Published in final edited form as:

J Neurochem. 2010 June ; 113(6): 1611–1620. doi:10.1111/j.1471-4159.2010.06722.x.

Regional differences in nerve terminal Na⁺ channel subtype expression and Na⁺ channel-dependent glutamate and GABA release in rat central nervous system

Robert I. Westphalen, Jieying Yu, Margarita Krivitski, Ting-Yu Jih, and Hugh C. Hemmings Jr.

Departments of Anesthesiology and Pharmacology, Weill Cornell Medical College, New York, NY 10065

Abstract

We tested the hypothesis that expression of presynaptic voltage-gated Na⁺ channel (Na_v) subtypes coupled to neurotransmitter release differs between transmitter types and CNS regions in a nerve terminal-specific manner. Na_v coupling to transmitter release was determined by measuring the sensitivity of 4-aminopyridine (4AP)-evoked [³H]glutamate and [¹⁴C]GABA release to the specific Na_v blocker tetrodotoxin (TTX) for nerve terminals isolated from rat cerebral cortex, hippocampus, striatum and spinal cord. Expression of various Na_v subtypes was measured by immunoblotting using subtype-specific antibodies. Potencies of TTX for inhibition of glutamate and GABA release were similar between CNS regions. However, the efficacies of TTX for inhibition of 4AP-evoked glutamate release were greater than for inhibition of GABA release in all regions except spinal cord. The relative nerve terminal expression of total Na_v subtypes as well as of specific subtypes varied considerably between CNS regions. The region-specific potencies of TTX for inhibition of 4AP-evoked glutamate release correlated with greater relative expression of total nerve terminal Na_v and Na_v1.2. Nerve terminal-specific differences in the expression of specific Na_v subtypes contribute to transmitter-specific and regional differences in pharmacological sensitivities of transmitter release.

Introduction

Calcium-triggered exocytosis of neurotransmitter-containing synaptic vesicles requires the coordinated activation of presynaptic voltage-gated ion channels including Na⁺ (Na_v) and Ca²⁺ (Ca_v) channels (Meir *et al.* 1999, Vacher *et al.* 2008). These members of the voltage-gated superfamily of ion channels are expressed as multiple subtypes with distinct functional roles, pharmacological properties, and tissue and developmental expression (Goldin 2001, Yu and Catterall 2003, Lai *et al.* 2004, Dib-Hajj and Priestly 2009). Compared to presynaptic Ca²⁺ channels coupled to neurotransmitter release (Catterall and Few 2008), relatively little is known about the expression and function of presynaptic Na⁺ channels. Nerve terminal Na⁺ channels are important in the amplification of presynaptic action potentials (Engel and Jonas, 2005). Inhibitors of Na⁺ and/or Ca²⁺ channels reduce neurotransmitter release from nerve terminals by reducing depolarization, Ca²⁺ entry, and Ca²⁺-dependent exocytosis (Wu and Saggau 1997). Transmitter-specific and regional differences in presynaptic Ca_v subtypes coupled to synaptic vesicle exocytosis lead to differences in the sensitivity of transmitter release to potent invertebrate neurotoxins (Bowman *et al.* 1993, Reid *et al.* 1997, Meir *et al.* 1999). A number of drugs including

anticonvulsants, neuroprotective agents, and volatile anesthetics selectively inhibit glutamate release over other transmitters apparently by preferential inhibition of presynaptic Na^+ channels relative to Ca^{2+} channels (Westphalen and Hemmings 2003, Sitges *et al.* 2007). The pharmacological selectivity of these Na_v blockers supports differences in ion channel mechanisms between excitatory glutamatergic and inhibitory GABAergic synapses (Prakriya and Mennerick 2000).

Presynaptic specializations leading to distinct pharmacological sensitivities for the release of various neurotransmitters could result from differential expression of presynaptic ion channel subtypes (Bowman *et al.* 1993, Reid *et al.* 1997, Mechaly *et al.* 2005), ion channel accessory subunits (Wynne *et al.* 2009), ionotropic receptors (MacDermott *et al.* 1999, Long *et al.* 2009, Westphalen *et al.* 2009), post-translational ion channel modulation (Hemmings 1998, MacDermott *et al.* 1999), excitation-secretion coupling mechanisms (Prakriya and Mennerick 2000), and/or synaptic vesicle fusion machinery (Südhof 2004). We explored the hypothesis that nerve terminal-specific differences in presynaptic expression of Na_v subtypes result in pharmacological differences in the Ca^{2+} -dependent release of specific neurotransmitters. The effects of the specific subtype-selective Na_v antagonists tetrodotoxin (Stuart and Sakmann 1994, Campos *et al.* 2004) and A-803467 (Jarvis *et al.* 2007) and of the subtype-selective Na_v agonist veratridine (Farrag *et al.* 2008) on evoked glutamate and GABA release were determined for nerve terminals isolated from four functionally distinct regions of rat central nervous system (CNS). For comparison, the relative expression of the major CNS Na_v subtypes was examined. There are 9 distinct Na_v pore-forming α -subunit subtypes ($\text{Na}_v1.1$ – 1.9) with distinct tissue distributions, electrophysiological properties, and toxin sensitivities (Goldin 2001, Strichartz 2008). Tetrodotoxin-sensitive (TTX-s) Na_v subtypes ($\text{Na}_v1.1$, 1.2, 1.3, 1.6, 1.7) are inhibited by TTX more potently than the TTX-resistant (TTX-r) subtypes ($\text{Na}_v1.4$, 1.5, 1.8, 1.9) (Goldin 2001, Lai *et al.* 2004), and are selectively activated by the phytotoxin veratridine (VTD, Farrag *et al.* 2008). We utilized these pharmacological distinctions to probe for differences between CNS regions in the regulation of glutamate and GABA release by specific Na_v subtypes. We hypothesized that differences in the presynaptic expression and/or function of Na_v subtypes coupled to transmitter release result in differential sensitivities of release to subtype-selective drugs.

Materials and methods

Materials

Tetrodotoxin (TTX), 4-aminopyridine (4AP), veratridine (VTD), and A-803467 were from Sigma-Aldrich Chemical Co. (St. Louis, MO). L-[^3H]Glutamate was from Amersham Radiochemical Centre (42 Ci/mmol; Buckinghamshire, UK). [^{14}C]GABA was from PerkinElmer Inc. (28 mCi/mmol; Boston, MA) or American Radiolabel Chemicals Inc. (55 mCi/mmol; St. Louis, MO).

Nerve terminal preparation

Experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals as approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee. Isolated nerve terminals (synaptosomes) from adult (200–300 g) male Sprague-Dawley rat (Charles River Laboratories, Troy, NY) cerebral cortex, hippocampus, striatum and spinal cord were prepared and demyelinated as described (Westphalen and Hemmings 2006a). Synaptosomes were incubated with 5–10 nM L-[^3H]glutamate and 400–500 nM [^{14}C]GABA for 15 min at 30°C in 10 ml Krebs-HEPES buffer (KHB, composition in mM: NaCl 140, KCl 5, HEPES 20, MgCl_2 1, Na_2HPO_4 1.2, NaHCO_3 5, EGTA 0.1, and D-glucose 10, pH 7.4 with NaOH).

Synaptosomes were collected by centrifugation for 10 min at $20,000 \times g$ at 4°C , resuspended in ice-cold 0.32 M sucrose, and loaded into release chambers.

Glutamate and GABA release

Simultaneous release of glutamate and GABA was assayed as described (Westphalen and Hemmings 2006b). Briefly, dual-labeled synaptosomes (0.3–0.8 mg protein) confined by Whatman (Maidstone, UK) GF/B glass fiber filter disks were superfused at 0.5 ml/min with KHB at 37°C using a customized Brandel SF12 superfusion apparatus (Gaithersburg, MD) set to collect 1 min fractions. Synaptosomes were superfused with TTX (0.1–3000 nM) 12 min prior to, during and following a 2 min depolarizing pulse of either 1 mM 4AP or 10 μM VTD in the absence or presence of 1.9 mM free extracellular Ca^{2+} (obtained by adding 2 mM CaCl_2 to KHB). In separate experiments, 4AP-evoked release was determined from synaptosomes exposed to 1 μM A-803467, a selective $\text{Na}_v1.8$ blocker (Jarvis *et al.* 2007), in the presence of 1 μM TTX to block TTX-s Na_v subtypes. Experiments were terminated by perfusion of 0.2 M perchloric acid to lyse synaptosomes. Radioactivity in each fraction was quantified by liquid scintillation spectrometry with dual isotope quench correction (Beckman Coulter LS 6000IC, Fullerton, CA) using BioSafe II scintillation cocktail (RPI, Mt. Prospect, IL).

Release of transmitter in each 1 min fraction of perfusate was expressed as a fraction of synaptosomal content of labeled transmitter prior to that fraction (fractional release [FR], Westphalen and Hemmings 2006b). The magnitude of evoked release was determined by subtracting baseline release (average of basal FR before and after stimulation) from cumulative FR values for each evoked release pulse to yield sum FR. Release data from each CNS region were generated from 2–5 (4AP-evoked) and 2–3 (VTD-evoked release) independent experiments. Each experiment included control (in duplicate), which was used to normalize data to the mean of all control values prior to curve fitting and/or statistical analysis. TTX concentration-effect data were fitted to a four-parameter logistic equation [$Y = I_{\text{max}} + (I_0 - I_{\text{max}}) / (1 + 10^{((X - \text{LogIC}_{50}) * \text{HillSlope}))})$] by least-squares analysis to estimate IC_{50} (Prism 5.0; GraphPad Software, San Diego CA). The curve fit parameters I_0 (upper plateau of the curve) and I_{max} (lower plateau of curve) were tested for significant differences from control or zero sum ΔFR , respectively. If no difference was found, I_0 was constrained to control and/or I_{max} was constrained to zero sum ΔFR . Maximal release inhibition ($I_0 - I_{\text{max}}$) was determined as percentage of control sum ΔFR . Significant differences between IC_{50} or I_{max} values were determined by F-test comparisons between best-fit values derived from separate curve fits to values derived from global fits with the tested parameter shared. The relative magnitude of control GABA vs. glutamate release was compared between secretagogues by subtracting sum ΔFR of glutamate from sum ΔFR of GABA and expressing the difference as percentage of evoked glutamate release. Mean sum ΔFR data were tested for differences between multiple groups by one-way analysis of variance (ANOVA) with Tukey *post hoc* test. Data compared between two groups used the Student t-test, with Welch correction if an F-test determined the group variances as not identical.

Quantitative immunoblotting

Immunoreactivity was analyzed in nerve terminal preparations using rabbit antibodies specific for the α -subunits of all Na_v1 subtypes (pan- Na_v1), or subtype-specific antibodies against rat amino acid residues 465–481 of $\text{Na}_v1.1$, 467–485 of $\text{Na}_v1.2$, 511–524 of $\text{Na}_v1.3$ (Sigma-Aldrich), 1042–1061 of $\text{Na}_v1.6$ (Millipore, Temecula, CA), 446–460 of $\text{Na}_v1.7$ (Alomone Labs, Jerusalem, Israel), 1943–1956 or 1943–1957 of $\text{Na}_v1.8$ (Millipore or Sigma-Aldrich), and 1748–1765 of $\text{Na}_v1.9$ (Millipore). Rat cerebral cortex, hippocampus, striatum and spinal cord synaptosomes were prepared as described above with the addition of a protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin,

bestatin, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, leupeptin, and pepstatin A; Sigma-Aldrich). Protein concentrations were determined by the method of Bradford (Bradford 1976) using bovine serum albumin as a standard before solubilization in 1% (w/v) sodium dodecyl sulfate (SDS). Aliquots containing equal protein amounts were separated by SDS-polyacrylamide (7.5%) gel electrophoresis, and proteins were transferred onto polyvinylidene difluoride membrane (0.45 μ m, Bio-Rad, Hercules, CA) as described (Hemmings *et al.* 1992). Protein amounts loaded were within the linear range of quantification for each antibody. Membranes were incubated in blocking buffer (5% dried milk powder (w/v) in PBST [10 mM Na-phosphate, 150 mM NaCl, 0.5% (v/v) Tween 20, pH 7.8]) for 1 h, probed with anti-pan Na_v1 (1:1000 dilution; 10–20 μ g protein load), anti-Na_v1.1 (1:400 dilution; 20–30 μ g protein load), anti-Na_v1.2 (1:200 dilution; 10–20 μ g protein load), anti-Na_v1.3 (1:400 dilution; 100 μ g protein load), anti-Na_v1.6 (1:200 dilution; 50–120 μ g protein load), anti-Na_v1.7 (1:100 dilution; 80–100 μ g protein load), anti-Na_v1.8 (1:200 dilution; 90–100 μ g protein load), anti-Na_v1.9 (1:100 dilution; 100 μ g protein load) for 1 h. Membranes were then washed 3 $\times$ 10 min with PBST and bound antibody was detected using horseradish peroxidase-conjugated goat-anti-rabbit antiserum and ECL (Amersham/GE Healthcare, Pittsburgh, PA).

Exposed films of immunoblots were scanned with a Typhoon Trio9410 variable mode imager (Amersham/GE Healthcare) using a 633 nm He-Ne laser and photomultiplier running at 600 V. Signal intensity was quantified using ImageQuant 5.2 software (Amersham/GE Healthcare) with local average background correction. Relative densities of immunoreactive bands were determined using 2–3 different amounts of protein per region to insure linearity. Immunoreactivity in each brain region was normalized to cerebral cortex on the same blot, and averaged from two exposure times. Relative expression in each CNS region were compared to cortex by repeated measures one-way ANOVA with Tukey *post hoc* test (Prism 5.0). Correlations of Na_v immunoreactivity (relative to cortex) with potencies (1/IC₅₀) of TTX for inhibition of 4AP-evoked transmitter release were tested for significance using the Pearson correlation (Prism 5.0).

Results

4AP-evoked release

The K⁺ channel blocker 4AP evokes transmitter release from isolated nerve terminals by a mechanism that requires sequential activation of Na⁺ and Ca²⁺ channels following transient membrane depolarizations (Tibbs *et al.* 1989). Stimulation of nerve terminals with a 2 min pulse of 1 mM 4AP in the presence of 1.9 mM free external Ca²⁺ evoked both L-[³H]glutamate and [¹⁴C]GABA release (Fig. 1). TTX inhibited 4AP-evoked glutamate and GABA release from all four CNS regions in a concentration-dependent manner (Fig. 1) with IC₅₀ values of 5–30 μ M (Fig. 2A). TTX inhibited 4AP-evoked glutamate release from hippocampal or striatal nerve terminals with greater potency than release from cortical nerve terminals. Inhibition of glutamate release from spinal cord nerve terminals was relatively low. TTX inhibited 4AP-evoked GABA release from cortical, striatal and spinal cord nerve terminals equipotently, and with less potency from hippocampal terminals. TTX inhibited glutamate and GABA release equipotently from cortical terminals, while potency for inhibition of glutamate release was greater than for GABA release from hippocampal and striatal terminals.

Glutamate and GABA release evoked by 4AP was not completely inhibited by TTX: the lower plateaus of all curve fits (I_{max}) did not reach zero (cortex, hippocampus and striatum, P < 0.0001; spinal cord, P < 0.05). Maximal inhibition (efficacy) by TTX expressed as a percent of control release was significantly greater for glutamate release than for GABA release in cortex, hippocampus and striatum, but similar in spinal cord (Fig. 2B). Maximal

inhibition by TTX of 4AP-evoked glutamate release from cortical, striatal, and spinal cord nerve terminals was similar, but less than that from hippocampal nerve terminals. Maximal inhibition by TTX of GABA release from cortex and spinal cord was less than that from hippocampal and striatal nerve terminals. Ca^{2+} -independent 4AP-evoked release of both glutamate and GABA release from cortex was inhibited by TTX as previously reported (Westphalen and Hemmings 2003 Westphalen and Hemmings 2006b), but not from spinal cord (Fig. 3). In spinal cord, TTX inhibited GABA but not glutamate release.

A role for selective $\text{Na}_v1.8$ expression leading to relatively lower potency for TTX inhibition of 4AP-evoked glutamate release from spinal cord nerve terminals was tested using the selective $\text{Na}_v1.8$ antagonist A-803467 (Jarvis *et al.* 2007). In the presence of TTX to inhibit TTX-s Na_v subtypes, A-803467 further inhibited 4AP-evoked glutamate release from spinal cord but not from cortical nerve terminals, but had no effect on GABA release (Fig. 3). The additive inhibition by A-803467 and TTX approached residual Ca^{2+} -independent glutamate release evoked by 4AP in spinal cord. A-803467 alone, at a high nonselective concentration (10 μM) that also inhibits TTX-s Na_v channels (Jarvis *et al.* 2007), inhibited 4AP-evoked glutamate and GABA release from cortical nerve terminals by $30 \pm 2\%$ ($n=3$, data not shown).

Control sum ΔFR of GABA was greater than that of glutamate for all four rat CNS regions tested (by 1.6 – 2.3-fold; $P < 0.001$; Fig. 4A), as reported previously for rat cortex (Westphalen and Hemmings 2003). Control glutamate release from cortical nerve terminals ($n=36$) was equivalent to release from hippocampal ($n=27$) and striatal terminals ($n=29$), and greater than that from spinal cord terminals ($n = 26$; $P < 0.001$). Control GABA release from cortical nerve terminals was equivalent to release from hippocampal terminals, and greater than that from striatal ($P < 0.001$) and spinal cord terminals ($P < 0.001$).

Veratridine-evoked release

Veratridine evokes transmitter release from isolated nerve terminals by effecting Na_v activation through impaired channel inactivation leading to sustained membrane depolarization (Ulbricht 1998). 4AP-evoked GABA release was consistently greater than glutamate release in all four CNS regions (Fig. 4A). In contrast, VTD-evoked release of glutamate and GABA from cortical and striatal nerve terminals was similar, while GABA release was greater than glutamate release from hippocampal (1.5-fold; $P < 0.01$) and spinal cord (3-fold; $P < 0.05$) nerve terminals (Fig. 4B). The difference between GABA and glutamate release from spinal cord nerve terminals was greater for VTD-evoked release compared to 4AP-evoked release, primarily due to the lower efficacy of VTD-evoked glutamate release.

Although TTX inhibition of VTD-evoked release was qualitatively similar to inhibition of 4AP-evoked release (Fig. 1), TTX completely inhibited VTD-evoked release from all four regions tested (represented by cortex; Fig. 5A). This is consistent with an absolute requirement for activation of presynaptic Na^+ channels for depolarization by VTD but not by 4AP. TTX inhibited VTD-evoked glutamate and GABA release from cortical, hippocampal, and striatal nerve terminals equipotently, but was far less potent in inhibiting glutamate release from spinal cord nerve terminals (Fig. 5B). The potency of TTX for inhibition of VTD-evoked GABA release did not differ between regions, and was greater compared to inhibition of spinal cord glutamate release (Fig. 5B).

Na^+ channel immunoreactivity

An antibody directed against a conserved epitope that recognizes all vertebrate $\text{Na}_v1 \alpha$ -subunits (Toledo-Aral *et al.* 1997) was used to probe nerve terminal membrane preparations

from rat cerebral cortex, hippocampus, striatum and spinal cord for total Na_v expression (all subtypes) normalized to total protein (Fig. 6). Immunoblots revealed the presence of immunoreactivity at a relative molecular mass of 260–280 kDa corresponding to the glycosylated ~260 kDa Na_v α-subunits present in adult rat brain (Messner and Catterall 1985). The relative expression of total Na_v1 immunoreactivity in nerve terminal preparations was striatum (1.4-fold higher; $P < 0.01$) > hippocampus = cortex > spinal cord (0.4-fold lower; $P < 0.001$) (Fig. 6). Subtype-specific antibodies were used to probe relative expression of the major Na⁺ channel α-subunits expressed in rat CNS: Na_v1.1, 1.2, 1.3, 1.6, 1.7, 1.8 and 1.9 (Goldin 2001). Cortical, hippocampal and striatal nerve terminals showed similar expression of Na_v1.1, while expression in spinal cord terminals was 1.8-fold greater ($P < 0.001$, Fig. 7A). Cortical and hippocampal nerve terminals expressed comparable amounts of Na_v1.2, while Na_v1.2 expression was 2.0-fold greater ($P < 0.001$) in striatum and very low amounts ($P < 0.001$) are expressed in spinal cord (Fig. 7B). Expression of Na_v1.6 was 3.5-fold ($P < 0.05$), 4.4-fold ($P < 0.01$) and 4.2-fold ($P < 0.05$) greater, respectively, in hippocampus, striatum and spinal cord relative to cortical nerve terminals (Fig. 7C). Cortical, hippocampal and striatal terminals expressed comparable amounts of Na_v1.7, while Na_v1.7 expression was 40-fold greater ($P < 0.001$) in spinal cord terminals (Fig. 7D). No Na_v1.3, Na_v1.8 or Na_v1.9 immunoreactivity was detected in the regions tested with the antibodies used (data not shown).

Immunoreactive Na_v1, Na_v1.1, Na_v1.2, and Na_v1.6 were enriched in nerve terminal preparations compared to whole tissue homogenates in hippocampus (by 2.4-, 1.4-, 1.7-, and 3.1-fold, respectively), striatum (by 52-, 3.1-, 63-, and 9.5-fold, respectively) and spinal cord (by 5.1-, 46-, 22-, and 120-fold, respectively), but not in cerebral cortex (1.2-, 0.7-, 0.5-, and 0.9-fold, respectively). Immunoreactive Na_v1.7 was enriched in striatal nerve terminal preparations compared to whole homogenates (1.5-fold), but not in cortex (1.1-fold), hippocampus (1.2-fold), or spinal cord (1.0-fold) preparations.

Relative expression of the TTX-s Na_v subtypes Na_v1.1, Na_v1.6, or Na_v1.7 correlated either negatively or did not correlate with potencies of TTX (1/IC₅₀) for inhibition of 4AP-evoked glutamate release between CNS regions (Table 1). Expression of the TTX-s Na_v1.2 and of total Na_v1 immunoreactivity correlated positively with potency of TTX for inhibition of glutamate release. There were no correlations between Na_v expression and potency of TTX for inhibition of GABA release.

Discussion

Voltage-gated Na⁺ channels are critical upstream regulators of depolarization-evoked Ca²⁺-dependent exocytosis of amino acid neurotransmitters from small synaptic vesicles (Tibbs *et al.* 1989). We hypothesized that presynaptic differences in Na_v subtype expression and/or function underlie neurotransmitter-specific and regional differences in the physiology and pharmacology of release. Although transmitter and regional differences are known for presynaptic Ca²⁺ channel subtypes (Bowman *et al.* 1993, Reid *et al.* 1997, Vacher *et al.* 2008), comparable differences have not been described for Na⁺ channels (Vacher *et al.* 2008, Dib-Hajj and Priestly 2009). Due to technical limitations in resolving presynaptic from postsynaptic ion channel localization using immunofluorescence microscopy, the localization of specific Na_v subtypes to typical small CNS nerve terminals has been difficult. We used neurochemical approaches to analyze expression of the major CNS Na_v subtype α-subunits in nerve terminals (synaptosomes) isolated from various CNS regions and correlations with transmitter release. Synaptosomes are a subcellular fraction highly enriched for functional nerve terminals capable of synthesizing, releasing, and re-accumulating various neurotransmitters depending on the region used for isolation (Nicholls 1993). We focused our analysis on the release of the abundant CNS neurotransmitters

glutamate and GABA, which are released from the majority of CNS nerve terminals (Raiteri 2008).

We observed significant regional differences in both the sensitivities of depolarization-evoked glutamate and GABA release to inhibition by the Na⁺ channel blocker TTX as well as in the expression of Na_v subtypes. The differences in TTX potency suggest nerve terminal-specific expression and/or function of specific Na_v subtypes, which vary in sensitivity to TTX (Goldin 2001, Lai *et al.* 2004). Relative expression of specific Na_v subtypes varied between CNS regions. Although isolated nerve terminal preparations can be contaminated by postsynaptic and glial membranes, they are highly enriched in presynaptic components (Dodd *et al.* 1981) and in Na_v subtype expression, supporting presynaptic Na_v expression. This is consistent with immunohistochemical studies of Na_v1.2 (Gong *et al.* 1999) and Na_v1.6 (Caldwell *et al.* 2000). Regional expression of Na_v typically correlated better with TTX inhibition potency for glutamate vs. GABA release. Expression of total Na_v1 and Na_v1.2 immunoreactivity correlated with potency of TTX for inhibition of 4AP-evoked glutamate release, suggesting that expression of Na_v1.2, and of overall nerve terminal Na_v1 contribute to region-specific differences in sensitivity of glutamate release to inhibition by Na_v blockers. Na_v1.2 expression is therefore closely coupled to Na⁺ channel-dependent 4AP-evoked glutamate release compared to other Na_v subtypes or to 4AP-evoked GABA release.

Fractional release of GABA evoked by 4AP was consistently greater than that of glutamate in all CNS regions. This demonstrates fundamental differences in release mechanisms that could lead to transmitter-selective pharmacological effects. TTX was generally less potent and consistently less efficacious in inhibiting 4AP-evoked GABA release compared to glutamate release, except in spinal cord. Spinal cord terminals exhibited distinct differences in potencies for TTX inhibition of transmitter release and in Na_v subtype expression compared to forebrain-derived nerve terminals. Less total transmitter release was evoked from spinal cord nerve terminals by VTD, which selectively opens TTX-s channels (Farrag *et al.* 2008), compared to 4AP, consistent with greater involvement of TTX-r subtypes in spinal cord release. This interpretation is supported by the ability of the Na_v1.8-selective inhibitor A-803467 to inhibit glutamate release in spinal cord but not in cortex, consistent with a component of Na_v1.8-mediated glutamate release in spinal cord. Na_v1.8 is highly resistant to TTX (Goldin 2001, Lai *et al.* 2004), so its expression could contribute to the lower potency of TTX inhibition of release. Na_v1.8 immunoreactivity in spinal cord has been detected (Arroyo *et al.*, 2002), but we were unable to detect it by immunoblotting. Together, our data indicate that quantitative and/or qualitative differences in expression of presynaptic Na_v subtypes coupled to transmitter release lead to transmitter-specific and regional differences in sensitivities of release to the Na_v subtype-selective inhibitors TTX and A-803467 and the agonist VTD.

Residual TTX-insensitive 4AP-evoked GABA release was consistently greater than residual glutamate release across regions except spinal cord. TTX-insensitive 4AP-evoked GABA release from cortical and spinal cord nerve terminals contains a Ca²⁺-dependent component, while 4AP-evoked glutamate release does not (Westphalen and Hemmings, 2006b). The presynaptic mechanisms contributing to these differences are unknown. Greater 4AP-evoked GABA vs. glutamate release was not evident in all CNS regions when release was evoked by selective activation of TTX-s Na⁺ channels using VTD (Farrag *et al.* 2008). This might be due to dissimilar contributions of Ca²⁺-dependent vs. Ca²⁺-independent glutamate and GABA release to release evoked by either 4AP or VTD. However, TTX completely inhibited VTD-evoked glutamate and GABA release with similar potencies across brain regions, and had greater efficacy for 4AP-evoked glutamate vs. GABA release inhibition across brain regions. These findings suggest that differences in the relative contributions of

TTX-s Na_v and of TTX-insensitive mechanisms to neurotransmitter release between GABAergic and glutamatergic terminals could determine their distinct pharmacological sensitivities.

The potency of TTX for inhibition of 4AP-evoked glutamate release varied between brain regions (IC_{50} =5–14 nM) in the range of potencies reported for TTX inhibition of Na^+ currents carried by TTX-s Na_v subtypes (Goldin 2001, Lai *et al.* 2004). These Na_v α -subunits exhibit distinct regional and developmental expression in mammalian CNS (Goldin 2001, Lai *et al.* 2004, Vacher *et al.* 2008): $\text{Na}_v1.1$, $\text{Na}_v1.2$ and $\text{Na}_v1.6$ are all widely expressed, $\text{Na}_v1.3$ is selectively expressed early in development, $\text{Na}_v1.7$ has more limited expression, while $\text{Na}_v1.8$ and $\text{Na}_v1.9$ are reported to be exclusively expressed in dorsal root ganglion (DRG) neurons. Consistent with these reports, isolated nerve terminals from rat cortex, hippocampus and striatum expressed only $\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.6$, and $\text{Na}_v1.7$ to varying degrees. The pharmacological identification of $\text{Na}_v1.8$ in spinal cord terminals is consistent with afferent projections from $\text{Na}_v1.8$ mRNA-rich DRG neurons to spinal cord (Ohshiro *et al.* 2007), the role of $\text{Na}_v1.8$ in transmitter release from DRG neurons (Medvedeva *et al.* 2009), and the detection of $\text{Na}_v1.8$ expression in rat spinal cord (Arroyo *et al.* 2002). Expression of $\text{Na}_v1.8$ in rat spinal cord was not detectable by immunoblotting possibly due to a low level of expression, despite positive and negative controls confirming antibody specificity (data not shown).

Rat brain Na_v α -subunit mRNA expression varies between cell types (Vacher *et al.* 2008). Although not directly attributable to presynaptic distribution, mRNA expression detected by *in situ* hybridization indicates that multiple subtypes are expressed in specific CNS regions (Black *et al.* 1994, Felts *et al.* 1997). Multiple Na_v α -subunit mRNA expression in individual neurons has been shown by *in situ* hybridization (Black *et al.* 1994) and single-cell RT-PCR (Mechaly *et al.* 2005). The specific Na_v subtypes expressed in presynaptic terminals and coupled to action potential-evoked neurotransmitter release probably vary between axon terminal types given the considerable differences in regional, neuronal, and subcellular differences in Na_v expression (Gong *et al.* 1999, Caldwell *et al.* 2000, Boiko *et al.* 2003, Engel and Jonas 2005). Our observations of differences between CNS regions in Na_v subtype expression and in sensitivity of release to Na_v -selective agents further supports such cellular and subcellular specialization in Na_v expression and function.

The greater potency of TTX to inhibit 4AP-evoked glutamate release from hippocampal and striatal vs. cortical nerve terminals is consistent with the greater expression of the highly TTX-sensitive $\text{Na}_v1.6$ subtype (IC_{50} =1 nM; Lai *et al.* 2004) in hippocampal and striatal terminals. This is supported by evidence for presynaptic expression of $\text{Na}_v1.2$ and $\text{Na}_v1.6$ obtained by patch-clamp electrophysiological recordings of hippocampal mossy fiber boutons (Engel and Jonas 2005). Lower relative expression of the least TTX-sensitive subtype $\text{Na}_v1.2$ (IC_{50} =18 nM; Lai *et al.* 2004) was found in cortical vs. hippocampal and striatal terminals, suggesting that the balance between Na_v subtype expression might determine presynaptic sensitivity to TTX. However, the TTX sensitivity of transmitter release depends not only on the TTX sensitivities of the expressed Na_v subtypes, but also on their functional coupling to depolarization and downstream release mechanisms. Moreover, the methods used do not allow resolution of the relative expression of Na_v subtypes within a particular nerve terminal type, but only of the average of the population of nerve terminals contained in each preparation.

In conclusion, our findings indicate that nerve terminal-specific differences in the expression of specific Na_v subtypes contribute to transmitter-specific and regional differences in the pharmacological sensitivities of transmitter release.

Acknowledgments

This study was supported by National Institutes of Health Grant GM 58055 and the Department of Anesthesiology, Weill Cornell Medical College. We gratefully acknowledge the expert statistical advice of Dr. James Root, Departments of Psychiatry and Anesthesiology, Weill Cornell Medical College.

Abbreviations

4AP	4-aminopyridine
ΔFR	sum fractional release
FR	fractional release
Na_v	voltage-gated sodium channel
VTD	veratridine
TTX	tetrodotoxin
TTX-s	tetrodotoxin-sensitive
TTX-r	tetrodotoxin-resistant

References

- Arroyo EJ, Xu T, Grinspan J, Lambert S, Levinson SR, Brophy PJ, Peles E, Scherer SS. Genetic dysmyelination alters the molecular architecture of the nodal region. *J Neurosci*. 2002; 22:1726–1737. [PubMed: 11880502]
- Black JA, Yokoyama S, Higashida H, Ransom BR, Waxman SG. Sodium channel mRNAs I, II and III in the CNS: cell-specific expression. *Brain Res Mol Brain Res*. 1994; 22:275–289. [PubMed: 8015385]
- Boiko T, Van Wart A, Caldwell JH, Levinson SR, Trimmer JS, Matthews G. Functional specialization of the axon initial segment by isoform-specific sodium channel targeting. *J Neurosci*. 2003; 23:2306–2013. [PubMed: 12657689]
- Bowman D, Alexander S, Lodge D. Pharmacological characterisation of the calcium channels coupled to the plateau phase of KCl-induced intracellular free Ca²⁺ elevation in chicken and rat synaptosomes. *Neuropharmacology*. 1993; 32:1195–1202. [PubMed: 8107973]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]
- Caldwell JH, Schaller KL, Lasher RS, Peles E, Levinson SR. Sodium channel Nav1.6 is localized at nodes of Ranvier, dendrites, and synapses. *Proc Natl Acad Sci USA*. 2000; 97:5616–5620. [PubMed: 10779552]
- Campos FV, Moreira TH, Beirão PS, Cruz JS. Veratridine modifies the TTX-resistant Na⁺ channels in rat vagal afferent neurons. *Toxicon*. 2004; 43:401–406. [PubMed: 15051403]
- Catterall WA, Few AP. Calcium channel regulation and presynaptic plasticity. *Neuron*. 2008; 59:882–901. [PubMed: 18817729]
- Dib-Hajj, S.; Priestly, T. Voltage-Gated Sodium Channels. In: Davies, C.; Kew, JN., editors. *Structure and Function of Ion Channels*. Oxford University Press; London: 2009. p. 131–171.
- Dodd PR, Hardy JA, Oakley AE, Edwardson JA, Perry EK, Delaunoy J-P. A rapid method for preparing synaptosomes: comparison, with alternative procedures. *Brain Res*. 1981; 226:107–118. [PubMed: 7296283]
- Engel D, Jonas P. Presynaptic action potential amplification by voltage-gated Na⁺ channels in hippocampal mossy fiber boutons. *Neuron*. 2005; 45:405–417. [PubMed: 15694327]
- Farrag KJ, Bhattacharjee A, Docherty RJ. A comparison of the effects of veratridine on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels in isolated rat dorsal root ganglion neurons. *Pflügers Arch*. 2008; 455:929–938.

- Felts PA, Yokoyama S, Dib-Hajj S, Black JA, Waxman SG. Sodium channel alpha-subunit mRNAs I, II, III, NaG, Na6 and hNE (PN1): different expression patterns in developing rat nervous system. *Brain Res Mol Brain Res*. 1997; 45:71–82. [PubMed: 9105672]
- Goldin AL. Resurgence of sodium channel research. *Ann Rev Physiol*. 2001; 63:871–894. [PubMed: 11181979]
- Gong B, Rhodes KJ, Bekele-Arcuri Z, Trimmer JS. Type I and type II Na(+) channel alpha-subunit polypeptides exhibit distinct spatial and temporal patterning, and association with auxiliary subunits in rat brain. *J Comp Neurol*. 1999; 412:342–352. [PubMed: 10441760]
- Hemmings HC Jr, Girault JA, Nairn AC, Bertuzzi G, Greengard P. Distribution of protein phosphatase inhibitor-1 in brain and peripheral tissues of various species: comparison with DARPP-32. *J Neurochem*. 1992; 59:1053–1061. [PubMed: 1353788]
- Hemmings HC Jr. General anesthetic effects on protein kinase C. *Toxicol Lett*. 1998; 100–101:89–95.
- Jarvis MF, Honore P, Shieh CC, et al. A-803467, a potent and selective Nav1.8 sodium channel blocker, attenuates neuropathic and inflammatory pain in the rat. *Proc Natl Acad Sci USA*. 2007; 104:8520–8525. [PubMed: 17483457]
- Lai J, Porreca F, Hunter JC, Gold MS. Voltage-gated sodium channels and hyperalgesia. *Annu Rev Pharmacol Toxicol*. 2004; 44:371–397. [PubMed: 14744251]
- Long P, Mercer A, Begum R, Stephens GJ, Sihra TS, Jovanovic JN. Nerve terminal GABA_A receptors activate Ca²⁺/calmodulin-dependent signaling to inhibit voltage-gated Ca²⁺ influx and glutamate release. *J Biol Chem*. 2009; 284:8726–37. [PubMed: 19141616]
- MacDermott AB, Role LW, Siegelbaum SA. Presynaptic ionotropic receptors and the control of transmitter release. *Annu Rev Neurosci*. 1999; 22:443–485. [PubMed: 10202545]
- Mechaly I, Scamps F, Chabbert C, Sans A, Valmier J. Molecular diversity of voltage-gated sodium channel alpha subunits expressed in neuronal and non-neuronal excitable cells. *Neuroscience*. 2005; 130:389–396. [PubMed: 15664695]
- Medvedeva YV, Kim M-S, Schnizler K, Usachev YM. Functional tetrodotoxin-resistant Na⁺ channels are expressed presynaptically in rat dorsal root ganglia neurons. *Neuroscience*. 2009; 159:559–569. [PubMed: 19162133]
- Meir A, Ginsburg S, Butkevich A, Kachalsky SG, Kaiserman I, Ahdut R, Demircoren S, Rahamimoff R. Ion channels in presynaptic nerve terminals and control of transmitter release. *Physiol Rev*. 1999; 79:1019–1088. [PubMed: 10390521]
- Messner DJ, Catterall WA. The sodium channel from rat brain. Separation and characterization of subunits. *J Biol Chem*. 1985; 260:10597–10604. [PubMed: 2411726]
- Nicholls DG. The glutamatergic nerve terminal. *Eur J Biochem*. 1993; 212:613–631. [PubMed: 8096460]
- Ohshiro H, Ogawa S, Shinjo K. Visualizing sensory transmission between dorsal root ganglion and dorsal horn neurons in co-culture with calcium imaging. *J Neurosci Methods*. 2007; 165:49–54. [PubMed: 17597226]
- Prakriya M, Mennerick S. Selective depression of low-release probability excitatory synapses by sodium channel blockers. *Neuron*. 2000; 26:671–682. [PubMed: 10896162]
- Raiteri M. Presynaptic metabotropic glutamate and GABA_B receptors. *Handb Exp Pharmacol*. 2008; 184:373–407. [PubMed: 18064420]
- Reid CA, Clements JD, Bekkers JM. Nonuniform distribution of Ca²⁺ channel subtypes on presynaptic terminals of excitatory synapses in hippocampal cultures. *J Neurosci*. 1997; 17:2738–2745. [PubMed: 9092595]
- Sitges M, Guameros A, Nekrasov V. Effects of carbamazepine, phenytoin, valproic acid, oxcarbazepine, lamotrigine, topiramate and vinpocetine on the presynaptic Ca²⁺ channel-mediated release of [³H]glutamate: comparison with the Na⁺ channel-mediated release. *Neuropharmacology*. 2007; 53:854–862. [PubMed: 17904592]
- Strichartz GR. Novel ideas of local anaesthetic actions on various ion channels to ameliorate postoperative pain. *Br J Anaesth*. 2008; 101:45–47. [PubMed: 18487246]
- Stuart GJ, Sakmann B. Active propagation of somatic action potentials into neocortical pyramidal cell dendrites. *Nature*. 1994; 367:69–72. [PubMed: 8107777]
- Südhof TC. The synaptic vesicle cycle. *Annu Rev Neurosci*. 2004; 27:509–547. [PubMed: 15217342]

- Tibbs GR, Barrie AP, Van Mieghem FJE, McMahon HT, Nicholls DG. Repetitive action potentials in isolated nerve terminals in the presence of 4-aminopyridine: Effects on cytosolic free Ca^{2+} and glutamate release. *J Neurochem.* 1989; 53:1693–1699. [PubMed: 2553862]
- Toledo-Aral JJ, Moss BL, He ZJ, et al. Identification of PN1, a predominant voltage-dependent sodium channel expressed principally in peripheral neurons. *Proc Natl Acad Sci USA.* 1997; 94:1527–1532. [PubMed: 9037087]
- Ulbricht W. Effects of veratridine on sodium currents and fluxes. *Rev Physiol Biochem Pharmacol.* 1998; 133:1–54. [PubMed: 9600010]
- Vacher H, Mohapatra DP, Trimmer JS. Localization and targeting of voltage-dependent ion channels in mammalian central neurons. *Physiol Rev.* 2008; 88:1407–1447. [PubMed: 18923186]
- Westphalen RI, Gomez RS, Hemmings HJ. Nicotinic receptor-evoked hippocampal norepinephrine release is highly sensitive to inhibition by isoflurane. *Br J Anaesth.* 2009; 102:355–360. [PubMed: 19189985]
- Westphalen RI, Hemmings HC Jr. Selective depression by general anesthetics of glutamate versus GABA release from isolated cortical nerve terminals. *J Pharmacol Exp Ther.* 2003; 304:1188–1196. [PubMed: 12604696]
- Westphalen RI, Hemmings HC Jr. Volatile anesthetic effects on glutamate versus GABA release from isolated rat cortical nerve terminals: basal release. *J Pharmacol Exp Ther.* 2006a; 316:208–215. [PubMed: 16174801]
- Westphalen RI, Hemmings HC Jr. Volatile anesthetic effects on glutamate versus GABA release from isolated rat cortical nerve terminals: 4-aminopyridine-evoked release. *J Pharmacol Exp Ther.* 2006b; 316:216–223. [PubMed: 16174800]
- Wu L-G, Saggau P. Presynaptic inhibition of elicited neurotransmitter release. *Trends Neurosci.* 1997; 20:204–212. [PubMed: 9141196]
- Wynne PM, Puig SI, Martin GE, Treistman SN. Compartmentalized beta subunit distribution determines characteristics and ethanol sensitivity of somatic, dendritic, and terminal large-conductance calcium-activated potassium channels in the rat central nervous system. *J Pharmacol Exp Ther.* 2009; 329:978–986. [PubMed: 19321803]
- Yu FH, Catterall WA. Overview of the voltage-gated sodium channel family. *Genome Biol.* 2003; 4:207. [PubMed: 12620097]

Figure 1.

Effects of tetrodotoxin on neurotransmitter release in various CNS regions. Data for inhibition of L-[^3H]glutamate and [^{14}C]GABA release evoked by 1 mM 4AP from rat cortical, hippocampal, striatal, and spinal cord synaptosomes were fitted to sigmoidal concentration-effect curves (mean $\pm$ SEM; n=24–33). IC₅₀ values and maximal inhibition values are shown in Fig 2. Control data are presented as mean $\pm$ SD (n=10–18).

Figure 2.

Potency and efficacy for inhibition of glutamate and GABA release from isolated nerve terminals by tetrodotoxin. **a:** IC₅₀ values ($\pm$ SEM) derived by fitting data to sigmoidal concentration-effect curves (Fig. 1) for release from cerebral cortex (Cx), hippocampus (Hip), striatum (Str) and spinal cord (SC). Statistical comparisons between cerebral cortex and other CNS regions (* $P < 0.05$) and between glutamate and GABA release ($\dagger P < 0.05$; $\dagger\dagger P < 0.001$) were analyzed by comparing logIC₅₀ values between sigmoidal curve fits by F-tests. **b:** Maximal inhibition by TTX determined as the lower plateau of sigmoidal concentration-effect curve fits (Fig. 1) expressed as $I_0 - I_{\max}/I_0 \times 100$ ($\pm$ SEM). Statistical comparisons between cerebral cortex and other CNS regions (** $P < 0.001$) and between glutamate and GABA release ($\dagger\dagger P < 0.01$; $\dagger\dagger\dagger P < 0.001$) were analyzed by comparing lower plateaus of curves by F-tests.

Figure 3.

Effects of tetrodotoxin and A-803467 on 4AP-evoked glutamate and GABA release from cortex and spinal cord nerve terminals. Ca²⁺-independent 4AP-evoked glutamate release from cortex, but not spinal cord, was inhibited by 1 μM tetrodotoxin (TTX). TTX inhibited Ca²⁺-independent 4AP-evoked GABA release from both cortex and spinal cord. The selective Na_v1.8 blocker A-803467 had no effect on TTX-insensitive glutamate or GABA release from cortical nerve terminals in the presence of Ca²⁺, but inhibited TTX-insensitive glutamate, but not GABA, release from spinal cord nerve terminals. *P < 0.05, **P < 0.01 by unpaired Student t-test with Welch correction (mean±SEM). Group sizes (n) for GABA are the same as for glutamate.

Figure 4.

Neurotransmitter release evoked by 4-aminopyridine or veratridine from various CNS regions. Release of L-[^{3} H]glutamate and [^{14} C]GABA from rat cortical (Cx), hippocampal (Hip), striatal (Str) and spinal cord (SC) nerve terminals evoked by 2 min pulses of 1 mM 4AP (**a**, $n=14-22$) or 10 μ M VTD (**b**, $n=10-31$). Statistical comparisons between cortex and other CNS regions ($***P < 0.001$), and between glutamate and GABA ($\dagger P < 0.05$; $\dagger\dagger P < 0.01$; $\dagger\dagger\dagger P < 0.001$) were analyzed by one-way ANOVA using Tukey's post-hoc testing (mean $\pm$ SD).

Figure 5.

Effects of tetrodotoxin on neurotransmitter release evoked by veratridine. **A:** Inhibition by tetrodotoxin of L-[³H]glutamate and [¹⁴C]GABA release evoked by 10 μ M VTD from rat cortical synaptosomes (n=20). **B:** IC₅₀ values for inhibition by tetrodotoxin of transmitter release from cortical nerve terminals determined by fitting data to sigmoidal concentration-effect curves. Statistical comparisons between cortex and other CNS regions (***) and between glutamate and GABA (††) release curves were analyzed by comparing log IC₅₀ values between sigmoidal curve fits by F-test (mean $\pm$ SEM).

Figure 6.

Relative expression of total voltage-gated Na⁺ channel immunoreactivity in rat CNS nerve terminal preparations. Representative immunoblot shown for pan-specific Na_v1 antibody labeling of cerebral cortical (Cx), hippocampal (Hip), striatal (Str), and spinal cord (SC) nerve terminals (*upper panel*). Relative expression of immunoreactive bands (260–280 kDa; *arrowheads*) was compared to cortex by repeated measures one-way ANOVA. **P < 0.01, ***P < 0.001 vs. cortex (mean±SEM; n=14).

Figure 7.

Relative expression of voltage-gated Na⁺ channel subtype immunoreactivity in rat CNS nerve terminal preparations. Representative immunoblots (*upper panels*) shown for **a**: Na_v1.1 (n=16), **b**: Na_v1.2 (n=16), **c**: Na_v1.6 (n=13), and **d**: Na_v1.7 (n=7) labeling of cortical (Cx), hippocampal (Hip), striatal (Str), and spinal cord (SC) nerve terminals. Relative expression of immunoreactive bands (260–280 kDa; *arrowheads*) was compared to cortex by repeated measures one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001 vs. cortex (mean±SEM).

Table 1
Relationship between Na_v immunoreactivity and tetrodotoxin potency

Correlation between regional expression of total Na_v1 and specific Na_v1 subtype immunoreactivity relative to cortex and the $-\log IC_{50}$ of tetrodotoxin for inhibition of 4AP-evoked glutamate or GABA release from nerve terminals prepared from cortex, hippocampus, striatum and spinal cord. Slope values were derived by linear regression and tested by Pearson's correlation

Immunoreactivity Na _v	Tetrodotoxin potency ($-\log IC_{50}$)			
	Glutamate release		GABA release	
	Slope	Correlation (r^2)	Slope	Correlation (r^2)
1	1.01	0.99 **	-0.11	0.08
1.1	-0.96	0.89	0.20	0.26
1.2	0.55	0.95 *	-0.05	0.05
1.6	0.01	<0.01	0.01	0.01
1.7	-0.02	0.77	0.01	0.18

* $P < 0.05$,

** $P < 0.01$.