The sialic acid component of the beta\textsubscript{1} subunit modulates voltage-gated sodium channel function

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Running Title: \(\beta_1\) sialic acids modulate \(\text{Na}^+\) channel gating

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Summary

Voltage-gated sodium channels (Nav) are responsible for initiation and propagation of nerve, skeletal muscle, and cardiac action potentials. Nav are composed of a pore-forming α subunit and often one to several modulating β subunits. Previous work showed that terminal sialic acid residues attached to α subunits affect channel gating. Here we show that the fully sialylated β1 subunit induces a uniform, hyperpolarizing shift in steady state and kinetic gating of the cardiac and two neuronal α subunit isoforms. Under conditions of reduced sialylation, the β1-induced gating effect was eliminated. Consistent with this, mutation of β1 N-glycosylation sites abolished all effects of β1 on channel gating. Data also suggest an interaction between the cis effect of α sialic acids and the trans effect of β1 sialic acids on channel gating. Thus, β1 sialic acids had no effect on the gating of the heavily glycosylated skeletal muscle α subunit. However, when glycosylation of the skeletal muscle α subunit was reduced through chimeragenesis such that α sialic acids did not impact gating, β1 sialic acids caused a significant hyperpolarizing shift in channel gating. Together, the data indicate that β1 N-linked sialic acids can modulate Nav gating through an apparent saturating electrostatic mechanism. A model is proposed in which a spectrum of differentially functionally sialylated Nav can directly modulate channel gating, thereby impacting cardiac, skeletal muscle, and neuronal excitability.
Introduction

The importance of voltage-gated sodium channels (Nav) in action potential initiation and propagation is well established. The orchestrated activation and inactivation gating of sodium channels is vital to normal neuronal signaling, skeletal muscle contraction, and regular heart rhythms. Even small syncopations from this normal gating rhythm may alter cellular excitability and whole animal physiology significantly, leading to such disorders as long QT syndrome and epilepsy(1-7).

Nav are complex transmembrane glycoproteins that are comprised of a large α subunit that forms the pore through which ions can pass (8-10). Ten α subunit isoforms have been cloned from excitable tissues, with orthologues present in a wide range of species (11). While the α subunit is sufficient to form functional channels when expressed alone, it is often associated with a β1 subunit that modulates sodium channel activity - the exact manner by which β1 alters channel function is still under investigation(12-15).

Isoforms of the α subunit undergo extensive glycosylation. Estimates indicate that 15-40% of the total Nav molecular weight is carbohydrate (16-18). Approximately 40-45% of the added carbohydrate residues are sialic acid moieties, resulting in the addition of an estimated 100 sialic acid residues per α subunit molecule (16;17). Recent studies have demonstrated that sialic acid is an important modulator of Nav α subunit gating. For example, enzymatically removing sialic acid or the entire glycosylation structure, from purified, transfected or endogenous Nav, shifts channel gating in the depolarized direction (19-22). Additionally, Nav gate at more depolarized potentials when expressed in a mutant CHO cell line that is deficient in its ability to
sialylate proteins(20;23). Interestingly, the effects of sialylation on Na, α subunit gating appear to show some degree of isoform specificity, with gating of one human α subunit isoform, Na,1.4, dependent on α subunit sialic acids, while gating of a second α isoform, Na,1.5, was independent of sialic acid as expressed in the same experimental system. The functional sialic acids attached to Na,1.4 were localized to the DIS5-S6 extracellular loop (23). Together, these studies demonstrate cis-regulation of Na, gating by α subunit sialic acids.

The β, subunit is predicted to have a single transmembrane spanning domain with an extracellular N-terminal end that contains four potential N-glycosylation sites and a single immunoglobulin-like fold (24). The small internal portion of the protein appears to be responsible for efficient association of β, with the Na, α subunit via ankyrin G (14;25). The external domain of β, has been shown to be critical for correct modulation of sodium channel gating mediated by different Na, isoforms (26-29). In general, β, causes a hyperpolarizing shift in the voltage-dependence of inactivation. In several studies, activation gating was also shifted in the hyperpolarized direction by β, (3;13;25;26;28-31). Conversely, β, was shown to cause a depolarizing shift in voltage-dependence of inactivation for Na, expressed in HEK cell lines (12;32;33). This may be caused by competition between endogenous and heterologous β, in this system. Regardless, these differences in the impact of β, on channel gating strongly suggest that the cellular milieu has a dramatic effect on the ability of the β, subunit to modulate Na, channel gating.

At least three of the four N-linked glycosylation sites present in the N-terminus of β, are thought to be glycosylated in the mature protein (24;34). Could sialic acids
attached to $\beta_1$ alter $\alpha$ subunit gating through a novel trans-regulatory mechanism? While the $\beta_1$ gene product expressed in heart, skeletal muscle, and brain is identical, the level of posttranslational modification appears to vary among cell types(35). In addition, expression of $\beta_1$ is tightly regulated over the course of development. Depending on the tissue, expression is first observed in the first four days after birth and increases to maximal, maintained, levels after 2-4 weeks (35-39). Thus, expression of $\beta_1$, an important modulator of $\text{Na}_v$ gating, is developmentally regulated. Also, $\beta_1$ is heavily and differentially glycosylated. If $\beta_1$ glycosylation alters $\alpha$ subunit gating, then channel gating might be modulated differently by various levels of $\beta_1$ sialic acid among excitable tissues and from one developmental stage to another.

Given that $\alpha$ subunit sialic acids impact channel gating, we wished to test the hypothesis that sialic acids attached to the $\beta_1$ subunit are involved in modulating channel function. To this end, we expressed four different $\text{Na}_v$ $\alpha$ subunit isoforms in the presence or absence of $\beta_1$ in a cell line that essentially fully sialylates proteins, and in a mutant daughter cell line that is unable to sialylate proteins. Our data indicate a novel mechanism by which $\beta_1$ can modulate $\text{Na}_v$ gating in a saturating, sialic acid-dependent manner, and that $\beta_1$ sialic acids account for all effects of $\beta_1$ on sodium channel gating. In addition, the data indicate for the first time, that within a single membrane, transmembrane protein function is modulated by the sugars attached to a second membrane protein.
Experimental Procedures

Vector construction and mutagenesis

The cDNA containing the rNa_v1.2 open reading frame (ORF) inserted into pRC-CMV (Invitrogen) was a kind gift of Dr. Alan Goldin. The hNa_v1.7 cDNA ORF was inserted into pcDNA3.1. Expression vectors containing hNa_v1.4 and hNa_v1.5 were as previously described(23). hβ1 was subcloned into the bi-cistronic vector, pIRES2-EGFP (Clontech), to ensure expression of β1 through visual inspection. The quadruple hβ1 mutant (hβ1-∆N) was created using the GeneEditor (Promega) site directed mutagenesis kit with hβ1 subcloned into pBluescript vector (Stratagene) as template. Each asparagine residue initiating an external N-linked consensus sequence, NX(S/T), was mutated to a serine residue through sequential mutagenesis. Constructs were sequenced to confirm successful mutagenesis and sequence fidelity. hβ1-∆N was then subcloned into pIRES2-EGFP for co-expression experiments. The hβ1 and hβ1-∆N ORFs minus their stop codons were amplified using PCR with the following oligonucleotides 5'-TCCGGCCACCTGGACGCCCG-3' and 5'-GCGCAGCACGCGCCGCGCAG-3'. PCR products were subcloned into pcDNA3.1/V5-His TOPO TA expression vector (Invitrogen). Both ORFs were subsequently subcloned into pEGFP-N1 (Clontech) to generate C-terminal, GFP tagged hβ1 and hβ1-∆N constructs.

Mammalian cell culture and transfection

Pro5 and Lec2 cells were grown as described previously(23). For transfections, cells were seeded onto 35 mm dishes 24 hours prior to exposure to 1 ml of Opti-mem medium containing 8 µl lipofectamine (Invitrogen) and 2.0-2.5 µg of DNA (85-92% α
sodium channel vector and 8-15% pGreen Lantern Fluorescent Protein (GFP, Gibco) or the β₁ subunit vector) and then incubated for 24 hours. The transfection mix was replaced with the appropriate growth medium and cells were incubated for a further 48 hours before use.

Electrophysiology and data analysis

\( I_{Na} \) were recorded at room temperature (~22°C) using established whole cell patch clamp techniques, pulse protocols, data analyses and solutions as previously described(20;23). For the Ca\(^{2+}\) perfusion studies, seals were formed in the bath solution containing 2.0 mM Ca\(^{2+}\). Cells were first perfused with the 2.0 mM Ca\(^{2+}\) bath solution, and then with the 0.2 mM Ca\(^{2+}\) bath solution to determine directly the shift in \( V_a \) with a 10-fold decrease in external Ca\(^{2+}\) concentration. Although series resistance was compensated 95-98% for all data, the smaller current produced using the low sodium solutions further minimized any remaining series resistance error, resulting in < 1mV error. All data shown are recorded at least 5 minutes after attaining whole cell configuration to assure complete dialysis of the intracellular solution. All solutions were filtered using Gelman 0.2 µm filters immediately prior to use.

Pulse protocols

Conductance-voltage (G-V) relationship

The cell was held at -100 mV or -120 mV. Voltages were stepped to various depolarized potentials (ranging from −100 to +70 mV in 10 mV increments) for 10 ms, and then returned to the holding potential. Consecutive pulses were stepped every 1.5 seconds and the data were leak subtracted using the P/4 method, stepping negatively
from the holding potential. At each test potential, steady-state whole cell conductance was determined by measuring the peak current at that potential and dividing by the driving force (i.e., difference between the membrane potential and the observed reversal potential). The maximum conductance generated by each cell was used to normalize the data for each cell to its maximum conductance by fitting the data to a single Boltzmann distribution (equation 1, solving for maximum conductance). The average $V_a +/-$ SEM values listed in Table 1 were determined from these single Boltzmann distributions. The normalized data were then averaged with those from other cells, and the resultant average conductance-voltage curve was fit via least squares using the following Boltzmann relation:

$$\text{Fraction of maximal conductance} = \left[1 + \exp\left(-\frac{(V-V_a)}{K_a}\right)\right]^{-1},$$
equation (1)

where $V$ is the membrane potential, $V_a$ is the voltage of half activation, and $K_a$ is the slope factor.

**Measurement of inactivation time constants**

Inactivation time constants were determined by fitting the current traces used to measure G-V relationships. Attenuating currents from 90 to 10% of the peak values were fit to a single exponential function to determine the time course of fast inactivation.
**Steady state inactivation curves ($h_{inf}$)**

Voltage dependence of steady state inactivation was determined by first prepulsing the membrane for 500 ms from the holding potential, then stepping to +60 mV for 5 ms, and then returning to the holding potential. The prepulse voltages ranged from -130 mV to +10 mV in 10 mV increments. The currents from each cell were normalized to the maximum current measured by fitting each single cell data to a single Boltzmann distribution (equation 2, solving for maximum current), from which the mean $V_i$ +/- SEM values listed in Table 1 were determined. The normalized data for many cells were then averaged and fit to equation (2), from which the average $h_{inf}$ curves describing steady state inactivation for the channel population were calculated.

\[
\text{Fraction of maximum current} = \left[1 + \exp\left(\frac{(V - V_i)}{K_i}\right)\right]^{-1} \quad \text{equation (2)}
\]

**Recovery from inactivation**

Cells were stepped to +60 mV for 10 ms from the holding potential and then returned to the recovery potential for varying duration ranging from 1 to 20 ms in 1 ms increments. Following this recovery pulse, the potential was again stepped to +60 mV for 10 ms. The peak current measured during the two +60 mV depolarizations were compared, and the fractional peak current remaining during the second depolarization was plotted as a function of the recovery pulse duration. This represents the fraction of channels that recovered from inactivation during the recovery interval. Time constants of recovery were determined by fitting the data to single exponential functions.
Results

$\beta_1$ modulates gating of three of four \( \text{Nav} \) \( \alpha \) subunits

The external domain of $\beta_1$ is critical for correct modulation of sodium current mediated by different \( \text{Nav} \) channel $\alpha$ subunit isoforms (26-29). Reports indicate effects of $\beta_1$ ranging from increased fast inactivation rate to hyperpolarizing and even small depolarizing shifts in the voltage dependence of steady state channel gating (3;12;13;31;32;40). These varied effects apparently depend on the $\alpha$ subunit and the cellular expression system used to study $\beta_1$ function. In order to minimize the variation observed among cellular expression systems, we co-expressed $\beta_1$ with one of four different $\alpha$ subunits in Chinese hamster ovary (CHO) cells to compare directly $\alpha$ subunit isoform-specific effects of $\beta_1$ on sodium current. Figure 1 shows the average \( \text{Nav} \) conductance voltage relationships (G-V) recorded from cells expressing one of four \( \text{Nav} \) $\alpha$ subunits: the adult skeletal muscle isoform (\( \text{Nav} \)1.4), the cardiac isoform (\( \text{Nav} \)1.5), a peripheral nerve isoform (\( \text{Nav} \)1.7), and a brain isoform (\( \text{Nav} \)1.2), in the presence or absence of $\beta_1$. Note that \( \text{Nav} \)1.4 activation is unaffected by $\beta_1$, whereas the G-V curves for \( \text{Nav} \)1.2, \( \text{Nav} \)1.5, and \( \text{Nav} \)1.7 are shifted in the hyperpolarized direction by 8-9 mV when $\beta_1$ is present. Figure 2 shows that the voltage dependence of steady state channel availability ($h_{\text{inf}}$) is similarly affected by $\beta_1$, with the voltages of half inactivation ($V_i$) for \( \text{Nav} \)1.2, \( \text{Nav} \)1.5, and \( \text{Nav} \)1.7 shifted 6-9 mV in the hyperpolarized direction in the presence of $\beta_1$, while $V_i$ for \( \text{Nav} \)1.4 is unaffected by $\beta_1$.

To describe more fully the effects of $\beta_1$ on $\alpha$ subunit gating, we examined the rates of fast inactivation and recovery from fast inactivation. As shown in Figures 3 and
4, the effects of $\beta_1$ on gating kinetics are similar to its effects on steady state parameters. Thus, $\beta_1$ had no effect on the gating of $\text{Na}_v1.4$, but caused a nearly uniform hyperpolarization of all measured gating parameters for $\text{Na}_v1.2$, $\text{Na}_v1.5$, and $\text{Na}_v1.7$. Mean values $\pm$ SEM as well as statistical analyses for all parameters and conditions measured in this study are shown in Table 1.

Modulation of $\text{Na}_v$ gating by $\beta_1$ requires $\beta_1$ sialic acids

Sodium channel $\alpha$ subunits undergo extensive glycosylation, often capped with sialic acid residues (SA) (16-18). Previous work showed that $\text{Na}_v$ $\alpha$ subunit sialylation is an important process by which sodium channel gating is modulated in an isoform- and developmental-dependent manner(21-23). The $\beta_1$ subunit is also heavily glycosylated, with four potential $N$-glycosylation motifs within its extracellular N-terminal domain(24).

To determine if the observed isoform-specific shifts in $\text{Na}_v$ gating produced by $\beta_1$ are caused by $\beta_1$ sialic acids, we co-expressed each of the $\alpha$ subunits with $\beta_1$ in two well-characterized CHO cell lines that produce proteins with differing amounts of attached sialic acids(41-43). The Pro5 cell line allows normal CHO cell protein sialylation while Lec2 cells, deficient in the CMP-sialic acid transporter, produce proteins that are essentially non-sialylated. Figures 1 and 2 show the G-V and $h_{\text{inf}}$ curves for each $\alpha$ subunit $\pm \beta_1 \pm \text{SA}$. When expressed in Lec2 cells, the less sialylated $\text{Na}_v1.4$ gates at more depolarized potentials than the fully sialylated $\text{Na}_v1.4$ expressed in Pro5 cells irrespective of the presence or absence of $\beta_1$. These data indicate that gating of $\text{Na}_v1.4$ is dependent on $\alpha$ subunit sialic acids but not on $\beta_1$ sialic acids. Gating of $\text{Na}_v1.2$, $\text{Na}_v1.5$ and $\text{Na}_v1.7$ are not significantly affected by sialylation in the absence of $\beta_1$,
suggesting that α subunit sialic acids do not alter gating of these isoforms significantly. There is a small, consistent, but generally insignificant, SA-dependent shift in Naᵥ1.2 gating, while Naᵥ1.5 and Naᵥ1.7 gating show no dependence on sialic acids. In contrast, β₁ expression induces a 6-10 mV hyperpolarizing shift in G-V and h_inf curves for Naᵥ1.2, Naᵥ1.5 and Naᵥ1.7 when expressed in the fully sialylating Pro5 cell line. This β₁ induced shift in gating is not present when β₁ sialylation is reduced. When β₁ is co-expressed with α in Lec2 cells, the observed G-V and h_inf relationships for each isoform are nearly identical to those measured for the α subunit alone. These data indicate that β₁ sialic acids can alter directly Naᵥ1.2, Naᵥ1.5, and Naᵥ1.7 gating. Figures 3 and 4 confirm that the β₁ induced shifts in all measured gating parameters for these three α subunits are caused by β₁ sialic acids.

**β₁ sialic acids can modulate the gating of an Naᵥ1.4 mutant with reduced glycosylation**

Here we observed that Naᵥ1.4 gating is not affected by the presence of β₁. Naᵥ1.4 is predicted to have more N-glycosylation than Naᵥ1.2 or Naᵥ1.7, and we showed previously that Naᵥ1.4 is more heavily glycosylated than Naᵥ1.5 in this system(23), consistent with work performed in other laboratories(44). The majority of Naᵥ1.4 N-glycosylation sites are present in the DIS5-S6 extracellular linker, and previous work demonstrated that the sialic acid effects on Naᵥ1.4 gating are localized to this domain(23). Given that β₁ can modulate gating of channels with putatively less glycosylation than Naᵥ1.4 (e.g., Naᵥ1.2, Naᵥ1.5, and Naᵥ1.7), we examined the effects of β₁ on the Naᵥ1.4 DIS5-S6 loop chimera, hSkM1P1, that is less glycosylated than wild-
type Na\textsubscript{v}1.4(23;27). As shown in Figure 5, gating of hSkM1P1 alone is not dependent on sialic acid. However, co-expression of β\textsubscript{1} imposes a sialic acid dependent hyperpolarizing shift in hSkM1P1 gating similar in magnitude and direction to the effects of β\textsubscript{1} sialic acids on gating of Na\textsubscript{v}1.2, Na\textsubscript{v}1.5, and Na\textsubscript{v}1.7. This, together with the lack of effect of β\textsubscript{1} on Na\textsubscript{v}1.4 gating observed here would suggest there is a potential saturating limit to the amount (location) of β\textsubscript{1} sialic acids that can affect α subunit channel gating. More precisely, apparently combinations of the sialic acids associated with α subunit IS5-S6 and with β\textsubscript{1} determine the overall impact of these sugars on channel gating, and this effect is saturating.

**β\textsubscript{1} sialic acids alter Na\textsubscript{v} gating by contributing to an apparent external surface potential**

To determine if β\textsubscript{1} sialic acids are contributing to a negative surface potential, we examined the effect of different external Ca\textsuperscript{2+} concentrations on channel gating. If β\textsubscript{1} sialic acids contribute to a negative surface potential, then α subunits that are sensitive to β\textsubscript{1} sialic acids would be predicted to be more sensitive to external Ca\textsuperscript{2+} concentration when co-expressed with β\textsubscript{1} than when expressed alone. As shown in Figure 6, the external Ca\textsuperscript{2+} dependent shift in sodium channel V\textsubscript{a} is largest when hSkM1P1 is co-expressed with the fully sialylated β\textsubscript{1} subunit. When hSkM1P1 is expressed alone (± SA) or when co-expressed with β\textsubscript{1} under conditions of reduced sialylation, the Ca\textsuperscript{2+} dependent shift in V\textsubscript{a} is significantly smaller and nearly identical for these three conditions.
\[ \beta_1 \text{N-linked sialic acids are sufficient to produce the full effect of } \beta_1 \text{ on Na}_v \text{ gating} \]

The \( \beta_1 \) subunit is predicted to have four \( N \)-linked glycosylation motifs and biochemical analysis has indicated the presence of three or four carbohydrate chains attached to the mature protein (24;34). To question whether \( \beta_1 \) \( N \)-linked sialylation is fully responsible for the effect of \( \beta_1 \) on channel gating, we deleted all four predicted \( N \)-linked glycosylation motifs through site-directed mutagenesis. The mutant \( \beta_1 \) subunit (\( \beta_1-\Delta N \)) does not modulate \( V_a \) for any of the tested \( \alpha \) subunit isoforms when co-expressed in fully sialylating Pro5 cells (Fig 7). Surface expression of \( \beta_1 \) and \( \beta_1-\Delta N \) were confirmed by tagging each with GFP; cell surface distribution of \( \beta_1 \) and \( \beta_1-\Delta N \) were indistinguishable (data not shown). Thus, all \( \beta_1 \) sialic acid dependent effects on \( \alpha \) subunit gating can be assigned to \( \beta_1 \) \( N \)-linked sialic acids.
Discussion

$\beta_1$ N-linked sialic acids fully account for effects of $\beta_1$ on Na$_\alpha$ gating

Here we sought to determine more directly the role of the $\beta_1$ subunit in voltage-gated sodium channel gating. We compared, in a single cellular system, the effects of $\beta_1$ on the gating of four different Na$_\alpha$ subunit isoforms, the adult skeletal muscle isoform (Na$_\alpha$1.4), the cardiac isoform (Na$_\alpha$1.5), a peripheral (Na$_\alpha$1.7) and central (Na$_\alpha$1.2) nervous system isoform. First, the effects of $\beta_1$ co-expression on the gating of the four $\alpha$ subunits were observed in a single CHO cell line, Pro5. As shown in Figures 1-4, $\beta_1$ induced a hyperpolarizing shift in all measured gating parameters for three of the four tested $\alpha$ subunits. Only Na$_\alpha$1.4 gating was unaffected by $\beta_1$. The data agree in general with previously published work, with most studies indicating that $\beta_1$ induces hyperpolarizing shift in gating of various $\alpha$ subunits (4;24;45-48).

Previous work from our laboratory demonstrated that the gating of Na$_\alpha$ channel $\alpha$ subunits expressed in CHO cells is altered by changes in sialylation levels (20;23). We recently described how Na$_\alpha$1.5 gating parameters were unaffected by $\alpha$ subunit sialic acids, whereas Na$_\alpha$1.4 gated at more depolarized potentials when expressed in essentially non-sialylating Lec2 cells versus fully sialylating Pro5 cells (23). Here, we extended this study to question the role of sialic acids in the gating of two neuronal $\alpha$ subunits, Na$_\alpha$1.2 and Na$_\alpha$1.7. As shown in Figures 1-4, much like that observed for Na$_\alpha$1.5 previously, reduced sialylation of Na$_\alpha$1.7 had no effect on gating. Na$_\alpha$1.2 gating was, in general, not significantly altered by $\alpha$ subunit sialic acids, although a consistent,
small, depolarizing shift in gating was observed for Naν1.2 expressed in Lec2 cells compared to gating in the fully sialylating Pro5 cells.

The β1 subunit is predicted to have four N-linked glycosylation sites, at least three of which are glycosylated (24;34). We tested the hypothesis that β1 sialic acids modulate Naν gating by co-expressing β1 with the four α subunits in the fully sialylating Pro5 cells and compared channel function with those observed in the non-sialylating Lec2 cells. As shown in Figures 1-4, all effects of β1 on α subunit gating measured were removed under conditions of reduced β1 sialylation. That is, β1 induced hyperpolarizing shifts in Naν1.2, Naν1.5, and Naν1.7 gating only under fully sialylating conditions. β1 expressed in the non-sialylating Lec2 cells had no effect on α subunit gating. Thus, β1 sialic acids can account for all effects of β1 on Naν gating observed here.

In addition to N-linked glycosylation, membrane proteins often have sugars attached through different linkages. For example, O-linked glycosylation (attached to serine or threonine residues) is prevalent. We sought to determine whether the β1 sialic acid dependent effects on α subunit gating were limited to N-linked sialic acids by mutating all four asparagine residues that initiate each N-glycosylation consensus sequence. The mutant β1, β1-ΔN, contains no N-linked sugars, but would maintain other posttranslational modifications, including O-linked sugars. As shown in Figure 7, fully sialylated β1-ΔN had no effect on the gating of any of the four α subunits previously shown to be affected by β1 sialic acids. Thus, the data confirm that β1 N-linked sialic acids are responsible fully for the observed shifts in α subunit gating.
β₁ sialic acids modulate Naᵥ channel gating through an apparent saturating electrostatic mechanism

We showed previously that α subunit sialic acids impose effects on gating through an apparent electrostatic mechanism. It is well established that Naᵥ gating is sensitive to changes in external Ca²⁺ concentrations, requiring a larger depolarization as external Ca²⁺ levels increase. A surface potential theory is often assigned to this phenomenon. Thus, external negative surface charges produce a surface potential that alters the voltage sensed by the channel gating mechanism(49). As Ca²⁺ levels increase, these charges will be screened effectively, minimizing their impact on gating. The voltage sensed by the gating mechanism of the channel becomes more negative, moving away from the voltage of activation, and thus a larger depolarization is required to activate the channel. If sialic acids contribute to such a negative surface potential, then sensitivity to screening by external Ca²⁺ should increase with the level of functional sialic acids. If β₁ sialic acids contribute to a negative surface potential, then gating of channels co-expressed with fully sialylated β₁ should be the most Ca²⁺ sensitive as shown in Figure 6. The shift in Vₐ with changing external Ca²⁺ levels for hSkM1P1 alone ± SA, and for hSkM1P1 + β₁ expressed in Lec2 cells, were similar and significantly smaller than the shift observed for the fully sialylated hSkM1P1 + β₁. Thus, β₁ sialic acids alter α subunit gating through an electrostatic mechanism, apparently contributing to an external negative surface potential.

Figures 1 -4 showed that gating of the heavily glycosylated Naᵥ1.4 was affected by α subunit sialic acids (cis effect), but not further altered by β₁ sialic acids. Conversely, gating of each of the putatively lesser glycosylated α subunits was not
dependent on $\alpha$ sialic acids, but was dependent on $\beta_1$ sialic acids (trans effect). These data suggest that in this system there is a saturating limit to the contribution of sialic acids to channel gating, with $\alpha_v1.4$ sialic acids likely achieving saturation. Figure 5 provides further supporting evidence, showing that gating of the less glycosylated $\alpha_v1.4$ chimera, hSkM1P1, is no longer sensitive to sialic acid, but is sensitive to $\beta_1$ sialic acids. These data suggest that with hSkM1P1, $\alpha_v1.4$ functional sialylation is reduced to levels below saturation such that $\beta_1$ sialic acids might contribute to channel gating. Thus, it appears the combined effects of cis $\alpha$ subunit DIS5-S6 and trans $\beta_1$ subunit functional sialic acids on channel gating are saturating (see Figure 8).

**The ability of the cell to produce differentially sialylated proteins leads to a spectrum of $\alpha_v$ functional sialic acid levels that directly modulate channel gating**

There have been numerous $\alpha$ and $\beta$ subunits identified to date. Each $\alpha$ subunit has a distinct glycosylation pattern that potentially can modulate current in an isoform specific manner (21;23). We now show that sialylation of the $\beta_1$ subunit also modulates $\alpha_v$ function. Expression of $\beta_1$ is regulated over the course of development, and is apparently differently processed among excitable tissues(35-38). As suggested by the model shown in Figure 8, it is possible to envisage a scenario in which different $\alpha_v \alpha \beta_1$ subunit combinations are differentially functionally sialylated in various tissues, over the course of development, perhaps pathologically, and/or even chronically within a single cell, potentially leading to modulation of $\alpha_v$ gating.

Thus, the cell would have two complementary methods to modulate sodium current through differential sialylation. Changing the level of sialylation through
regulation of sialyltransferase activity might alter the function of some channels chronically. For example, sodium currents in adult rat cortical and dorsal root ganglion neurons are less sensitive to sialic acid than sodium currents from neonatal neurons (22;50). A developmental decrease in Na\textsubscript{v} glycosylation was observed in one study(22). Recent work from our laboratory indicates that adult ventricular myocyte Na\textsubscript{v} are more heavily sialylated and gate at more hyperpolarized potentials than do neonate ventricular myocyte Na\textsubscript{v} (Stocker and Bennett, unpublished data). Thus there are tissue specific changes in sialylation over the course of development.

Secondly, control of \(\beta_1\) expression (and the specific \(\alpha\) subunit expressed) causes acute changes in functional sialic acid levels with the additional \(\beta_1\) sialic acids potentially altering the gating of the \(\alpha\) subunits with which they associate. Thus, gating of a specific \(\alpha\) subunit could be modulated by varying the expression of \(\beta_1\) over time in combination with manipulating the extent of \(\alpha\) and \(\beta_1\) posttranslational modification, producing a spectrum of differentially sialylated Na\textsubscript{v}. As a result, the excitability of the cell would be affected in a controlled manner that is potentially more efficient than directly manipulating the channel primary structure. Such negative shifts in gating imposed by \(\beta_1\) sialic acids would shift the voltage range of the window current, causing persistent channel activity to occur at more hyperpolarized potentials. There are several reports that show point mutations of SCN1A (Na\textsubscript{v}1.1) and SCN5A (Na\textsubscript{v}1.5) cause shifts in the window current voltage range that may be responsible for such maladies as epilepsy and arrhythmias (LQTS)(1;4;5). In addition, \(\beta_1\) sialic acids slow the rate to recovery from fast inactivation which would directly affect action potential relative refractory periods. That is, if \(\beta_1\) sialic acids induce slower recovery rates, then
at a short time following initialization of a Na\textsubscript{v} kinetic cycle, the percentage of inactivated channels will increase. This will directly affect the rate at which subsequent depolarizations might lead to activation of Na\textsubscript{v} sufficient to cause an action potential to fire. Thus, we report novel findings that are relevant to the modulation of voltage-gated sodium channel activity that will directly impact how one’s heart, muscle, and brain function.

**Trans-regulation of membrane proteins by carbohydrate structures attached to a second protein: a unique process?**

In addition, a more global phenomenon can be described for the first time. Our data indicate that $\beta_1$ sialic acids directly alter $\alpha$ subunit function, consistent with a mechanism by which transmembrane protein function can be modulated solely and directly by the sugars attached to a second protein. Because nearly all transmembrane proteins are glycosylated, such a phenomenon may be involved in processes other than the modulation of Na\textsubscript{v} gating, with significant potential for sugar residues to impose direct functional effects on many transmembrane proteins.
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Figure legends

Figure 1: β₁ sialic acids cause a hyperpolarizing shift in the peak conductance-voltage relationships for three α subunits.

Conductance-voltage (G-V) relationships for four voltage-gated sodium channel α subunits ± β₁ as expressed in two CHO cell lines with varying abilities to sialylate proteins. Expression in Pro5 cells allows normal CHO cell sialylation, while expression in Lec2 cells prevents sialylation. Data are the mean normalized peak conductance ± SEM at a membrane potential, and curves are fits of the data to single Boltzmann relationships. n = 9-13 for each condition tested (see Table 1). Significant differences among conditions tested throughout are listed in Table 1. Subunit schematics demarcate the location and number of N-glycosylation consensus sequences along each α subunit. Circles, Solid Lines: α subunit alone. Squares, Dashed Lines: α + β₁. Filled symbols: in Pro5 cells. Open symbols: in Lec2 cells.

A: Nav1.4.
B: Nav1.5.
C: Nav1.7.
D: Nav1.2.

Figure 2: β₁ sialic acids cause a similar hyperpolarizing shift in channel availability curves.

Steady state channel availability (h_infinity) curves for the four α subunits ± β₁ ± sialic acid. Data are the mean normalized peak current (Ī)±/SEM measured during a maximally
depolarizing test pulse following a 500 ms prepulse to the plotted potentials. Symbols, lines, sample numbers, and panels are as described in Figure 1.

**Figure 3:** $\beta_1$ sialic acids increase the rate of fast inactivation.

Data are the mean ± SEM time constants for fast inactivation ($\tau_h$) as a function of membrane potential. Lines are non-theoretical, point-to-point. Symbols, lines, sample numbers, and panels are as described in Figure 1. Inset to Panel C: Representative normalized whole cell Na$^+$ current traces measured at -20 mV for Na$_v$1.7. Note that the rate at which the current attenuates (inactivates) is much faster in the presence of $\beta_1$ sialic acids, consistent with the observed shift in $\tau_h$ along the voltage axis. Scale shown is for Na$_v$1.7 + $\beta_1$ + SA current traces to which the other current traces were normalized.

**Figure 4:** $\beta_1$ sialic acids slow the rate to recovery from fast inactivation causing a similar negative shift in the voltage dependence of recovery rate.

$\beta_1$ sialic acids slow the rate of recovery from fast inactivation. Data are the time constants for recovery from fast inactivation ($\tau_{rec}$) ± SEM measured for each construct at three recovery potentials. Lines are non-theoretical point-to-point. Symbols, lines, sample numbers, and panels are as described in Figure 1. Inset to Panel C: Typical plot of the fractional recovery measured using a standard twin pulse protocol to determine the rate of recovery from fast inactivation at a -120 mV recovery potential for Na$_v$1.7 + $\beta_1$ + SA. Data are the mean ± SEM fractional current measured during a second depolarizing test pulse following the plotted interval at -120 mV from the original
depolarizing test pulse. Lines are exponential fits of the data from which the $\tau_{\text{rec}}$ were determined.

**Figure 5:** $\beta_1$ sialic acids can modulate the gating of an Na$_v$1.4 mutant with reduced glycosylation.

Gating of hSkM1P1 is sensitive to $\beta_1$ sialic acids. Voltage dependent steady state and kinetic gating for hSkM1P1 $\pm \beta_1 \pm$ SA. Circles: hSkM1P1 expressed alone. Squares: hSkM1P1 $+ \beta_1$. Filled symbols: in Pro5 cells. Open symbols: in Lec2 cells. n = 8-11 for each condition (see Table 1). A schematic of hSkM1P1 structure illustrates that the chimera consists of Na$_v$1.4 with the less glycosylated Na$_v$1.5 DIS5-S6 loop replacing the Na$_v$1.4 DIS5-S6.

**A:** G-V relationship.

**B:** Steady state channel availability.

**C:** Fast inactivation time constants.

**D:** Time constants for recovery from fast inactivation.

**Figure 6:** The effect of $\beta_1$ sialic acids on gating is consistent with an electrostatic mechanism.

$\beta_1$ sialic acids apparently contribute to an external negative surface potential that affects Na$_v$ gating. Bar graph of the observed hyperpolarizing shifts in $V_a$ for hSkM1P1 $\pm \beta_1 \pm$ SA with a ten-fold decrease in external Ca$^{2+}$ concentration used to differentially screen external negative surface charges. The $V_a$ shifts by about -15 mV when extracellular Ca$^{2+}$ is changed from 2.0 to 0.2 mM for hSkM1P1 alone $\pm$ SA, and for hSkM1P1 $+ \beta_1$ -
SA (in Lec2 cells). However, the Ca^{2+} dependent shift in V_a under conditions of fully sialylated \( \beta_1 \) is a much larger, significant (p<0.02), -25 mV. \( n = 3-4 \) for each condition.

**Figure 7:** \( \beta_1 \) N-linked sialic acids account fully for \( \beta_1 \) modulation of Na_v gating.

G-V relationships for hSkM1P1 (Panel A), Na_v1.5 (Panel B), Na_v1.7 (Panel C), and Na_v1.2 (Panel D) under fully sialylated conditions alone (Filled Circles; Solid Lines; \( n = 9-13 \) for each), co-expressed with \( \beta_1 \) (Filled Squares; Dashed Lines; \( n = 9-12 \) for each), or with \( \beta_1-\Delta_N \), a mutant \( \beta_1 \) in which all four putative N-linked glycosylation asparagine residues were mutated to serine residues (Filled Triangles; Dotted Lines; \( n = 4-6 \) for each). Removal of \( \beta_1 \) N-linked sugars prevents fully the \( \beta_1 \) sialic acid induced hyperpolarizing shifts in channel gating. Data are the mean \( \pm \) SEM peak conductance at a membrane potential. Lines are fits of the data to single Boltzmann relationships. \( \beta_1-\Delta_N \): Filled triangles and dotted line. All other symbols, lines, and sample numbers are as previously described. Schematics of the wild type \( \beta_1 \), with its four putative N-glycosylation structures attached, and \( \beta_1-\Delta_N \), with all four carbohydrate structures missing, are pictured in Panel A.

**Figure 8:** Model predicting the putative saturating effects of \( \beta_1 \) sialic acids on \( \alpha \) subunit gating.

A: Examples of possible interactions of \( \beta_1 \) sialic acids with Na_v1.4 (left) and the glycosylation-deficient chimera, hSkM1P1 (right). Data suggest that \( \beta_1 \) sialic acids cannot contribute further to the gating of Na_v1.4, but do contribute to the gating of the other \( \alpha \) subunits through an apparent electrostatic mechanism. Thus, the left panel shows
ineffectual $\beta_1$ sialic acids as distant from Nav1.4, while the right panel illustrates that fewer $\alpha$ subunit functional DIS5-S6 sialic acids may allow $\beta_1$ sialic acids to interact more intimately with the $\alpha$ subunit and contribute to channel gating.

**B:** A theoretical concentration response curve comparing relative contributions to $V_a$ by functional sialic acids associated with various $\alpha\beta_1$ combinations. A typical sigmoidal curve is shown, with the various $\alpha\beta_1$ subunit combinations studied here aligned along the curve – the location of each is not precise, but consistent, relatively, with the data shown here. For example, Nav1.4 must contain essentially saturating levels of functional sialic acids in this experimental system. Thus, Nav1.4 alone and Nav1.4 + $\beta_1$ are pictured along the maximum of the curve. In a second example, Nav1.7 alone can not contain many DIS5-S6 functional sialic acids, and therefore Nav1.7 expressed alone must lie somewhere along the minimum portions of the curve. When $\beta_1$ is co-expressed with Nav1.7, the levels of functional sialic acids increase, causing a hyperpolarization in $V_a$, and hence, Nav1.7 + $\beta_1$ is shown somewhere along the rising slope of the curve.

While the data presented here show uniform effects of $\beta_1$ sialic acids on measured voltage dependent Nav gating parameters, there is no reason, *a priori*, for this to occur. The impact of $\beta_1$ sialic acids on specific Nav gating parameters will depend on several factors including the extent of coupling among channel kinetic states and/or any inherent voltage dependence of the transition from one state to another. Thus, for example, the interaction of $\beta_1$ sialic acids with a specific $\alpha$ subunit may place the channel at a different position along the concentration response curves for shifts in $V_a$ versus shifts in $V_i$. This would likely be observed as differences in the impact of $\beta_1$ sialic acids on $V_a$ and $V_i$. 
Table 1: The measured gating parameters for α ± β1 ± sialic acid

| Channel construct | n  | $V_a$ (mV) | $V_i$ (mV) | $\tau_h$ (ms) | $\tau_{rec}$ (-120 mV) (ms) |
|-------------------|----|------------|------------|--------------|-----------------|
| $Na_v1.4 + SA$    | 9  | -31.3 ± 2.0 | -71.5 ± 3.3 | 2.4 ± 0.4     | 1.8 ± 0.05      |
| $Na_v1.4 - SA$    | 9  | -16.7 ± 1.7 | -60.8 ± 1.5 | 7.9 ± 1.1     | 1.3 ± 0.03      |
| $Na_v1.4 + \beta_1 + SA$ | 11 | -29.2 ± 1.6 | -70.0 ± 2.3 | 2.9 ± 0.5     | 1.7 ± 0.02      |
| $Na_v1.4 + \beta_1 - SA$ | 9  | -16.3 ± 1.1 | -63.1 ± 1.8 | 8.1 ± 0.2     | 1.4 ± 0.03      |
| $Na_v1.5 + SA$    | 13 | -29.0 ± 2.2 | -78.7 ± 2.5 | 2.8 ± 0.4     | 4.0 ± 0.1       |
| $Na_v1.5 - SA$    | 10 | -29.5 ± 1.6 | -79.5 ± 1.9 | 2.4 ± 0.2     | 4.1 ± 0.1       |
| $Na_v1.5 + \beta_1 + SA$ | 11 | -37.4 ± 1.6 | -86.1 ± 3.2 | 2.0 ± 0.1     | 5.6 ± 0.3       |
| $Na_v1.5 + \beta_1 - SA$ | 9  | 28.6 ± 0.9  | -78.8 ± 1.6 | 2.9 ± 0.2     | 4.1 ± 0.1       |
| $Na_v1.7 + SA$    | 10 | -15.1 ± 1.2 | -70.0 ± 2.4 | 4.8 ± 0.8     | 5.5 ± 0.09      |
| $Na_v1.7 - SA$    | 9  | -14.4 ± 1.7 | -70.0 ± 3.7 | 5.2 ± 1.2     | 5.5 ± 0.06      |
| $Na_v1.7 + \beta_1 + SA$ | 12 | -23.8 ± 1.8 | -76.2 ± 2.0 | 3.0 ± 0.4     | 7.8 ± 0.2       |
| $Na_v1.7 + \beta_1 - SA$ | 9  | -13.4 ± 1.3 | -68.3 ± 1.5 | 5.1 ± 0.8     | 5.5 ± 0.2       |
| $Na_v1.2 + SA$    | 9  | -14.6 ± 1.6 | -60.5 ± 3.1 | 3.3 ± 0.7     | 2.5 ± 0.08      |
| $Na_v1.2 - SA$    | 10 | -11.7 ± 1.8 | -62.7 ± 3.1 | 3.1 ± 0.5     | 2.2 ± 0.04      |
| $Na_v1.2 + \beta_1 + SA$ | 12 | -20.8 ± 0.7 | -68.1 ± 2.7 | 2.3 ± 0.3     | 3.1 ± 0.07      |
| $Na_v1.2 + \beta_1 - SA$ | 9  | -11.9 ± 1.0 | -62.2 ± 2.8 | 3.2 ± 0.6     | 2.2 ± 0.06      |
| hSkM1P1 + SA      | 10 | -23.5 ± 2.3 | -70.4 ± 2.5 | 3.4 ± 0.5     | 1.8 ± 0.07      |
| hSkM1P1 - SA      | 8  | -26.7 ± 1.2 | -68.8 ± 2.5 | 3.4 ± 0.8     | 1.8 ± 0.05      |
| hSkM1P1 + $\beta_1 + SA$ | 9  | -32.7 ± 2.0 | -75.9 ± 1.6 | 2.3 ± 0.4     | 2.4 ± 0.06      |
| hSkM1P1 + $\beta_1 - SA$ | 11 | -24.0 ± 1.5 | -69.2 ± 1.5 | 3.7 ± 0.6     | 1.9 ± 0.04      |

Data are the mean parameter value ± SEM. For $\tau_h$, data were measured at -40 mV for $Na_v1.4$, $Na_v1.5$, and hSkM1P1, and at -30 mV for $Na_v1.2$ and $Na_v1.7$. To determine the effects of $\beta_1$ sialic acids, significance was tested using a two-tailed student's t test, with each condition compared to the parameter measured for the fully sialylated α subunit alone. Significance is demarcated as follows: * = significant (p<0.1); ** = highly significant (p<0.005).
Figure 1

A

Na\textsubscript{1.4}

mV

-80 -60 -40 -20 0 20 40

0.00

0.25

0.50

0.75

1.00

B

Na\textsubscript{1.5}

-80 -60 -40 -20 0 20 40

C

Na\textsubscript{1.7}

-80 -60 -40 -20 0 20 40

D

Na\textsubscript{1.2}

-80 -60 -40 -20 0 20 40
Figure 2

A

Na\textsubscript{1.4}

B

Na\textsubscript{1.5}

C

Na\textsubscript{1.7}

D

Na\textsubscript{1.2}
Figure 3

(A) $\tau_h$ (ms) for $\text{Na}_v1.4$

(B) $\tau_h$ (ms) for $\text{Na}_v1.5$

(C) $\tau_h$ (ms) for $\text{Na}_v1.7$

(D) $\tau_h$ (ms) for $\text{Na}_v1.2$
Figure 5
Figure 6

Shift in $V_a$ (mV)

- $\alpha + SA$
- $\alpha - SA$
- $\alpha + \beta_1 + SA$
- $\alpha + \beta_1 - SA$

*
Figure 8

A

B

Relative Contribution to \( V_m \)

[Functional Sialic Acids]
The sialic acid component of the beta{sub}1 subunit modulates voltage-gated sodium channel function
Daniel Johnson, Marty L. Montpetit, Patrick J. Stocker and Eric S. Bennett

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