Neurosteroids Allopregnanolone Sulfate and Pregnanolone Sulfate Have Diverse Effect on the α Subunit of the Neuronal Voltage-gated Sodium Channels $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ Expressed in *Xenopus* Oocytes

Takafumi Horishita, M.D., Ph.D., Nobuyuki Yanagihara, Ph.D., Susumu Ueno, M.D., Ph.D., Yuka Sudo, Ph.D., Yasuhito Uezono, M.D., Ph.D., Dan Okura, M.D., Tomoko Minami, M.D., Takashi Kawasaki, M.D., Ph.D., Takeyoshi Sata, M.D., Ph.D.

ABSTRACT

Background: The neurosteroids allopregnanolone and pregnanolone are potent positive modulators of γ-aminobutyric acid type A receptors. Antinociceptive effects of allopregnanolone have attracted much attention because recent reports have indicated the potential of allopregnanolone as a therapeutic agent for refractory pain. However, the analgesic mechanisms of allopregnanolone are still unclear. Voltage-gated sodium channels (Na_v) are thought to play important roles in inflammatory and neuropathic pain, but there have been few investigations on the effects of allopregnanolone on sodium channels.

Methods: Using voltage-clamp techniques, the effects of allopregnanolone sulfate (APAS) and pregnanolone sulfate (PAS) on sodium current were examined in *Xenopus* oocytes expressing $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ α subunits.

Results: APAS suppressed sodium currents of $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ at a holding potential causing half-maximal current in a concentration-dependent manner, whereas it markedly enhanced sodium current of $Na_v1.8$ at a holding potential causing maximal current. Half-maximal inhibitory concentration values for $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ were 12 ± 4 (n = 6), 41 ± 2 (n = 7), and 131 ± 15 (n = 5) μmol/l (mean ± SEM), respectively. The effects of PAS were lower than those of APAS. From gating analysis, two compounds increased inactivation of all α subunits, while they showed different actions on activation of each α subunit. Moreover, two compounds showed a use-dependent block on $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$.

Conclusion: APAS and PAS have diverse effects on sodium currents in oocytes expressing four α subunits. APAS inhibited the sodium currents of $Na_v1.2$ most strongly. **(ANESTHESIOLOGY 2014; 121:620-31)**

NEUROSTEROIDS are neuroactive steroids synthesized from cholesterol in both central and peripheral nervous systems, and they accumulate in the nervous system.[1] They rapidly alter neuronal excitability by mediating actions through ion-gated neurotransmitter receptors, but not through classic steroid hormone nuclear receptors.[2] Many of them are converted to sulfated metabolites by hydroxysteroid sulfotransferases, and neurosteroid sulfates are also known to regulate physiological processes. They are thought to be potentially therapeutic because of their many pharmacological properties.[3,4]

Two 3α-hydroxylated metabolites of progesterone, allopregnanolone (3α-hydroxy-5α-pregnane-20-one) and pregnanolone (3α-hydroxy-5β-pregnane-20-one), are known to be positive modulators at γ-aminobutyric acid type A ($GABA_A$) receptors with high potency.[5] These neurosteroids have been shown to have greater anesthetic potencies than those of other intravenous anesthetics that are clinically used, and not to cause acute tolerance that are observed in other

What We Already Know about This Topic

- Sodium channels are important targets for analgesic actions in the spinal cord, but their role in neurosteroid analgesia is unclear
- The effects of two sulfated neurosteroids with analgesic and anesthetic properties were tested on heterologously expressed rat voltage-gated sodium channel function

What This Article Tells Us That Is New

- The neurosteroids tested produced voltage and use-dependent block of all the subtypes tested, with more potent effects on $Na_v1.2$
- Inhibition of $Na_v1.2$ in the spinal cord by allopregnanolone is a plausible mechanism for its analgesic effects if confirmed in neuronal preparations and pain models

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anesthetics, suggesting usefulness of these neurosteroids as general anesthetics.[6,7] On the contrary, allopregnanolone was shown to have the most potent analgesic effects among all neurosteroids in pain models.[8] Recent studies demonstrated its analgesic effects in neuropathic pain models. Allopregnanolone alleviates thermal and mechanical hyperalgesia by ligation of the sciatic nerve in rats,[9] produces analgesic effects on formalin-induced pain in rats,[10] and prevents anticancer drug oxaliplatin-induced cold and mechanical allodynia and hyperalgesia.[11] In addition, it was suggested that stimulation of allopregnanolone synthesis might be involved in the antinociceptive effects of several analgesic drugs in neuropathic pain models.[12–14] Its effect on $GABA_A$ receptors may be important for its antinociceptive properties because GABA is involved in pain pathways in the nervous systems, and drugs targeting subtypes of GABA receptors have analgesic effects in chronic pain.[15] However, these two neurosteroids, allopregnanolone and pregnanolone, also act on other ion channels in pain signaling pathways, including T-type calcium channels[16] and *N*-methyl-D-aspartate receptors.[17]

Voltage-gated sodium channels (Na_v) have an important role in action potential initiation and propagation in excitable nerve and muscle cells. Nine α subunits ($Na_v1.1$ to $Na_v1.9$) and four auxiliary β subunits have been identified in mammals.[18,19] Each pore-forming α subunit has a different pattern of development and localization and has distinct physiological and pathophysiological roles. Sodium channel α subunits expressed in the dorsal root ganglion are considered possible targets for analgesics for inflammatory and neuropathic pain.[20–22] However, there has been little investigation on the effects of allopregnanolone on sodium channel function. It is important to examine these effects because they may be useful in clarifying the mechanisms of the analgesic effects of allopregnanolone and developing natural and safe neurosteroid-based analgesics for refractory pain. In addition, our recent report demonstrated the importance of neurosteroid sulfonation for regulation of ion channels because of more potent effects of sulfated steroid than those of nonsulfated steroids.[23] Here, we investigate the effects of two sulfated neurosteroids, allopregnanolone sulfate (APAS) and pregnanolone sulfate (PAS) (fig. 1), on several sodium channel α subunits, including $Na_v1.2$, which is expressed in the central nervous system; $Na_v1.6$, which is expressed in the central nervous system and dorsal root ganglion neurons; and $Na_v1.7$ and $Na_v1.8$, which are expressed in dorsal root ganglion neurons.

Materials and Methods

This study was approved by the Animal Research Committee of the University of Occupational and Environmental Health, Kitakyushu, Japan.

Drugs

Allopregnanolone sulfate and PAS were purchased from Steraloids, Inc. (Newport, RI).

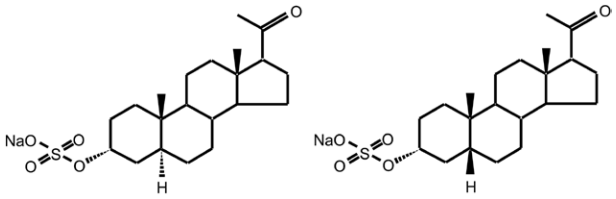

Fig. 1. Structures of allopregnanolone sulfate (APAS) and pregnanolone sulfate (PAS).

Plasmids

Rat $Na_v1.2$ α subunit complementary DNA (cDNA) was a gift from Dr. William A. Catterall, Ph.D. (Professor, Department of Pharmacology, University of Washington, Seattle, Washington). Rat $Na_v1.6$ α subunit cDNA was a gift from Dr. Alan L. Goldin, M.D., Ph.D. (Professor, Department of Anatomy and Neurobiology, University of California, Irvine, California). Rat $Na_v1.7$ α subunit cDNA was a gift from Gail Mandel, Ph.D. (Professor, Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon). Rat $Na_v1.8$ α subunit cDNA was a gift from Dr. Armen N. Akopian, Ph.D. (Assistant Professor, University of Texas Health Science Center, San Antonio, Texas), and human β_1 subunit cDNA was a gift from Dr. Alfred L. George, Jr., M.D. (Professor, Department of Pharmacology, Vanderbilt University, Nashville, Tennessee). The percentages of homology between rat and human protein of $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ are 98, 99, 93, and 83%, respectively, suggesting the possible limitations imposed by using rat α subunit for only $Na_v1.8$ to make conclusions in humans.

Complementary RNA (cRNA) Preparation and Oocyte Injection

After linearization of cDNA with *Cla*I ($Na_v1.2$ α subunit), *Not*I ($Na_v1.6$, 1.7 α subunits), *Xba*I ($Na_v1.8$ α subunit), and *EcoR*I (β_1 subunit), cRNAs were transcribed using SP6 (Na_v 1.8 α, β_1 subunits) or T7 ($Na_v1.2$, 1.6, and 1.7 α subunits) RNA polymerase from the mMESSAGE mMACHINE kit (Ambion, Austin, TX). Adult female *Xenopus laevis* frogs were obtained from Kyudo Co., Ltd. (Saga, Japan). *X. laevis* oocytes and cRNA microinjection were prepared as described previously.[24] Na_v α subunit cRNAs were coinjected with β_1 subunit cRNA at a ratio of 1:10 (total volume was 20 to 40 ng/50 nl) into *Xenopus* oocytes (all α subunits were coinjected with the β_1 subunit) that were randomly assigned to four α subunit groups for injection. Injected oocytes were incubated at 19°C in incubation medium, and 2 to 6 days after injection, the cells were used for electrophysiological recordings.

Electrophysiological Recordings

All electrical recordings were performed at room temperature (23°C). Oocytes were placed in a 100-μl recording chamber

and perfused at 2 ml/min with Frog Ringer's solution containing 115 mmol/l NaCl, 2.5 mmol/l KCl, 10 mmol/l HEPES, 1.8 mmol/l $CaCl_2$, pH 7.2, using a peristaltic pump (World Precision Instruments Inc., Sarasota, FL). Recording electrodes were prepared, and the whole-cell voltage clamp and recordings were achieved as described previously.[24] Transients and leak currents were subtracted using the P/N procedure, in which N subsweeps each 1/Nth of the amplitude of the main stimulus waveform (P) are applied. APAS and PAS stocks were prepared in dimethylsulfoxide and diluted in Frog Ringer's solution to a final dimethylsulfoxide concentration not exceeding 0.05%. APAS and PAS were perfused for 3 min to reach equilibrium. All recordings were performed by the experimenters who were blind to the type of compound.

The voltage dependence of activation was determined using 50-ms depolarizing pulses from a holding potential causing maximal current (V_{max}) (–90 mV for $Na_v1.2$ and $Na_v1.6$, –100 mV for $Na_v1.7$ and $Na_v1.8$) and from a holding potential causing half-maximal current ($V_{1/2}$) (from approximately –40 mV to –70 mV) to 60 mV in 10-mV increments. V_{max} and $V_{1/2}$ holding potentials induce resting and inactivated states of sodium channels. Because the effects of many analgesics in the inactivated state are known to be important for analgesic action,[25] we used these two different holding potentials to compare the effects of compounds in the resting and inactivated states. Normalized activation curves were fitted to the Boltzmann equation as described previously[24]: briefly, $G/G_{max} = 1/(1 + \exp(V_{1/2} - V)/k)$, where G is the voltage-dependent sodium conductance, G_{max} is the maximal sodium conductance, G/G_{max} is the normalized fractional conductance, $V_{1/2}$ is the potential at which activation is half maximal, and k is the slope factor. To measure steady-state inactivation, currents were elicited by a 50-ms test pulse to –20 mV for $Na_v1.2$ and $Na_v1.6$, –10 mV for $Na_v1.7$, and +10 mV for $Na_v1.8$ after 200 ms (500 ms for only $Na_v1.8$) prepulses ranging from –140 to 0 mV in 10-mV increments from a holding potential of V_{max}. Steady-state inactivation curves were fitted to the Boltzmann equation: $I/I_{max} = 1/(1 + \exp(V_{1/2} - V)/k)$, where I_{max} is the maximal sodium current, I/I_{max} is the normalized current, $V_{1/2}$ is the voltage of half-maximal inactivation, and k is the slope factor. To investigate a use-dependent sodium channel block, currents were elicited at 10 Hz by a 20-ms depolarizing pulse of –20 mV for $Na_v1.2$ and $Na_v1.6$, –10 mV for $Na_v1.7$, and +10 mV for $Na_v1.8$ from a $V_{1/2}$ holding potential in both the absence and presence of 100 μmol/l APAS and PAS. Peak currents were measured and normalized to the first pulse and plotted against the pulse number. Data were fitted to the monoexponential equation $I_{Na} = \exp(-\tau_{use} \cdot n) + C$, where n is pulse number, C is the plateau I_{Na}, and τ_{use} is the time constant of use-dependent decay.

Statistical Analysis

The GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) was used to perform the statistical analysis, and a statistical power analysis was performed using G*Power software. All values are presented as means ± SEM. The n values refer to the number of oocytes examined. Each experiment was performed with oocytes taken from at least two frogs. Data were statistically evaluated by paired t test (two-tailed). We assessed the inhibitory effects at different APAS concentrations in the concentration–response curve, using one-way ANOVA followed by Dunnet *post hoc* test for multiple comparisons. Hill slope, half-maximal inhibitory concentration (IC_{50}), and half-maximal effective concentration

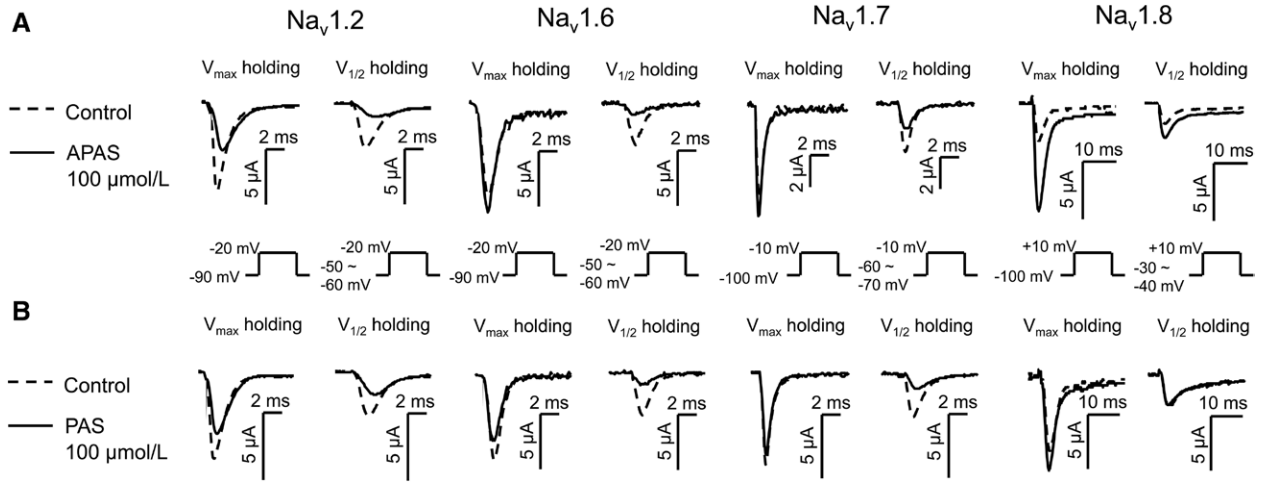

Fig. 2. Effects of allopregnanolone sulfate (APAS) (*A*) and pregnanolone sulfate (PAS) (*B*) on peak sodium inward currents in *Xenopus* oocytes expressing $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, or $Na_v1.8$ α subunits with β_1 subunits at two holding potentials. Representative traces are shown. Sodium currents were evoked by 50-ms depolarizing pulses to –20 mV for $Na_v1.2$ and $Na_v1.6$, –10 mV for $Na_v1.7$, and +10 mV for $Na_v1.8$ from V_{max} or $V_{1/2}$ in both the absence and presence of 100 μmol/l of the compounds. Na_v = voltage-gated sodium channel; V_{max} holding = holding potential causing maximal current; $V_{1/2}$ holding = holding potential causing half-maximal current.

(EC_{50}) values were also calculated. *P* value less than 0.05 was considered to indicate a significant difference.

Results

Effects of APAS and PAS on Peak Na^+ Inward Currents Elicited from Two Different Holding Potentials

Currents were elicited using a 50-ms depolarizing pulse to −20 mV for $Na_v1.2$ and $Na_v1.6$, −10 mV for $Na_v1.7$, and +10 mV for $Na_v1.8$ applied every 10 s from a V_{max} or $V_{1/2}$ holding potential in both the absence and presence of 100 μmol/l APAS and PAS (fig. 2). The amplitude of expressed sodium currents was typically 2 to 15 μA, and oocytes that showed a maximal current greater than 20 μA were not included in the data collection in all the following experiments. APAS had dual effects on sodium currents depending on the holding potential and α subunit (figs. 2 and 3). At $V_{1/2}$, APAS reduced the peak I_{Na} (sodium current) induced by $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ by 79 ± 1%, 71 ± 2%, and 49 ± 3%, respectively. At V_{max}, APAS also reduced I_{Na} induced by $Na_v1.2$ by 60 ± 4%, whereas it enhanced I_{Na} induced by $Na_v1.6$ and $Na_v1.7$ by 15 ± 6% and 14 ± 1%, respectively, although these effects were small. In contrast, APAS greatly enhanced I_{Na} induced by $Na_v1.8$ at both $V_{1/2}$ and V_{max} by 112 ± 34% and 202 ± 14%, respectively (fig. 3A). PAS reduced I_{Na} induced by $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ at $V_{1/2}$ by 54 ± 4%, 71 ± 1%, and 48 ± 2%, respectively. Effects of PAS on I_{Na} at V_{max} were smaller than those at $V_{1/2}$, and the magnitudes of inhibitory effects on $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ were 31 ± 5%, 10 ± 1%, and 6 ± 1%, respectively. While PAS enhanced I_{Na} induced by $Na_v1.8$ at V_{max} by 39 ± 6%, it did not affect I_{Na} induced by $Na_v1.8$ at $V_{1/2}$ (fig. 3B). In summary, PAS inhibited I_{Na} induced by $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ at both $V_{1/2}$ and V_{max} holding potentials. APAS had inverse effects on $Na_v1.6$ and $Na_v1.7$ according to the different holding potentials, whereas it suppressed I_{Na} induced by $Na_v1.2$ at both $V_{1/2}$ and V_{max}. Moreover, APAS markedly enhanced I_{Na} induced by $Na_v1.8$ at both $V_{1/2}$ and V_{max}.

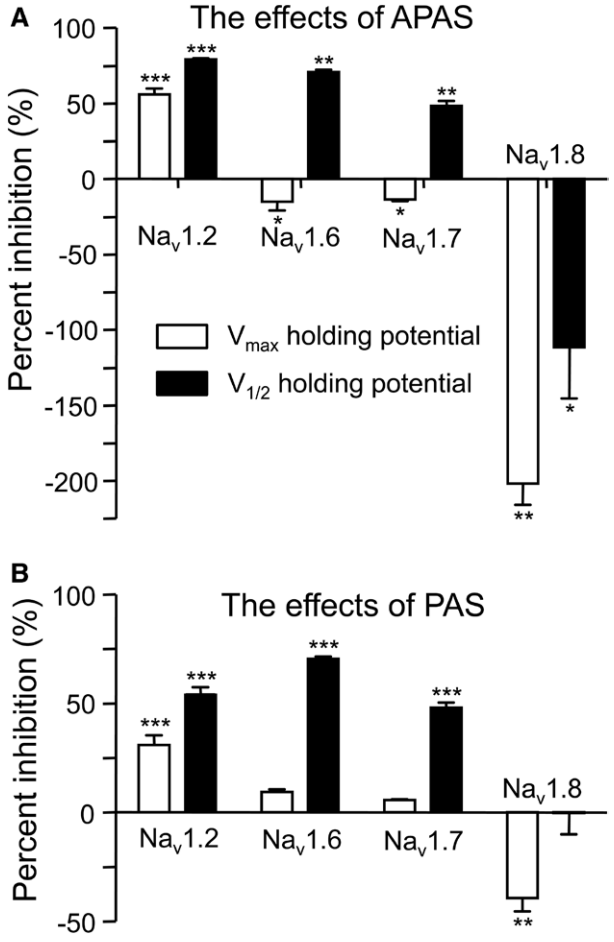

Fig. 3. Percentage inhibition of sodium currents of allopregnanolone sulfate (APAS) (n = 6) (*A*) and pregnanolone sulfate (PAS) (n = 5) (*B*) were calculated. *Open columns* represent the effect at V_{max} holding potential, and *closed columns* indicate the effect at $V_{1/2}$. Data are presented as means ± SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with the control, based on paired *t* test (two-tailed). Na_v = voltage-gated sodium channel; V_{max} holding potential = holding potential causing maximal current; $V_{1/2}$ holding potential = holding potential causing half-maximal current.

Next, we examined the concentration–response relationship for suppression of the peak I_{Na} induced through $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ by APAS and PAS at $V_{1/2}$ holding potential because suppression by both neurosteroids of these α subunits at $V_{1/2}$ was more potent than that at V_{max} (fig. 4, A and B). In addition, we investigated the concentration–response relationship for potentiation of the peak I_{Na} of $Na_v1.8$ by APAS and PAS at V_{max}, because both neurosteroids showed potent enhancement of I_{Na} at V_{max} compared with that at $V_{1/2}$ (fig. 4C). IC_{50} values, EC_{50} values, and Hill slopes calculated from nonlinear regression analyses of the dose–response curves are shown in table 1. From these analyses, the effect of APAS on $Na_v1.2$ was the most potent among the two neurosteroids and four α subunits.

Effects of APAS and PAS on Activation of Sodium Currents

We examined the effects of APAS and PAS on four α subunits in sodium current activation. Voltage dependence of activation was determined using 50-ms depolarizing pulses from a holding potential of V_{max} to 50 mV in 10-mV increments or from a holding potential of $V_{1/2}$ to 60 mV in 10-mV increments for $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ in both the absence and presence of 100 μmol/l APAS and PAS (fig. 5). Activation curves were derived from the I–V curves (see Electrophysiological Recordings under Materials and Methods). At V_{max}, APAS greatly reduced the peak I_{Na} induced by $Na_v1.2$, whereas it greatly enhanced the peak I_{Na} induced by $Na_v1.8$ in the depolarizing region where channel opening begins. It also enhanced the peak I_{Na} induced by $Na_v1.6$ and $Na_v1.7$, similar to its effects on $Na_v1.8$, although both effects were small. At $V_{1/2}$, APAS greatly suppressed the peak I_{Na} induced by $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$,

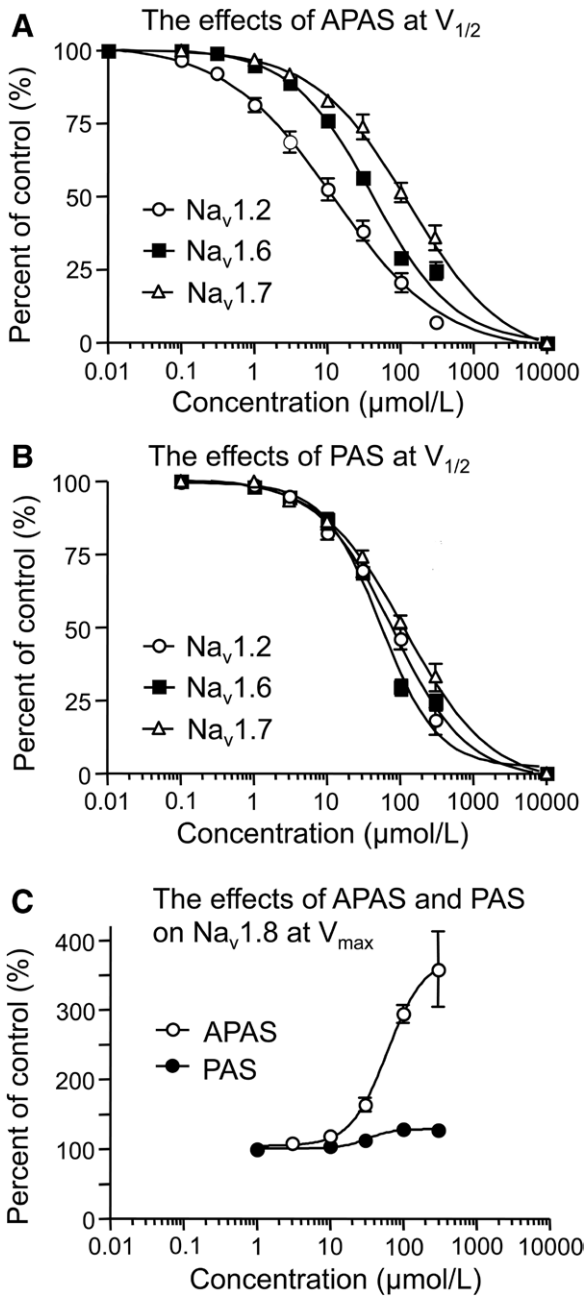

Fig. 4. Concentration–response curves for two-compound suppression of sodium currents elicited by 50-ms depolarizing pulses to –20 mV for $Na_v1.2$ (n = 6) and $Na_v1.6$ (n = 7) and –10 mV for $Na_v1.7$ (n = 5) from $V_{1/2}$ holding potential (*A* and *B*) and those for two-compound potentiation of sodium currents elicited by 50-ms depolarizing pulses to +10 mV for $Na_v1.8$ (n = 5) from V_{max} (*C*). The peak current amplitude in the presence of two compounds was normalized to that of the control, and the effects are expressed as percentages of the control. Hill slopes, IC_{50} values, and EC_{50} values are shown in table 1. Data are presented as means ± SEM. Data were fitted to the Hill slope equation to give the Hill slopes, IC_{50} values, and EC_{50} values. Hill slopes, IC_{50} values, and EC_{50} values were calculated using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). APAS = allopregnanolone sulfate; Na_v = voltage-gated sodium channel; PAS = pregnanolone sulfate; V_{max} = holding potential causing maximal current; $V_{1/2}$ = holding potential causing half-maximal current.

but it enhanced the peak I_{Na} induced by $Na_v1.8$, similar to its effects on $Na_v1.8$ at V_{max}. PAS reduced I_{Na} induced by $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ at both $V_{1/2}$ and V_{max}, whereas it enhanced I_{Na} induced by $Na_v1.8$ in the depolarizing region at V_{max}, but had no effect at $V_{1/2}$.

At V_{max} holding potential, APAS significantly shifted the midpoint of the steady-state activation ($V_{1/2}$) in a depolarizing direction for $Na_v1.2$, but it significantly shifted $V_{1/2}$ in a hyperpolarizing direction for $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$. At $V_{1/2}$, APAS also shifted $V_{1/2}$ in a similar direction as the shift at V_{max}, although the shift was small and not significant, except for $Na_v1.8$. The shifts of $V_{1/2}$ by PAS were smaller than those by APAS. PAS significantly shifted $V_{1/2}$ in a depolarizing direction for $Na_v1.2$ and $Na_v1.6$ at $V_{1/2}$, but it had no or slight effects on all α subunits at V_{max}, and on $Na_v1.7$ and $Na_v1.8$ at $V_{1/2}$ (fig. 6 and tables 2 and 3).

Effects of APAS and PAS on Inactivation of Sodium Currents

We also investigated the effects of APAS and PAS on steady-state inactivation. Currents were elicited by a 50-ms test pulse to –20 mV for $Na_v1.2$ and $Na_v1.6$, –10 mV for $Na_v1.7$, and +10 mV for $Na_v1.8$ after 200 ms (500 ms for only $Na_v1.8$) prepulses ranging from –140 mV to 0 mV in 10-mV increments from V_{max} holding potential. Steady-state inactivation curves were fitted to the Boltzmann equation (see Electrophysiological Recordings under Materials and Methods). APAS and PAS significantly shifted the midpoint of steady-state inactivation ($V_{1/2}$) in the hyperpolarizing direction for all α subunits; APAS shifted by 8.0, 8.9, 6.7, and 8.9 mV and PAS shifted by 4.5, 8.0, 6.6, and 10.2 mV for $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$, respectively (fig. 7 and tables 2 and 3). The effects of APAS and PAS in the hyperpolarizing range were consistent with the effects of these two neurosteroids on the peak I_{Na} at V_{max} and their effects on the I–V curves in the hyperpolarizing range at V_{max}.

Use-dependent Block of Sodium Currents by APAS and PAS

The use-dependent block of sodium currents by APAS and PAS was also investigated. Currents were elicited at 10 Hz by a 20-ms depolarizing pulse of –20 mV for $Na_v1.2$ and $Na_v1.6$ and –10 mV for $Na_v1.7$ from a $V_{1/2}$ holding potential in both the absence and presence of 100 μmol/l APAS and PAS. Peak currents were measured and normalized to the first pulse and plotted against the pulse number (fig. 8, A–D). Data were fitted by the monoexponential equation (see Electrophysiological Recordings under Materials and Methods). APAS significantly reduced the plateau I_{Na} amplitude of $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ from 0.80 ± 0.03 to 0.57 ± 0.03, 0.89 ± 0.01 to 0.49 ± 0.07, and 0.89 ± 0.02 to 0.62 ± 0.06, respectively (fig. 8E). PAS also reduced the plateau I_{Na} amplitudes of $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ from 0.81 ± 0.2 to 0.70 ± 0.03, 0.94 ± 0.01 to 0.73 ± 0.02, and 0.91 ± 0.02 to 0.75 ± 0.01, respectively, and the reductions

Table 1. Fitted Parameters for Effects of APAS and PAS

	APAS			PAS		
	IC_{50}	EC_{50}	Hill Slope	IC_{50}	EC_{50}	Hill Slope
$Na_v1.2$	12.2 ± 3.5		0.58 ± 0.07	78.4 ± 9.8		0.86 ± 0.03
$Na_v1.6$	40.6 ± 1.9		0.77 ± 0.03	53.8 ± 3.2		1.12 ± 0.03
$Na_v1.7$	130.7 ± 14.7		0.67 ± 0.06	117.8 ± 19.0		0.74 ± 0.04
$Na_v1.8$		61.3 ± 8.5	1.72 ± 0.10		32.7 ± 3.4	2.45 ± 0.47

IC_{50} values, EC_{50} values, and Hill slopes calculated from nonlinear regression analyses of the dose–response curves shown in figure 4. Data are given as mean ± SEM; n = 6 ($Na_v1.2$), 7 ($Na_v1.6$), 5 ($Na_v1.7$), and 5 ($Na_v1.8$).

APAS = allopregnanolone sulfate; EC_{50} = half-maximal effective concentration; IC_{50} = half-maximal inhibitory concentration; Na_v = voltage-gated sodium channel; PAS = pregnanolone sulfate.

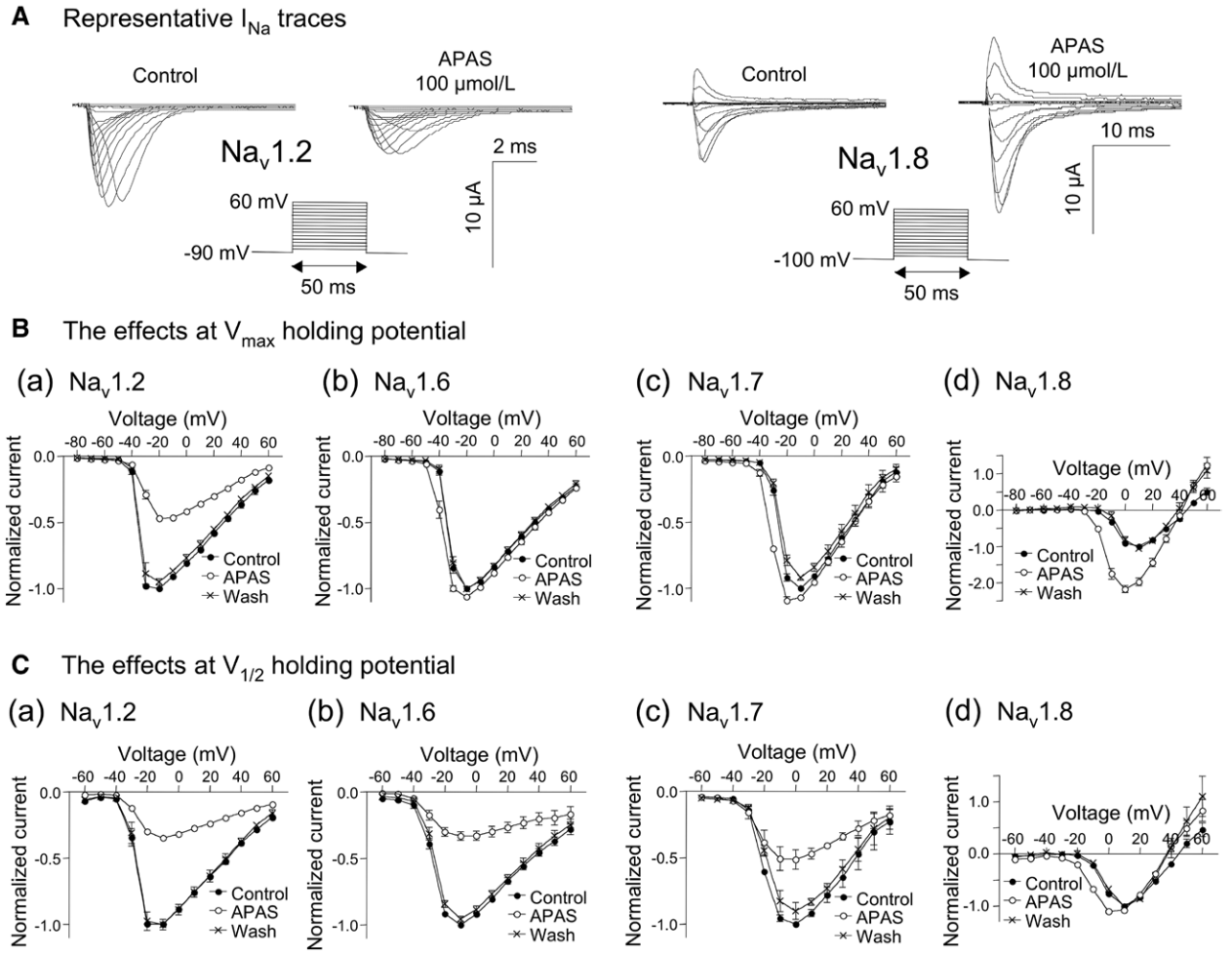

Fig. 5. Effects of allopregnanolone sulfate (APAS) on I–V curves of sodium currents in oocytes expressing $Na_v1.2$ (*a*) (n = 5), $Na_v1.6$ (*b*) (n = 7), $Na_v1.7$ (*c*) (n = 5), or $Na_v1.8$ (*d*) (n = 6) α subunits with β_1 subunits. Currents were elicited using 50-ms depolarizing steps between –80 and 60 mV in 10-mV increments from a V_{max} holding potential and elicited using 50-ms depolarizing steps between –60 and 60 mV in 10-mV increments from a $V_{1/2}$ holding potential. (*A*) Representative I_{Na} traces from oocytes expressing $Na_v1.2$ (*left*) and $Na_v1.8$ (*right*) with the β_1 subunit in both the absence and presence of 100 μmol/l of APAS at V_{max} holding potential are shown. The effects of APAS on normalized I–V curves elicited from V_{max} (*B*) and $V_{1/2}$ holding potentials (*C*) are shown (*closed circles*, control; *open circles*, neurosteroids; *cross*, washout). Peak currents were normalized to the maximal currents observed from –20 to +10 mV. Data are presented as means ± SEM. Na_v = voltage-gated sodium channel; V_{max} holding potential = holding potential causing maximal current; $V_{1/2}$ holding potential = holding potential causing half-maximal current; Wash = washout.

were significant except for $Na_v1.2$ (fig. 8F). These results demonstrated a use-dependent block of APAS and PAS on sodium channels, and the block by APAS was more potent than that by PAS.

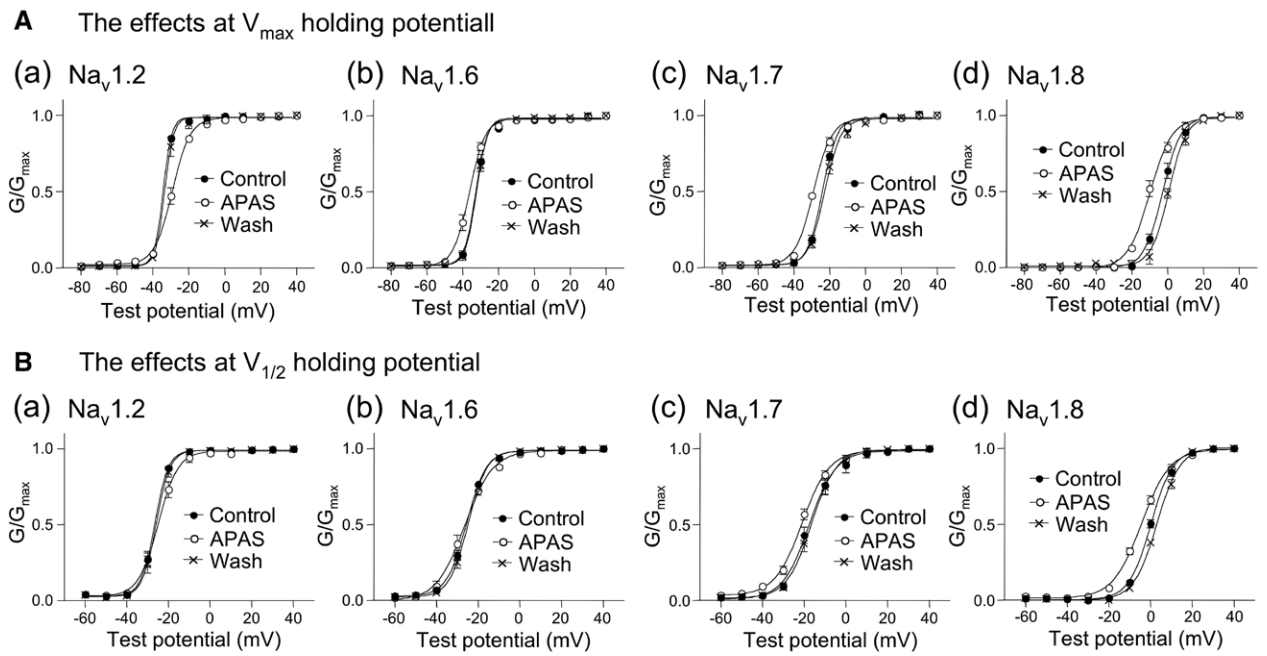

Fig. 6. Effects of allopregnanolone sulfate (APAS) on channel activation in oocytes expressing $Na_v1.2$ (*a*) (n = 5), $Na_v1.6$ (*b*) (n = 7), $Na_v1.7$ (*c*) (n = 5), or $Na_v1.8$ (*d*) (n = 6) α subunits with β_1 subunits from V_{max} (*A*) or $V_{1/2}$ holding potentials (*B*). *Closed circles*, *open circles*, and *cross* represent control, the effect of neurosteroids, and washout, respectively. Data are expressed as means ± SEM. Activation curves were fitted to the Boltzmann equation; $V_{1/2}$ is shown in table 2. Na_v = voltage-gated sodium channel; V_{max} holding potential = holding potential causing maximal current; $V_{1/2}$ holding potential = holding potential causing half-maximal current; Wash = washout.

Table 2. Effects of APAS on Activation and Inactivation

	$V_{1/2}$ (mV)					
	Holding V_{max}			Holding $V_{1/2}$		
	Control	APAS	Shift	Control	APAS	Shift
Activation						
$Na_v1.2$	−34.2 ± 0.5	−29.1 ± 1.0**	+5.1	−26.4 ± 0.8	−24.8 ± 1.1	+1.6
$Na_v1.6$	−32.5 ± 0.6	−36.3 ± 0.9***	−3.8	−25.6 ± 0.6	−26.7 ± 1.3	−1.1
$Na_v1.7$	−23.9 ± 0.6	−29.0 ± 0.3***	−5.1	−17.2 ± 1.7	−20.9 ± 0.9	−3.7
$Na_v1.8$	−2.7 ± 1.1	−9.8 ± 1.2***	−7.1	0.3 ± 0.6	−4.2 ± 0.8**	−4.5
Inactivation						
$Na_v1.2$	−50.1 ± 1.0	−58.1 ± 1.1***	−8.0			
$Na_v1.6$	−57.8 ± 0.5	−66.7 ± 0.7***	−8.9			
$Na_v1.7$	−72.3 ± 1.6	−79.0 ± 1.8***	−6.7			
$Na_v1.8$	−37.0 ± 2.2	−45.9 ± 1.7***	−8.9			

$V_{1/2}$ is calculated from nonlinear regression analyses of activation and inactivation curves shown in figures 6 and 7. Data are given as mean ± SEM; n = 5 ($Na_v1.2$), 7 ($Na_v1.6$), 5 ($Na_v1.7$), and 6 ($Na_v1.8$).

**P < 0.01; ***P < 0.001 compared with control, based on paired t test (two-tailed).

APAS = allopregnanolone sulfate; Holding V_{max} = holding potential causing maximal current; Holding $V_{1/2}$ = holding potential causing half-maximal current; Na_v = voltage-gated sodium channel; $V_{1/2}$ = the potential at which activation is half maximal for activation curve, and the voltage of half-maximal inactivation for inactivation curve.

Discussion

In the current study, we demonstrated that APAS and PAS differentially affected I_{Na} induced by four α subunits at both V_{max} and $V_{1/2}$ holding potentials. Moreover, we found that both neurosteroids suppress $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ at $V_{1/2}$ in a concentration-dependent manner. IC_{50} values indicated that the effect of APAS on $Na_v1.2$ was most potent among the two compounds and three α subunits. To the best of our knowledge, this is the first direct evidence of the various effects of these two neurosteroids on neuronal sodium channel α subunits. It is thought that APAS is synthesized from allopregnanolone by 3α-hydroxysteroid

Table 3. Effects of PAS on Activation and Inactivation

	$V_{1/2}$ (mV)					
	Holding V_{max}			Holding $V_{1/2}$		
	Control	PAS	Shift	Control	PAS	Shift
Activation						
$Na_v1.2$	−33.4 ± 0.6	−30.5 ± 1.5	+2.9	−26.1 ± 0.9	−23.7 ± 1.1**	+2.4
$Na_v1.6$	−32.1 ± 0.5	−32.4 ± 0.8	−0.3	−24.8 ± 0.8	−20.7 ± 1.4**	+4.1
$Na_v1.7$	−23.2 ± 0.5	−23.9 ± 0.6	−0.7	−18.7 ± 1.0	−18.0 ± 0.9	+0.7
$Na_v1.8$	−1.4 ± 2.1	−2.3 ± 1.7	−0.9	−0.2 ± 0.8	−1.1 ± 0.9	−0.9
Inactivation						
$Na_v1.2$	−49.9 ± 0.8	−54.4 ± 1.5**	−4.5			
$Na_v1.6$	−57.5 ± 0.5	−65.5 ± 0.5***	−8.0			
$Na_v1.7$	−72.3 ± 1.0	−78.9 ± 1.0***	−6.6			
$Na_v1.8$	−36.0 ± 1.3	−46.2 ± 1.4**	−10.2			

$V_{1/2}$ is calculated from nonlinear regression analyses of activation and inactivation curves (not shown). Data are given as mean ± SEM; n = 6 ($Na_v1.2$), 7 ($Na_v1.6$), 5 ($Na_v1.7$), and 6 ($Na_v1.8$).

** $P < 0.01$; *** $P < 0.001$ compared with control, based on paired *t* test (two-tailed).

Holding V_{max} = holding potential causing maximal current; Holding $V_{1/2}$ = holding potential causing half-maximal current; Na_v = voltage-gated sodium channel; PAS = pregnanolone sulfate; $V_{1/2}$ = the potential at which activation is half maximal for activation curve, and the voltage of half-maximal inactivation for inactivation curve.

sulfotransferase *in vivo*, because 3α-hydroxysteroid sulfotransferase has been isolated *in vivo*.[26] Therefore, allopregnanolone likely exerts a portion of its effects through APAS, which is its metabolite.

It was reported that the level of endogenous allopregnanolone changes in many physiological and pathological situations within a serum concentration range of 1 to 10 nmol/l.[27,28] However, it is not clear whether allopregnanolone has an analgesic effect in physiological concentrations. A recent study demonstrated that 1 and 10 μmol/l of allopregnanolone reduced mechanical allodynia and thermal heat hyperalgesia in normal and neuropathic pain models in rats after 10-μl intrathecal injection.[29] Another investigator reported that intrathecal administration of 10 μmol/l of allopregnanolone showed antihyperalgesic effects in hyperalgesic rats after spinal nerve ligation.[30] From these previous studies, concentrations approximately 1 μmol/l allopregnanolone at receptive fields are estimated to have an analgesic effect. In the current study, APAS tended to, *albeit* not significantly, suppress the I_{Na} of $Na_v1.2$ at 0.3 μmol/l by 8% and significantly ($P < 0.01$) inhibited it at 1 μmol/l by 19 ± 2%. The IC_{50} value of $Na_v1.2$ inhibition by APAS was 12 μmol/l. It was reported that relatively small degrees of sodium channel inhibition could have profound effects on the neuronal firing rate because a 10% inhibition of sodium current reduces the number of action potentials to 10 from a control response of 21 in 750 ms.[24] Therefore, APAS may reduce neuronal firing for $Na_v1.2$ at a concentration exhibiting the antinociceptive effects of allopregnanolone in animal models, whereas the effects of APAS and PAS on another three α and four α subunits, respectively, may not be pharmacologically relevant because these effects were observed at concentrations over 10 μmol/l. In addition, the effects of highly hydrophobic compounds—such as neurosteroids—we used tend to be attenuated in the voltage-clamp techniques with *Xenopus* oocytes, compared with the whole-cell voltage-clamp methods using mammalian cells. Indeed, it was reported that the enhancing effect by allopregnanolone on $GABA_A$ receptor combination ($\alpha_1\beta_2\gamma_{2L}$) was more potent in the human embryonic kidney 293 cells system (EC_{50}; 41 ± 2 nmol/l)[31] than that in the *Xenopus* oocyte system (EC_{50}; 177 ± 2 nmol/l).[32] This may be a limitation of experiments using the *Xenopus* oocyte expression system; this limitation indicates that APAS might inhibit function of $Na_v1.2$ more potently in a mammalian cell system than in the oocyte system, however, it also could potentiate $Na_v1.8$ function more potently in a mammalian cell. Therefore, further investigation is needed to consider the roles of these α subunits in humans.

Analysis of gating revealed common characteristics but also some differences in the effects of APAS and PAS on different α subunits. A common effect on all α subunits was enhancement of inactivation. Because of this enhancement effect, the inhibitions by two compounds at $V_{1/2}$ holding potentials could be interpreted as stronger effects because they shift inactivation curve to the hyperpolarizing direction, which makes the channel into further inactivated state. In contrast, APAS enhanced peak I_{Na} at V_{max}, shifted activation in the hyperpolarizing direction, and increased sodium currents in the hyperpolarizing range of the inactivation curves for $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$. These changes indicate that APAS shifts channel gating equilibrium toward the open channel state and activates sodium channels. This action might attenuate the effects on the inactivated state and, especially, lead to enhancement of I_{Na} even in the inactivated state ($V_{1/2}$ holding potential) for $Na_v1.8$ in spite of the great enhancement of inactivation. However, for $Na_v1.2$, APAS profoundly suppressed peak I_{Na} at V_{max}, shifted activation in the depolarizing direction at V_{max}, and greatly decreased

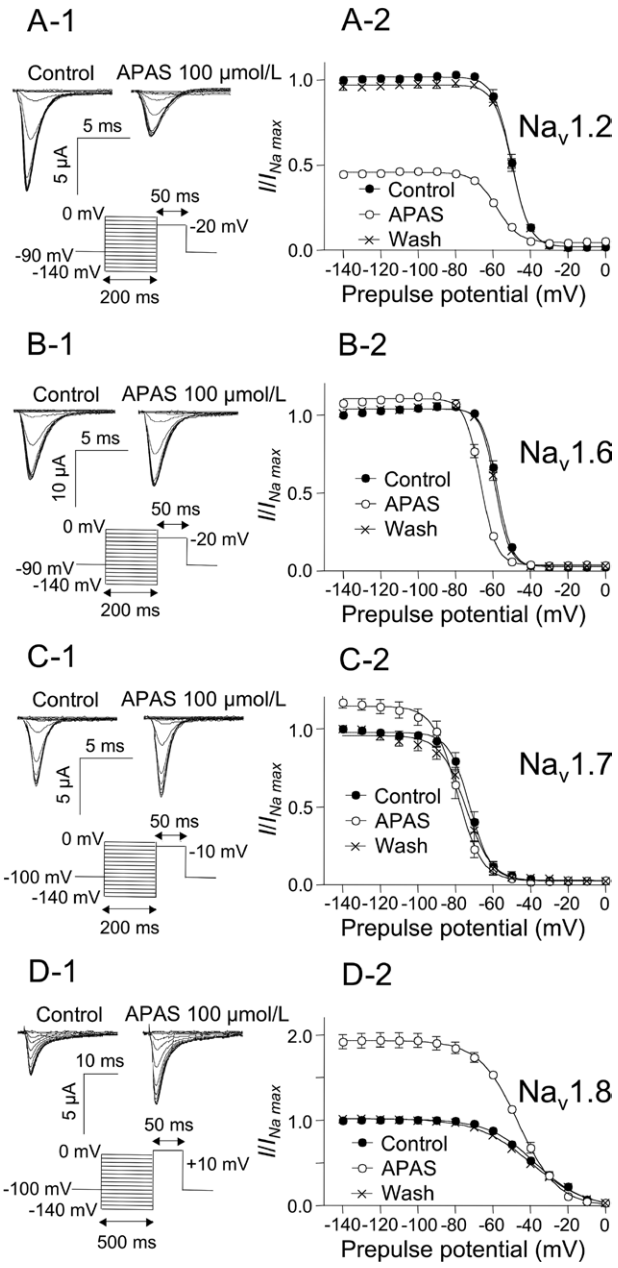

Fig. 7. Effects of allopregnanolone sulfate (APAS) on inactivation curves in oocytes expressing $Na_v1.2$ (*A*) (n = 6), $Na_v1.6$ (*B*) (n = 7), $Na_v1.7$ (*C*) (n = 5), or $Na_v1.8$ (*D*) (n = 6) α subunits with β_1 subunits. Currents were elicited by a 50-ms test pulse to −20 mV for $Na_v1.2$ and $Na_v1.6$, −10 mV for $Na_v1.7$, and +10 mV for $Na_v1.8$ after 200 ms (500 ms for only $Na_v1.8$) prepulses ranging from −140 mV to 0 mV in 10-mV increments from a V_{max} holding potential. Representative I_{Na} traces in both the absence and presence of APAS are shown in *A-1*, *B-1*, *C-1*, and *D-1*. Effects of APAS on inactivation curves (*closed circles*, control; open circles, neurosteroids; cross, washout) are shown in *A-2*, *B-2*, *C-2*, and *D-2*. Steady-state inactivation curves were fitted to the Boltzmann equation, and the $V_{1/2}$ values are shown in table 2. Data are expressed as means ± SEM. Na_v = voltage-gated sodium channel; Wash = washout.

sodium currents in the hyperpolarizing range of the inactivation curve, indicating that resting channel block is an important mechanism of APAS inhibition for only $Na_v1.2$. Both compounds demonstrated use-dependency for inhibition of $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$, suggesting the ability to slow the recovery time from inactivation.[33] Many investigators have shown that sodium channel blockers, including local anesthetics, tricyclic antidepressants, and volatile anesthetics, enhance steady-state inactivation with no effect on activation and exhibit use-dependent block.[34–36] We demonstrated that APAS enhances inactivation and shows use-dependent block similar to other sodium channel blockers, yet it also has diverse effects on activation according to differences in α subunits. These actions suggest that APAS may have different binding sites or allosteric conformational mechanisms to change sodium channel function, although further investigation with site-directed mutagenesis is needed to rule out nonspecific membrane effects. PAS may have common binding sites with APAS, because it shows similar effects, although these changes were small.

The α subunit consists of four homologous domains (I to IV) containing six transmembrane segments (S1 to S6), and one reentrant P-region connecting S5 to S6 (SS1/SS2). Tetrodotoxin-sensitive α subunits, $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$, are phylogenetically related and show 70 to 80% amino acid sequence identity. In contrast, tetrodotoxin-resistant α subunits, $Na_v1.8$, are phylogenetically distant and show only 55 to 56% sequence identity to the other three α subunits. In addition, the lengths of amino acid sequences of four α subunits differed within the range of 1957 to 2005 residues. Therefore, these differences would result in the diversity in neurosteroid action, especially in the effects on channel activation. Indeed, the longest extracellular regions in the α subunit (IS5 to SS1) are 93, 77, 73, and 66 amino acid residues in $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$, respectively. The diversity in sequence and differences in the effects on activation according to α subunit may be important for clarifying binding sites and the mechanism of $Na_v1.2$ inhibition by APAS in further investigations.

γ-Aminobutyric acid type A receptors have been considered to be important for the analgesic effects of allopregnanolone because it has high potency as a positive $GABA_A$ modulator compared with other neurosteroids. Pregnanolone also affects $GABA_A$ receptors in a manner similar to that of allopregnanolone; nevertheless, its analgesic effect is weak. In fact, pregnanolone was shown to reduce mechanical allodynia without reduction of thermal heat hyperalgesia in a neuropathic pain model in contrast to attenuation of both by allopregnanolone.[28] The investigators suggested that the partial analgesic effects of pregnanolone are caused by suppression of glycine receptors by demonstrating that pregnanolone had a significant analgesic effect only in animals displaying a strychnine-induced allodynia in two types of allodynia models induced by bicuculline and strychnine.[28] Moreover, a recent report demonstrated that

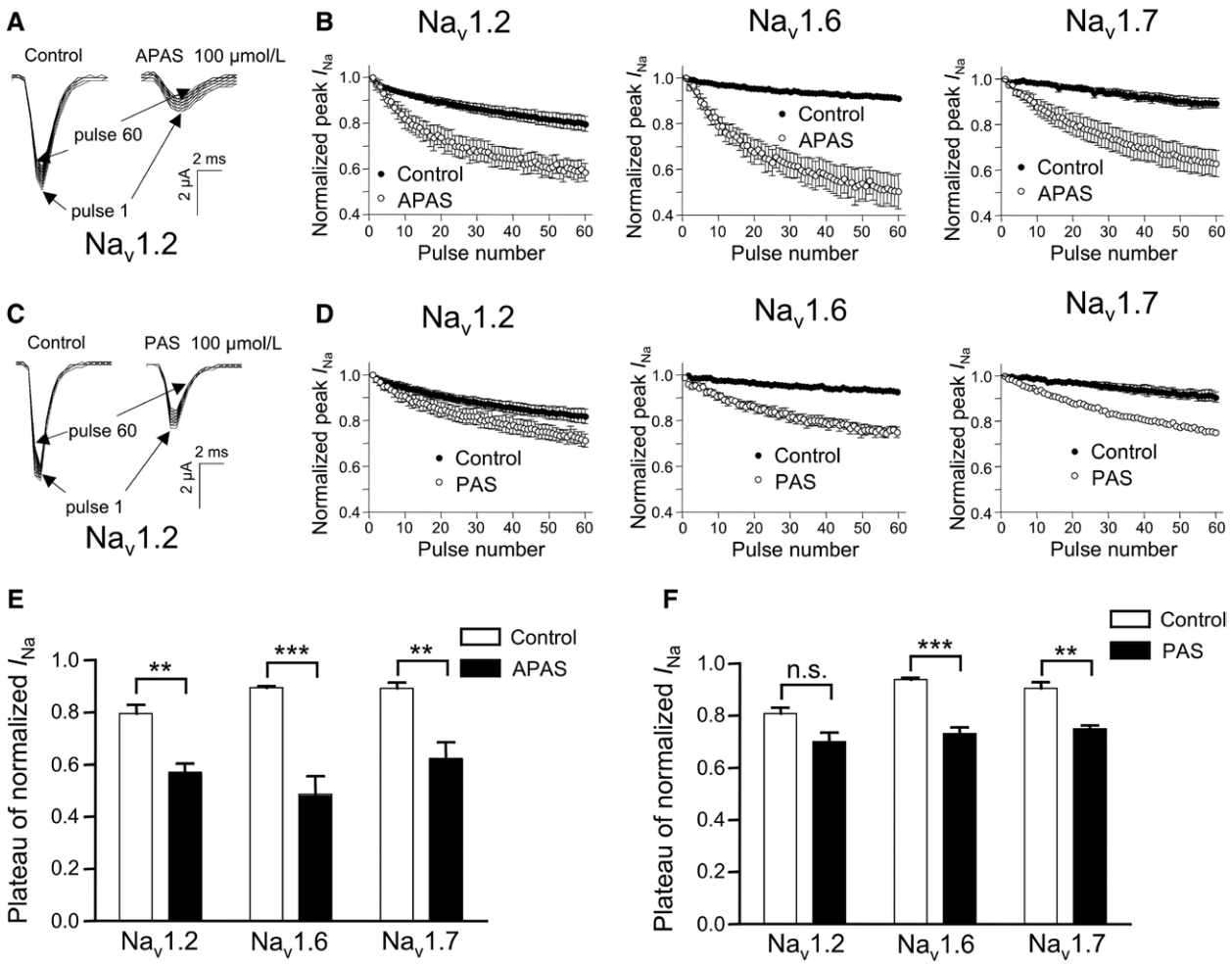

Fig. 8. Use-dependent blockage of sodium channels on Na$_v$1.2 (n = 5), Na$_v$1.6 (n = 6), and Na$_v$1.7 (n = 5) α subunits with β_1 subunits by allopregnanolone sulfate (APAS) and pregnanolone sulfate (PAS). Currents were elicited at 10 Hz by a 20-ms depolarizing pulse of –20 mV for Na$_v$1.2 and Na$_v$1.6 and –10 mV for Na$_v$1.7 from a $V_{1/2}$ holding potential in both the absence and presence of 100 μmol/l of the two compounds; representative I_{Na} traces in both the absence and presence of the two compounds (*A* and *C*). Peak currents were measured and normalized to the first pulse and plotted against the pulse number (*B*, the effects of APAS; *D*, the effects of PAS). *Closed circles* and *open circles* represent control and the effect of neurosteroids, respectively. Data were fitted to the monoexponential equation, and values for fractional blockage of the plateau of normalized I_{Na} are shown in *E* and *F*. Data are expressed as means ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control, based on paired *t* test (two-tailed). Na$_v$ = voltage-gated sodium channel.

allopregnanolone shows analgesic effects in rats through suppression of T-type Ca^{2+} currents and potentiation of $GABA_A$ currents.[16] These previous reports indicate several mechanisms underlying the analgesic effect of allopregnanolone likely exist, as well as potentiation of $GABA_A$ receptors.

Sodium channel α subunits expressed in the dorsal root ganglion (Na$_v$1.7, Na$_v$1.8, and Na$_v$1.9) are thought to be involved in the pathogenesis of inflammatory and neuropathic pain. A recent study reported that Na$_v$1.2 also plays an important role in pain signaling. It was reported that Na$_v$1.2 and Na$_v$1.3 predominantly compose functional sodium channel currents within lamina I/II (dorsal horn) neurons, which mediate acute and chronic nociceptive signals from peripheral nociceptors to pain-processing regions in the brain.[37] Another recent report showed that mutations in Na$_v$1.2 are associated with seizures and pain characterized by headaches and back pain.[38] A disubstituted succinamide, a potent sodium channel blocker, was reported to attenuate nociceptive behavior in a rat model of tonic pain and was demonstrated to potently block Na$_v$1.2, as well as Na$_v$1.7 and Na$_v$1.8, with a potency two orders of magnitude higher than anticonvulsant and antiarrhythmic sodium channel blockers currently used to treat neuropathic pain.[39] Other investigators demonstrated that four sodium channel blockers, including lidocaine, mexiletine, benzocaine, and ambroxol, which are used clinically to treat pain, suppressed recombinant Na$_v$1.2 currents as well as tetrodotoxin-resistant Na^+ channel currents in rat sensory neurons, which comprised mostly Na$_v$1.8 currents. The authors suggested that these sodium channel blockers would induce analgesia according

to the amount of sodium channel blocking, including $Na_v1.2$ and $Na_v1.8$.[40] These recent reports support that suppression of $Na_v1.2$ function by APAS might be a mechanism underlying the analgesic effects of allopregnanolone.

In conclusion, APAS and PAS have diverse effects on $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ α subunits expressed in *Xenopus* oocytes, with differences in the effects on sodium channel gating. In particular, only APAS inhibited sodium currents of $Na_v1.2$ at pharmacologically relevant concentrations. These results raise the possibility that suppression of $Na_v1.2$ by APAS may be important for pain relief by allopregnanolone and provide a better understanding of the mechanisms underlying the analgesic effects of allopregnanolone. However, further studies are needed to clarify the relevance of sodium channel inhibition by APAS.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Horishita: Department of Anesthesiology, School of Medicine, University of Occupational and Environmental Health, 1-1 Isegaoka, Yahatanisiku, Kitakyushu 807–8555, Japan. thori@med.uoeh-u.ac.jp. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Baulieu EE: Neurosteroids: A novel function of the brain. Psychoneuroendocrinology 1998; 23:963–87
2. Compagnone NA, Mellon SH: Neurosteroids: Biosynthesis and function of these novel neuromodulators. Front Neuroendocrinol 2000; 21:1–56
3. Morrow AL: Recent developments in the significance and therapeutic relevance of neuroactive steroids—Introduction to the special issue. Pharmacol Ther 2007; 116:1–6
4. Brinton RD: Neurosteroids as regenerative agents in the brain: Therapeutic implications. Nat Rev Endocrinol 2013; 9:241–50
5. Majewska MD, Harrison NL, Schwartz RD, Barker JL, Paul SM: Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. Science 1986; 232:1004–7
6. Van Hemelrijck J, Muller P, Van Aken H, White PF: Relative potency of eltanolone, propofol, and thiopental for induction of anesthesia. ANESTHESIOLOGY 1994; 80:36–41
7. Zhu D, Wang MD, Bäckström T, Wahlström G: Evaluation and comparison of the pharmacokinetic and pharmacodynamic properties of allopregnanolone and pregnanolone at induction of anaesthesia in the male rat. Br J Anaesth 2001; 86:403–12
8. Kavaliers M, Wiebe JP: Analgesic effects of the progesterone metabolite, 3α-hydroxy-5α-pregnan-20-one, and possible modes of action in mice. Brain Res 1987; 415:393–8
9. Pathirathna S, Todorovic SM, Covey DF, Jevtovic-Todorovic V: 5α-Reduced neuroactive steroids alleviate thermal and mechanical hyperalgesia in rats with neuropathic pain. Pain 2005; 117:326–39
10. Ocvirk R, Pearson Murphy BE, Franklin KB, Abbott FV: Antinociceptive profile of ring A-reduced progesterone metabolites in the formalin test. Pain 2008; 138:402–9
11. Meyer L, Patte-Mensah C, Taleb O, Mensah-Nyagan AG: Allopregnanolone prevents and suppresses oxaliplatin-evoked painful neuropathy: Multi-parametric assessment and direct evidence. Pain 2011; 152:170–81
12. Kawano T, Soga T, Chi H, Eguchi S, Yamazaki F, Yokoyama M: The involvement of the neurosteroid allopregnanolone in the antihyperalgesic effect of paroxetine in a rat model of neuropathic pain. Neuroreport 2011; 22:984–8
13. Sasso O, Russo R, Vitiello S, Raso GM, D'Agostino G, Iacono A, Rana GL, Vallée M, Cuzzocrea S, Piazza PV, Meli R, Calignano A: Implication of allopregnanolone in the antinociceptive effect of *N*-palmitoylethanolamide in acute or persistent pain. Pain 2012; 153:33–41
14. Aouad M, Petit-Demoulière N, Goumon Y, Poisbeau P: Etifoxine stimulates allopregnanolone synthesis in the spinal cord to produce analgesia in experimental mononeuropathy. Eur J Pain 2014; 18:258–68
15. Jasmin L, Wu MV, Ohara PT: GABA puts a stop to pain. Curr Drug Targets CNS Neurol Disord 2004; 3:487–505
16. Pathirathna S, Brimelow BC, Jagodic MM, Krishnan K, Jiang X, Zorumski CF, Mennerick S, Covey DF, Todorovic SM, Jevtovic-Todorovic V: New evidence that both T-type calcium channels and $GABA_A$ channels are responsible for the potent peripheral analgesic effects of 5α-reduced neuroactive steroids. Pain 2005; 114:429–43
17. Kussius CL, Kaur N, Popescu GK: Pregnanolone sulfate promotes desensitization of activated NMDA receptors. J Neurosci 2009; 29:6819–27
18. Catterall WA: From ionic currents to molecular mechanisms: The structure and function of voltage-gated sodium channels. Neuron 2000; 26:13–25
19. Catterall WA, Goldin AL, Waxman SG: International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacol Rev 2005; 57:397–409
20. Wood JN, Boorman JP, Okuse K, Baker MD: Voltage-gated sodium channels and pain pathways. J Neurobiol 2004; 61:55–71
21. Cummins TR, Sheets PL, Waxman SG: The roles of sodium channels in nociception: Implications for mechanisms of pain. Pain 2007; 131:243–57
22. Wang W, Gu J, Li YQ, Tao YX: Are voltage-gated sodium channels on the dorsal root ganglion involved in the development of neuropathic pain? Mol Pain 2011; 7:16
23. Horishita T, Ueno S, Yanagihara N, Sudo Y, Uezono Y, Okura D, Sata T: Inhibition by pregnenolone sulphate, a metabolite of the neurosteroid pregnenolone, of voltage-gated sodium channels expressed in *Xenopus* oocytes. J Pharmacol Sci 2012; 120:54–8
24. Horishita T, Eger EI II, Harris RA: The effects of volatile aromatic anesthetics on voltage-gated Na^+ channels expressed in *Xenopus* oocytes. Anesth Analg 2008; 107:1579–86
25. Scholz A, Kuboyama N, Hempelmann G, Vogel W: Complex blockade of TTX-resistant Na+ currents by lidocaine and bupivacaine reduce firing frequency in DRG neurons. J Neurophysiol 1998; 79:1746–54
26. Driscoll WJ, Martin BM, Chen HC, Strott CA: Isolation of two distinct 3-hydroxysteroid sulfotransferases from the guinea pig adrenal. Evidence for 3α-hydroxy *versus* 3 β-hydroxy stereospecificity. J Biol Chem 1993; 268:23496–503
27. Schlichter R, Keller AF, De Roo M, Breton JD, Inquimbert P, Poisbeau P: Fast nongenomic effects of steroids on synaptic

transmission and role of endogenous neurosteroids in spinal pain pathways. J Mol Neurosci 2006; 28:33–51

28. Mellon SH: Neurosteroid regulation of central nervous system development. Pharmacol Ther 2007; 116:107–24
29. Charlet A, Lasbennes F, Darbon P, Poisbeau P: Fast non-genomic effects of progesterone-derived neurosteroids on nociceptive thresholds and pain symptoms. Pain 2008; 139:603–9
30. Kawano T, Soga T, Chi H, Eguchi S, Yamazaki F, Kumagai N, Yokoyama M: Role of the neurosteroid allopregnanolone in the hyperalgesic behavior induced by painful nerve injury in rats. J Anesth 2011; 25:942–5
31. Akk G, Li P, Bracamontes J, Reichert DE, Covey DF, Steinbach JH: Mutations of the $GABA_A$ receptor α1 subunit M1 domain reveal unexpected complexity for modulation by neuroactive steroids. Mol Pharmacol 2008; 74:614–27
32. Lambert JJ, Belelli D, Harney SC, Peters JA, Frenguelli BG: Modulation of native and recombinant $GABA_A$ receptors by endogenous and synthetic neuroactive steroids. Brain Res Brain Res Rev 2001; 37:68–80
33. Wang GK, Russell C, Wang SY: State-dependent block of voltage-gated Na^+ channels by amitriptyline *via* the local anesthetic receptor and its implication for neuropathic pain. Pain 2004; 110:166–74
34. Ragsdale DS, McPhee JC, Scheuer T, Catterall WA: Molecular determinants of state-dependent block of Na^+ channels by local anesthetics. Science 1994; 265:1724–8
35. Poyraz D, Bräu ME, Wotka F, Puhlmann B, Scholz AM, Hempelmann G, Kox WJ, Spies CD: Lidocaine and octanol have different modes of action at tetrodotoxin-resistant Na^+ channels of peripheral nerves. Anesth Analg 2003; 97:1317–24
36. Ouyang W, Herold KF, Hemmings HC Jr: Comparative effects of halogenated inhaled anesthetics on voltage-gated Na^+ channel function. Anesthesiology 2009; 110:582–90
37. Hildebrand ME, Mezeyova J, Smith PL, Salter MW, Tringham E, Snutch TP: Identification of sodium channel isoforms that mediate action potential firing in lamina I/II spinal cord neurons. Mol Pain 2011; 7:67
38. Liao Y, Anttonen AK, Liukkonen E, Gaily E, Maljevic S, Schubert S, Bellan-Koch A, Petrou S, Ahonen VE, Lerche H, Lehesjoki AE: SCN2A mutation associated with neonatal epilepsy, late-onset episodic ataxia, myoclonus, and pain. Neurology 2010; 75:1454–8
39. Priest BT, Garcia ML, Middleton RE, Brochu RM, Clark S, Dai G, Dick IE, Felix JP, Liu CJ, Reiseter BS, Schmalhofer WA, Shao PP, Tang YS, Chou MZ, Kohler MG, Smith MM, Warren VA, Williams BS, Cohen CJ, Martin WJ, Meinke PT, Parsons WH, Wafford KA, Kaczorowski GJ: A disubstituted succinamide is a potent sodium channel blocker with efficacy in a rat pain model. Biochemistry 2004; 43:9866–76
40. Weiser T: Comparison of the effects of four Na^+ channel analgesics on TTX-resistant Na^+ currents in rat sensory neurons and recombinant $Na_v1.2$ channels. Neurosci Lett 2006; 395:179–84