**Expression of the Sialyltransferase, ST3Gal4, Impacts Cardiac Voltage-Gated Sodium Channel Activity, Refractory Period and Ventricular Conduction**

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**Supplementary Data**

**Materials and Methods**

**Generation of the ST3Gal4-/- Transgenic Strain**

ST3Gal4 is a β-Galactoside α2,3-Sialyltransferase (ST3Gal) that is 1 of 20 sialytransferases and 1 of 6 ST3Gals[[1](#_ENREF_1), [2](#_ENREF_2)]. The ST3Gal4 deficient strain is healthy, viable and displays no obvious phenotype[[3](#_ENREF_3)]. Among other functions, the enzyme is an important mediator in hemostasis, leukocyte arrest and selectin ligand formation[[3-10](#_ENREF_3)]. Creation and characterization of the ST3Gal4-/- mouse was performed by others previously[[3](#_ENREF_3)] and generously provided by Dr. Jamey Marth. In order to genotype the animals used in this study, genomic DNA was extracted from tail snips using the REDExtract-N-Amp tissue PCR kit (Sigma XNATR) following the manufactures directions. Genotypes were determined using standard PCR methods with the following three primers: GAC GCC ATC CAC CTA TGA G, GGC TGC TCC CAT TCC ACT and GGC TCT TTG TGG GAC CAT CAG.

**Cardiac Myocyte Isolation**

Adult (12-14 weeks old) male mice homozygous for the normal ST3Gal4 gene (WT) and mice homozygous for the ST3Gal4 null-transgene (ST3Gal4-/-) were anaesthetized using isoflurane (5%) then euthanized by cervical dislocation. Hearts were rapidly excised from the body and cannulated through the aorta. Using a modified Langendorff apparatus, each heart was perfused and digested with the following solutions for the indicated times at 36±1° C: 5 minutes with Hank’s Balanced Salt Solution (HBSS) (Cellgro 21-022) supplemented with 10 millimoles per liter (mM) of N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) from a 1 molar stock (Cellgro 25-060), 1 mM MgCl2 from a 1 molar stock (Ambion AM9530G) and 1 mM CaCl2, pH 7.4; 10 minutes with HBSS supplemented with 10 mM HEPES and 1 mM MgCl2, pH 7.4; 20 minutes with HBSS supplemented with 10 mM HEPES, 1 mM MgCl2, 0.1% bovine serum albumin (BSA), 0.025 mM CaCl2, 20 mM taurineand type 2 collagenase (Worthington CLS-2), pH 7.4; and 5 minutes with a high K+ solution containing 100 mM K+ glutamate, 10 mM K+ aspartate, 25 mM KCl, 10 mM KH2PO4, 2 mM MgSO4, 20 mM taurine, 5 mM creatine base, 0.5 mM ethylene glycol tetra acetic acid (EGTA), 5 mM HEPES, 0.1% BSA, and 20 mM glucose, pH 7.2 as described by others[[11](#_ENREF_11)]. At the completion of the perfusion scheme described above, the bottom 2 millimeters of the left ventricular apex (LVA) was cut and dispersed in the high K+ solution for approximately 10 minutes to obtain individual myocytes. The suspension was filtered and cells were allowed to sediment by gravity. Myocytes were maintained at room temperature in the high K­+ solution until use 1-4 hours following isolation.

#### ****Electrophysiology****

#### **0.2-0.5 ml of the myocyte suspension was added to a recording chamber (Warner RC-26) mounted on a Nikon TE200 microscope. Cells were allowed to settle for 5 minutes on laminin (BD** 354232**) coated cover slips. Once the cells adhered, approximately 5 volumes of extracellular recording solution (ECS) were perfused through the chamber. Patch pipettes were fabricated using a horizontal pipette puller (Sutter P97), filled with an intracellular recording solution (ICS) and mounted on a headstage (**Axon Instruments CV203BU). Immediately **prior to seal formation, the solid/liquid junction offset was zeroed manually using the pipette offset on an Axopatch 200B amplifier with β set to 0.1. Following seal formation of at least one gigaohm, the whole cell-configuration of the patch clamp method was obtained[**[**12**](#_ENREF_12)**]. Analog signals recorded by the amplifier were low-pass filtered at 5 kilohertz (kHz) then digitized at a rate of 50 kHz using a** Digidata 1440A analog/digital interface connected to a personal computer. **For recording INa in voltage-clamp mode, cell size was determined by integrating the capacitance of the cell following a 25 millisecond (ms) 10 millivolt (mV) step in voltage from a holding potential of -100 mV. After measuring cell size, resistance and capacitance were minimized manually using the amplifier and oscilloscope found in the axon Clampex 10.2 software. Patch Pipettes (Warner G150F-3) had mean resistances of approximately 1.4 ± 0.1 (n=24) megaohms (MΩ) when filled with an ICS consisting of the following in mM: CsCl 25, CsF 110,** EGTA 10, HEPES 10, magnesium adenosine triphosphate (MgATP) 5, glucose 5 and NaCl 5; pH 7.2 CsOH**. Series resistance was compensated as necessary but at a value of at least 80%. The maximum mean voltage errors from uncompensated series resistance were approximately -6.5 ± 0.6 and -6.0 ±** **0.7 mV when recording from control (n=11) and ST3Gal4-/- (n=13) LVA myocytes respectively and were ignored**. The ECS used to record INa **contained in mM: CsCl 130, CaCl2 0.5, glucose 10, HEPES 10, MgCl2 1, CdCl2 0.3 and NaCl 11; pH 7.35 CsOH. An ICS/ECS junction potential of -0.5 mV was calculated using the junction potential calculator in Clampex and was also ignored. For recording APs in current-clamp mode, patch pipettes (Chase 502) had mean resistances of approximately 2.5 ± 0.03 (n=26) MΩ when filled with an ICS that consisted of in mM: K+ aspartate 110, KCl 20, NaCl 8, MgATP 5, Hepes 10 and glucose 5; pH 7.2 NaOH. The AP ECS consisted of in mM: NaCl 130, KCl 5.4, Na2HPO4 0.33, CaCl2 1, MgCl2 1, HEPES 10 and glucose 10; pH7.4 NaOH. The AP ICS/ECS junction potential was calculated to be approximately -21 mV and was added off-line.** Voltage and current clamp protocols were written and executed using Clampex 10.2 and the ICS was allowed to dialyze for 5 minutes following cell rupture.

#### ****Voltage Clamp Protocols****

#### ***Current density and conductance-voltage relationships***

#### **Cells were held at -100 mV then depolarized by a series of voltage steps beginning at -85 mV and ending at 30 mV for 120 ms in 5 mV increments. Each voltage step was separated by 1.5 s and the current was leak subtracted using the P/5 method from the holding potential and in the opposite polarity as the current waveform. The current elicited from each test pulse was divided by the cell capacitance to report current density as pA/pF or the current was divided by the driving force using the following equation to determine conductance (*G*):**

#### ***G = I / (Vpot -Vrev)***

#### **where I is the current at each test potential, Vpot is the test potential and Vrev is the Nernst equation-derived reversal potential at 22° C (20.05 mV). Conductance-voltage relationships were fit with a Boltzmann equation of the form:**

#### ***Fraction of Maximum Conductance (G/Gmax) = [1+ (e –((Vpot -Va)/Ka))] -1***

#### **where Gmax is the maximum theoretical conductance, Va is the voltage of half-activation and Ka is the slope of the Boltzmann fit for activation as previously described[**[**13**](#_ENREF_13)**].** The **Gmax,Va, and Ka** for each cell were determined by fitting the single cell data to a single Boltzmann distribution, and were used to determine the mean parameter values among cells as listed in Table 1. Also, the Gmax was used to normalize cellular data**.**

#### ***Voltage-dependent inactivation***

#### **Cells were held at -100 mV and subjected to conditioning voltage pulses ranging from -140 mV to -65 mV in 5 mV increments for 500 ms. Following the conditioning pulses, the cells were depolarized to -20 mV for 10 ms. Currents were normalized to the maximum current and the data was fit with the following Boltzmann equation:**

#### ***Fraction of Maximum Current (I/Imax) = [1+ (e –((Vpot-Vi)/Ki))] -1***

#### **where I/Imax is the ratio of each current to the maximum current, Vpot is the potential of each conditioning pulse, Vi is the voltage of half-inactivation and Ki is the slope of the Boltzmann fit for inactivation as previously described[**[**13**](#_ENREF_13)**].** The Imax, Vi, and Ki for each cell were determined by fitting the single cell data to a single Boltzmann distribution, and were used to determine the mean parameter values among cells as listed in Table 1. Also, the Imax was used to normalize cellular data**.**

#### ***Time constants of fast inactivation (inact)***

#### **The attenuating portion of the current traces recorded during the current density/conductance-voltage measurements were fit with a single exponential function with the form:**

#### ***f(t) = Ae-t/ + C***

#### **where A is the amplitude in picoamps (pA), t is the time in ms, tau is the time in ms it takes for the current to inactivate one exponential unit and C is a constant.**

#### ***Time constants for recovery from fast inactivation (rec)***

#### **Cells were held at a voltage of -100 mV then depolarized to -20 mV for 50 ms. Following the initial pulse to -20 mV, the membrane potential was changed to the recovery potential (-90, -100, or -140 mV) for a duration of initially 10 ms then at a delta of 10 ms followed by a second pulse to -20 mV for 50 ms (see pulse protocol, Fig. 2A). Between each pulse the membrane potential was returned to -100 mV for 4 seconds. The current elicited from the second pulse to -20 mV was normalized to the current from the initial pulse to -20 mV for each sweep. The data were fit with a single term exponential with the form:**

#### ***I2/I1 = A(1-e-t/) + C***

#### **where I2/I1 is the ratio of the current elicited from the second -20 mV pulse to the current elicited from the initial -20 mV pulse, A is the asymptote of the curve, t is the time in ms,  is the time it takes the curve to rise one exponential unit and C is a constant. In some cases, curve fitting was marginally improved when a bi-exponential function was used. However, in many cases such a fit provided nonsensical results; therefore, a single exponential was used in these studies.**

#### ****Current Clamp Protocols****

#### F**ollowing cell rupture, the membrane potential was measured. Cells were injected with positive current for a duration of 4 ms with an initial current step of 100 pA followed by subsequent current steps with a delta of 50 pA. Each sweep was separated by 2 s. Threshold was determined by the first overshooting AP that preceded at least two more similar action potentials. The threshold current was calculated and a current amount of 125% of threshold was used in a two pulse protocol to estimate the cellular refractory period. Cells were stimulated with a current pulse of 125% of threshold for 4 ms followed by a second pulse 5 ms later and then each additional pulse came with a delta of 8 ms. The pulse number that elicited an overshooting AP that preceded at least 2 more similar APs was used to estimate the refractory period.**

**Membrane Protein Enrichment**

Adult male WT and ST3Gal4-/- mice between the ages of 12 and 14 weeks were anaesthetized using isoflurane (5%) then euthanized by cervical dislocation. The lower 2/3 of the heart consisting of both the right and left ventricles was separated and snap frozen in liquid nitrogen. The following protocol, which was previously described by others[[14](#_ENREF_14)], and solutions were all performed and maintained at 4° C. Two hearts per group were homogenized using glass douncers and sonication in 3 ml of extraction solution consisting of 20 mM trishydroxymethylaminomethane (Tris)-base, 5 mM Ethylenediaminetetraacetic acid(EDTA), 0.8 mM benzamidine, 1 mM iodacetamide, 1.1 μM leupeptin, 0.7 μM pepstatin, 76.8 nM aprotinin and 0.5 mM phenylmethanesulfonylfluoride; pH 7.5. The suspension was centrifuged at 1000xG for ten minutes and the supernatant was aspirated. The pellet was re-homogenized in 2.5 ml of extraction solution and centrifuged at 1000xG for ten minutes. Both supernatants were pooled and centrifuged at 40,000xG for ten minutes. The resultant pellet was suspended in extraction solution supplemented with 0.6 molar KCl for ten minutes to dissociate the cytoskeleton. The suspension was centrifuged at 40,000xG for ten minutes and the pellet was suspended in extraction solution; this was repeated twice to ensure the removal of KCl. The final pellet was solubilized in extraction solution supplemented with 2% Triton X-100 for 1 hour with periodic sonication. The suspension was centrifuged at 10,000xG for 10 minutes and the supernatant was aspirated. Protein concentrations were determined using a BCA assay (Pierce 23237) and the protein solutions were stored at -80° C until use.

**Western Blotting and Immunodetection**

Western blotting for densitometry analysis was performed using protocols adapted from Invitrogen’s Novex system. Membrane enriched protein was mixed 1:1 in a sample buffer that contained 106 mM Tris HCl, 141 mM Tris-base, 2% lithium dodecyl sulfate (LDS), 10% Glycerol, 0.51 mM EDTA, 0.075% Coomassie Blue R250 and 0.025% phenol red, pH 8.5. Nav are large proteins with 24 hydrophobic-rich transmembrane segments that can aggregate when exposed to high temperatures; therefore, protein was denatured at 37°C and 20µg of protein was loaded into the wells of a 4% Bis-Tris stacking gel. Protein was separated electrophoretically through an 8% Bis-Tris resolving gel using a 3-N-morpholinopropanesulfonic acid (MOPS)-SDS buffer, pH 7.7 supplemented with 5mM sodium bisulfite at 200 V. Following electrophoresis, protein was transferred to nitrocellulose membranes (GE Healthcare RPN3032D) using a tank transfer system (Bio-Rad 170-3930) at 4° C for 2 hour at 50 V. The transfer solution consisted of Bis-Tris 25mM, bicine 25mM, EDTA 1mM, sodium bisulfite 5 mM, 20% methanol and 0.0375% SDS. Blots were rinsed briefly then dried at room temperature and either used immediately or stored at 4°C.

Following western blotting, Nav visualization was performed using Millipore’s SNAP ID system following the manufacturer’s recommendations. Membranes were blocked with 0.5% non-fat dairy milk. A pan-Nav antibody, which was described previously[[13](#_ENREF_13), [15](#_ENREF_15)], was used as the primary antibody and a goat anti-rabbit horseradish-peroxidase (HRP) conjugated antibody (Millipore AP307P) was used as the secondary. Nav protein levels were normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression using a mouse monoclonal antibody raised against GAPDH (Santa Cruz SC32233) as the primary and a HRP conjugated goat anti-mouse antibody as the secondary (Millipore AP308P). Blots were incubated with a chemiluminescent substrate following the manufacturer's recommendations (Pierce 34080). **A Tris buffered solution (TBST) consisting of 50 mM Tris, 0.154 mM NaCl and 0.1% tween, pH 7.5 was used as the diluent and wash solution for the immunodetection protocol. Following the chemiluminescent reaction, bands were visualized with autoradiography film (Midsci BX57), scanned and densitometry was performed using the National Institute of Health's ImageJ software application.**

**Gel-Shift Analysis**

Sarcolemmal protein was enriched and isolated as described above. Approximately 25 µg of protein was diluted to 10 µl with 20 mM Tris-base (pH 7.5) along with 0.5% LDS, 2X HALT protease inhibitor cocktail (Pierce 87785) and either 1 U/ml of sialidase A (Prozyme GK80040) or a equal volume of 20 mM Tris-base/25 mM NaCl. The solution was incubated for 2 hours at 37°C. Following incubation, the solution was mixed 1:1 with a Lammeli-style sample buffer consisting of 62.5 mM Tris-base pH 6.8, 25% glycerol, 2% LDS, 0.01% bromomethyl blue and 5% betamercaptoethanol and denatured at 37°C. Denatured protein was added to a 4.5% Tris/glycine gel and separated electrophoretically as previously described[[13](#_ENREF_13)]. Protein was then blotted onto nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad 170-3940) at 15 V for 26 minutes in a transfer buffer consisting of Tris-base 48 mM, glycine 39 mM, 20% methanol and 0.0375% SDS. Following transfer, blots were rinsed briefly and allowed to air dry at room temperature. To determine differences in apparent molecular weight (MW), scanned film was analyzed using ImageJ. The log of each MW marker was plotted versus its distance in arbitrary units traveled into the gel. A linear regression was then performed and used to calculate the MW of sialidase-treated and untreated protein.

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