one hour at 4^{o}C; extracts were clarified by high speed centrifugation as noted above, diluted tenfold with 20 mM HEPES, pH 7.4, 150 mM NaCl and protease inhibitors, and incubated with FLAG M2 beads overnight at 4^{o}C. FLAG beads were collected in a five ml column and washed with five 1 ml volumes of buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% n-dodecyl beta-D-maltoside (DDM) and protease

inhibitors; the column was then brought to room temperature, and 100 μl of the previously mentioned buffer containing 0.5 mg/ml FLAG peptide (also at room temperature) was added to the column and collected. The column was then capped, and a second 100 μl was added and let incubate at room temperature for 15 minutes; after which time the volume was collected, and subsequent 100 μl volumes were added to elute the remainder of the $_{6HIS/FLAG}$Cav3.2 channel.

SDS-PAGE and Immunoblotting- Samples were prepared for separation by SDS-PAGE by incubation with sample buffer at room temperature for 1-2 hours; samples were not boiled. Protein separations were achieved either using a 4-15% precast gels (BioRad) or 12% mini-gels. Immunoblots from 4-15% precast gels were transferred overnight at 30 volts onto PVDF membrane at 4^{o}C; alternatively, immunoblots from 12% mini-gels were transferred for one hour at 100 volts onto nitrocellulose at 4^{o}C. Membranes were probed with monoclonal M2 FLAG antibody (Sigma) at a 1:1000 dilution. Immunoreactivity was detected with horseradish peroxidase coupled secondary antibody in conjunction with enhanced chemiluminescence. Polyacrylamide gels used for generation of samples for mass spectrometric analysis were prepared by 0.22 μM filtration of the separating and stacking solutions, as well as the running buffer; this step is important for removal of common protein contaminants, such as keratin, that can obscure the detection of sample proteins. Gels were stained in a 0.1% Coomassie Brilliant blue solution of 45:45:10 methanol:water:acetic acid, followed by destaining in a 45:45:10 methanol:water:acetic acid solution. Once protein bands were adequately visualized, gels were stored in a 10% acetic acid solution.

SUPPLEMENTARY DATA

Supplementary Table 1. Neuraminidase (NEU) does not have significant effects on passive membrane properties of acutely dissociated smaller DRG cells from Ob/Ob mice. Control cells (n=7) were treated with saline and experimental group (n=8) with 1.5 U/cc of NEU for 3 hours.

	Ob/Ob Control (n=7) mean ± SEM	Ob/Ob NEU (n=8) mean ± SEM
Soma diameter (μm)	32.4 ± 1.0	30.5 ± 1.0
Capacitance (pF)	36.4 ± 4.5	27.8 ± 5.2
Resting membrane potential (mV)	-51.5 ± 4.5	-50.5 ± 3.9
Input Resistance (MΩ)	160.7 ± 30.8	176.4 ± 26.2