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Multi-site Phosphorylation of Voltage-Gated Sodium Channel α Subunits from Rat Brain

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Abstract

Reversible phosphorylation of ion channels underlies cellular plasticity in mammalian neurons. Voltage-gated sodium or Nav channels underlie action potential initiation and propagation, dendritic excitability, and many other aspects of neuronal excitability. Various protein kinases have been suggested to phosphorylate the primary α subunit of Nav channels, affecting diverse aspects of channel function. Previous studies of Nav α subunit phosphorylation have led to the identification of a small set of phosphorylation sites important in mediating aspects of Nav channel function. Here we use nanoflow liquid chromatography tandem mass spectrometry (nano-LC MS/MS) on Nav α subunits affinity-purified from rat brain with two distinct monoclonal antibodies to identify 15 phosphorylation sites on Nav1.2, 12 of which have not been previously reported. We also found 3 novel phosphorylation sites on Nav1.1. In general, commonly used phosphorylation site prediction algorithms did not accurately predict these novel *in vivo* phosphorylation sites. Our results demonstrate that specific Nav α subunits isolated from rat brain are highly phosphorylated, and suggest extensive modulation of Nav channel activity in mammalian brain. Identification of phosphorylation sites using monoclonal antibody-based immunopurification and mass spectrometry is an effective approach to define the phosphorylation status of Nav channels and important membrane proteins in mammalian brain.

Keywords

voltage-gated sodium channels; brain; phosphorylation; tandem mass spectrometry; immunopurification; monoclonal antibody; nanoflow liquid chromatography

Introduction

The electrical excitability of cells is mediated by the expression of a large number of different types of plasma membrane ion channels. In mammalian neurons the distinct patterns of

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Supporting Information: Figures of peptide spectra for the Nav1.2 phosphopeptides, Mascot scores for the Nav1.1 and Nav1.2 phosphopeptides, summary of individual experiments, figures of peptide spectra for the Nav1.1 phosphopeptides, and results from analysis of Nav1.2 with consensus phosphorylation site prediction algorithms. This material is available free of charge via the Internet at <http://pubs.acs.org>.

expression, localization and activity of different ion channels generates a wide array of firing patterns, determining the input-output relationship of neurons and their subcellular compartments. As such, dynamic modulation of ion channels by reversible phosphorylation underlies plasticity in neuronal signaling. Among ion channels, voltage-gated sodium or Nav channels generate a depolarizing inward current and determine the threshold for axonal action potential generation¹, as well as the excitability of specific dendritic branches², and aspects of synaptic integration³. Nav channels consist of a highly posttranslationally modified α subunit of approximately 2000 amino acids ($M_r \approx 260$ kDa), comprising four internally repeated homologous domains or pseudosubunits (I-IV), each of which contains six transmembrane segments (S1–S6) that act as the voltage-sensing and pore-forming domains¹. The short intracellular linker connecting pseudosubunits III and IV (*i.e.*, the interdomain or ID III-IV linker) serves as the inactivation gate, folding into the channel structure and blocking the pore from the inside during sustained depolarization of the membrane¹. Nav α subunits are associated with one or two smaller auxiliary β subunits with a molecular weight of 30–40 kDa⁴. Although the α subunit is sufficient for functional expression⁵, β subunits modify the kinetics and voltage dependence of channel gating⁴.

Ten genes located on four chromosomes encode the different subtypes of Nav channel α subunits¹. At least nine of these genes are expressed in the nervous system⁶, with the exception of Nav1.4, which is expressed predominantly in adult skeletal muscle⁷. Of the different channel subtypes, Nav1.1, Nav1.2, Nav1.3 and Nav1.6 are prominently expressed in the mammalian brain, with different spatial and temporal patterns of expression⁶. Among these Nav1.2 is the most abundant α subunit expressed in the central nervous system, comprising approximately 80% of the total rat brain Nav α subunit pool⁸. Based on its prominent localization in axons and nerve terminals^{9, 10}, Nav1.2 is expected to control conductance of axonal action potentials and neurotransmitter release in presynaptic terminals¹¹. In contrast, Nav1.1 is primarily localized in the soma and proximal dendrites^{9, 10}, where it plays a role in neuronal excitability through integration of synaptic impulses to set the threshold for action potential initiation and propagation in dendritic and axonal compartments, and in axon initial segments^{12, 13}.

Nav channels are phosphorylated by various protein kinases, leading to modulation of channel activity and cellular excitability¹⁴. A number of phosphorylation sites have been described on Nav α subunits, with no sites yet detected on Nav β subunits¹⁴. Two-dimensional phosphopeptide mapping revealed that cAMP-dependent protein kinase (PKA) phosphorylates Ser residues S573, S610, S623 and S687 within the ID I-II linker¹⁵. PKC phosphorylates S554, S573, S576 and S1506¹⁶. S1506 is located in the ID III-IV linker that forms the inactivation gate¹⁷. The calcium-dependent protein phosphatase calcineurin can dephosphorylate S573 and S623, while protein phosphatase 2A is more active at S610, and the two phosphatases are equally effective at S687¹⁵. Increased phosphorylation is generally associated with a reduction in Nav currents, and a slowing of inactivation¹⁸, through phosphorylation of sites in ID I-II, and ID III-IV linkers, respectively [reviewed in 14]. Phosphorylation of Nav1.2 (and Nav1.1) in Purkinje neurons also affects open channel block by β 4 auxiliary subunits¹⁹.

Nav α subunit phosphorylation may also regulate the interaction of Nav channels with proteins important in expression, localization and function. Interaction between ankyrin-G and Nav1.2 at the axon initial segment is modulated by protein kinase CK2 *via* phosphorylation of S1112, S1124 and S1126 in the ID II-III linker²⁰. Nav1.2 is also modulated by phosphorylation of Tyr residues by Src-family Tyr kinases, through multi-site phosphorylation that affects binding of Fyn to Nav1.2 *via* SH2 and SH3 domains, and altered inactivation due to ID III-IV linker phosphorylation²¹. Nav1.1 phosphorylation has been studied to a lesser extent relative to that on Nav1.2. Nevertheless, a study of Nav1.1 and Nav1.2 expressed in *Xenopus* oocytes showed that current amplitudes were reduced for both ion channel subtypes after PKA phosphorylation²². Dynamic modulation of Nav channels by phosphorylation has also been proposed to

underlie changes in the biophysical and pharmacological properties of Nav currents in epileptic versus normal hippocampal neurons²³.

To better understand the complexity of Nav channel phosphorylation in mammalian brain, we used two different monoclonal antibodies (mAbs) to immunopurify Nav α subunits from rat brain. We then took an unbiased approach using nanoflow tandem mass spectrometry (nano-LC MS/MS) to systematically analyze the *in vivo* phosphorylation sites on the purified Nav α subunits. We found that the richness of Nav channel phosphorylation is greater than appreciated from previous studies, with fourteen *in vivo* phosphorylation sites identified on Nav1.2, and three on Nav1.1. These data provide novel insights into the molecular basis for modulation of Nav channel activity and cellular plasticity in mammalian brain.

Experimental Details

Immunoaffinity purification of Nav channels from rat brain

We immunopurified Nav α subunits from a crude rat brain membrane (RBM) fraction prepared from freshly isolated adult whole rat brains as described²⁴. RBM were solubilized at 1 mg protein/ml in lysis buffer (1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 10 mM sodium azide, 10 mM Tris-HCl, pH 8.0, 2 mM NaF, 1 mg/ml BSA, 1.5 μ g/ml aprotinin, 10 μ g/ml antipain, 10 μ g/ml leupeptin, 0.1 mg/ml benzamidine, 1 mM PMSF) on a tube rotator at 4°C for 30 min. The insoluble fraction was removed by centrifugation at 4°C/16,000 $\times$ g/30 min. Two different sets of immunoaffinity purifications were performed in this study. The first employed mouse mAb K69/3, raised against a recombinant fusion protein corresponding to amino acids 1882–2005 of the cytoplasmic C-terminus of rat Nav1.2. The specificity of this mAb was previously determined by ELISA against recombinant fusion proteins corresponding to the C-termini of Nav1.1, Nav1.2, Nav1.3 and Nav1.6, and immunofluorescence staining of heterologous cells expressing Nav1.1, Nav1.2, Nav1.4 and Nav1.6²⁵. We also used mouse mAb K58/35, raised against a synthetic peptide corresponding to the highly conserved ID III-IV linker and predicted to recognize all vertebrate Nav α subunits²⁶. Nav α subunits were affinity-purified by incubation of 10 mg RBM lysate with 2.5 μ g/ml mAb at 4°C overnight on a tube rotator. This was followed by immobilization of mAb-Nav complexes on protein-G agarose beads (500 μ l of a 50% slurry) at 4°C for 2 hrs on a tube rotator. Beads were washed 6x in lysis buffer without BSA and eluted with reducing sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8) by incubation at 37°C for 15 min¹⁰.

In-gel digestion and enrichment of phosphopeptides

Immunoaffinity-purified Nav channels were size-fractionated on 6% polyacrylamide-SDS gels and visualized by staining with Coomassie G-250. The Nav α subunit band of $M_r \approx 260$ kDa was excised, diced into small pieces and washed twice with 50% acetonitrile (ACN)/50 mM ammonium bicarbonate by vortexing for 10 min. After drying in a speed vacuum concentrator, Cys residues were reduced by incubation of the gel pieces in 10 mM DTT at 56°C for 1 hr and alkylated by incubation in 55 mM iodoacetamide at RT for 45 min. Gel pieces were then washed 10 min in 50 mM ammonium bicarbonate, dehydrated in 50% ACN/50 mM ammonium bicarbonate for 10 min and dried to complete dryness. Dried gel pieces were swollen with 100 μ l of 50 mM ammonium bicarbonate containing 10 ng/ μ l trypsin (Promega, Madison, WI) and incubated overnight at 37°C. Digested peptide mixtures were extracted with 50% ACN/5% formic acid for 30 min, dried to complete dryness and stored at –20°C until further processing.

The peptide mixtures were either directly used for MS-analyses or further processed to enrich for phosphopeptides using PHOS-Select Iron Affinity Gel beads (P9740, Sigma-Aldrich, Saint Louis, USA). PHOS-Select beads were equilibrated by washing twice in 250 mM acetic acid/30% ACN (washing buffer). Dried peptides were resuspended in 500 μ l washing buffer and

immobilized on the beads by rotating at RT for 30 min. Beads were washed twice and phosphopeptides were eluted by incubation for 5 min with 500 μ l 400 mM ammonium hydroxide. The eluant was dried completely and stored at -20°C until further processing.

Mass spectrometry, data processing and analysis

Nav channel α subunits affinity-purified with the mAbs K69/3 and K58/35 were analyzed with an LTQ ion trap or LTQ-FT hybrid mass spectrometer (Thermo-Fisher, San Jose CA) connected to a Waters Nano-Aquity UPLC system (Waters, Milford, MA). Dried peptide samples were resuspended in 2% ACN/0.1% TFA and concentrated on a Waters Symmetry C18 280 $\mu\text{m} \times 20$ mm nanoAcquity trap column at a loading flow rate of 15 $\mu\text{l}/\text{min}$. Peptides were then eluted from the trap and separated by a Waters BEH C18 1.7 μm , 100 $\mu\text{m} \times 10$ cm UPLC column using a 80 minute gradient of 1–70% Buffer B (Buffer A = 0.1% formic acid, Buffer B = 95% ACN/0.1% formic acid) at a flow rate of 400 nL/min, and sprayed into a LTQ-FT ion trap mass spectrometer through a nano-electrospray source. The MS survey scan was acquired using the LTQ or FTICR mass analyzer and then the top four ions in each survey scan were subjected to automatic low energy CID for MS/MS scans.

MS/MS spectra were extracted using the program Extract msn v.4.0 of the Bioworks software v.3.3 (Thermo Finnigan, San Jose, CA, USA) with default parameters, and interpreted with Mascot v.2.2 (Matrix Science, London, UK) by searching against the SwissProt mammalian database (02/18/09; 410,518 sequences; 148,080,998 residues) and the IPI rat database (01/21/2009; 40,288 sequences; 20,569,234 residues). Individual ions scores > 31 indicate identity or extensive homology ($p < 0.05$). Database searches were performed with a peptide mass tolerance of 20 ppm, MS/MS tolerance of 0.6 Da, strict tryptic specificity (cleavage after lysine and arginine) allowing one missed cleavage site; carbamidomethylation of Cys was set as a fixed modification, whereas oxidation (M) and phosphorylation (S, T, Y) were considered as variable modifications. Every MS/MS spectrum exhibiting possible phosphorylation was manually checked and validated based on the existence of a 98 Da mass loss ($-\text{H}_3\text{PO}_4$: phosphopeptide-specific CID neutral loss) for both precursor and fragmented ions.

Results

MS analyses of rat brain Nav α subunits

Proteomic analyses of Nav α subunits immunoaffinity purified from detergent extracts of total rat brain membrane preparations were performed in this study. We followed a general approach similar to that used in our previous studies of phosphorylation of ion channels immunopurified from mammalian brain [Kv2.1 27, Kv1.2 28, BK α 29 and Kv4.2 30]. One set of analyses focused on Nav α subunits immunopurified from rat brain with the Nav1.2-specific mouse mAb K69/3, which binds at the cytoplasmic C-terminus of this α subunit (Figure 1). The other set of analyses focused on Nav channels immunopurified from rat brain with the mouse mAb K58/35, raised against the highly conserved ID III-IV linker region within Nav α subunits (Figure 1) such that it recognizes all mammalian Nav isoforms. Both mAbs were effective in affinity purification yielding amounts of Nav protein sufficient for mass spectrometry-based phospho-profiling. Immunopurification from a detergent extract obtained from 10 mg of a crude whole rat brain membrane fraction, followed by fractionation on SDS gels and Coomassie blue staining, resulted in a Nav α subunit band of $M_r \approx 260$ kDa and containing approximately 1 μg of purified Nav α subunits (Figure 2A). Nav α subunit bands were excised, in-gel digested with trypsin, and the resulting tryptic peptides analyzed by nanoflow liquid chromatography tandem MS (nano-LC MS/MS) using a linear ion trap or high resolution FT-ICR mass spectrometer.

We performed sixteen independent immunopurification/nano-LC MS/MS analyses using Nav1.2-specific mAb K69/3. Nav channel α subunits contain highly conserved regions, mainly within the pseudosubunits, from which tryptic peptides exhibiting 100% identity between different Nav isoforms are obtained, and unique regions, mainly from ID linkers, from which isoform-specific tryptic peptides are obtained. The analyses of the Nav α subunit pool immunopurified with mAb K69/3 in these sixteen different experiments yielded numerous examples of peptides whose sequences were identical in more than one Nav α subunit (Table 1). In addition to these conserved peptides, a total of 929 peptides corresponding to sequences unique to a given Nav α subunit were obtained. Of these, 892 (96.0%) were specific to Nav1.2. A total of only 37 peptides across all sixteen experiments could be assigned to other Nav α subunits, the majority (20/37 or 2.1% of the peptides unique to a given Nav α subunit) corresponded to Nav1.6-specific peptides (Table 1).

These results are in sharp contrast to those obtained from nano-LC MS/MS analyses of sixteen parallel immunopurifications performed with the pan-Nav channel mAb K58/35. In this case a total of 1483 peptides unique to a given Nav channel were identified, with $\approx$ 54% from Nav1.2, $\approx$ 23% from Nav1.1, $\approx$ 15% from Nav1.6, and $\approx$ 7% from Nav1.3 (Table 1). As such mAb K69/3 represents a reliable reagent for selective immunopurification of Nav1.2 from mammalian brain, and mAb K58/35 for immunopurification of all brain Nav channel isoforms.

Proteomic analysis of the primary structure of rat brain Nav1.2

Based on the substantial yield of Nav1.2 from our immunoaffinity purifications, mass spectrometry analyses retrieved up to 155 Nav1.2-specific peptides in a given experiment. Mascot scores for Nav1.2 ranging from 1508 to 7609 were obtained from these sixteen independent experiments. Together, we obtained $\sim$ 41% (816/2005 amino acids or a.a.) overall coverage (Figure 3) of the deduced Nav1.2 a.a. sequence (Swiss-Prot: P04775.1 or SP, identical to NCBI Reference Sequence: NP_036779.1). Among the major cytoplasmic domains, coverage was 76% for the N-terminus (96/127 a.a.), 71% for interdomain I-II (235/329 a.a.), 40% for interdomain II-III (88/219 a.a.), 62% for interdomain III-IV (32/50 a.a.) and 77% for the C-terminus (174/226 a.a.). Overall coverage within these cytoplasmic domains was $\approx$ 66% (625/951 a.a.), with many of the uncovered regions corresponding to tryptic peptides that were outside the mass range amenable to LC MS/MS analysis [between MWs of 500 and 3500 Da; 31]. Among the four pseudosubunit domains coverage was 26% for domain I (79/301 a.a.), 12% for domain II (27/231 a.a.), 23% for domain III (64/273 a.a.) and 9% for domain IV (22/249 a.a.). Overall coverage in all four pseudosubunit domains was 18% (192/1054 a.a.).

MS/MS searches against two different databases revealed proteomic evidence for expression of two different Nav1.2 isoforms in rat brain, the canonical Nav1.2 sequence (Swiss-Prot: P04775.1 or SP, identical to NCBI Reference Sequence: NP_036779.1) and a variant (IPI00476429.2, or IP) exhibiting 98.7% pairwise identity. The major difference in primary sequences is within the segment corresponding to positions 130–143 in both sequences. Peptides covering this sequence, as well as a site of an additional difference (at position 189) located within the S1 transmembrane segment within domain I could not be recovered in the tryptic peptides amenable to the MS analysis. Two peptides within both sequences show a difference in one amino acid (at positions 538 and 579), namely GFQFSLEGSR *versus* GFRFSLEGSR, and ASLFNFKGR *versus* ASLFSFKGR, in the SP *versus* IP Nav1.2 sequences, respectively. Interestingly, in the nano-LC MS/MS analyses matches for these two peptides were found for the IP (GFRFSLEGSR, found in 8/18 experiments; ASLFSFKGR, found in 16/18 experiments), but not the SP, version of Nav1.2 (Figure 3). Another inconsistency between these sequences is within the ID III-IV linker (a.a. 1483–1485; SP: KKMSQDIFM; IP: KKFGGQDIFM). In this case we detected the SP but not IP version of Nav1.2.

For simplicity, in spite of the fact that peptides derived from two different Nav1.2 sequences were identified in this study, all data presented here concerning amino acid positions correspond to those in the canonical Nav1.2 sequence (Swiss-Prot: P04775.1; NCBI Reference Sequence: NP_036779.1) as originally described by Numa and colleagues ³².

Analysis of Nav1.1 primary structure

Immunoaffinity purification of Nav α subunits with the pan-Nav channel mAb K58/35 yielded 338 peptides specific for Nav1.1 (Swiss-Prot: P04774.1; NCBI Reference Sequence: NP_110502). Mascot scores for this protein varied between 284 and 2740 across the sixteen independent experiments. Overall sequence coverage from all sixteen MS experiments for the Nav1.1 amino acid sequence (Figure 4) was ~37% (742/2009 a.a.). Sequence coverage was 83% for the N-terminal cytoplasmic domain (100/121 a.a.), 50% for the ID I-II linker (167/337 a.a.), 31% for the ID II-III linker (69/221 a.a.), 53% for the ID III-IV linker (31/58 a.a.), and 80% for the C-terminus (180/223 a.a.). The overall coverage of these sequences within these cytoplasmic domains was 59%. Among the four pseudosubunit domains coverage was 27% for domain I (81/302 a.a.), 12% for domain II (27/230 a.a.), 24% for domain III (64/270 a.a.) and 10% for domain IV (25/249 a.a.). Overall coverage in all four Nav1.1 pseudosubunit domains was 18% (197/1051 a.a.).

In vivo phosphorylation sites on rat brain Nav α subunits

We next investigated the phosphorylation status of Nav1.2 by MS analyses of tryptic peptide fragments without and with enrichment of phosphopeptides. Nano-LC MS/MS analyses led to identification of a total of 15 serine/threonine (Ser/Thr) phosphorylation sites on Nav1.2 (Figures 1, 3). Of these sites, pS579 was unique to IP isoform peptides obtained from the search of the IPI rat database, the remaining 14 sites were present in both the SP and IP Nav1.2 isoforms. Two (pS468, pS610) of the 15 phosphorylation sites were only observed in experiments employing iron affinity resin for enrichment of phosphorylated peptides. Phosphorylated residues at thirteen sites were unambiguously assigned based on phosphopeptide MS/MS spectra. Among the 15 identified phospho-sites, only pS4 is located in the Nav1.2 N-terminus. Eleven phospho-sites (pS468, pS471, pS484, pS528, pS554, pS579, pS610, pS623, pS687, pS688 and pS721) are clustered on the ID I-II linker. The remaining three sites (pS1930, T1966 and S1971) are located on the cytoplasmic C-terminus (Figure 1). Figure 2B shows a representative MS/MS-spectrum for a doubly charged, singly phosphorylated Nav1.2 peptide at m/z 726.36 that was fragmented to produce a tandem mass spectrum with b - and y -ion series that describe the sequence FSpSPHQSLLSIR (a.a. 552–563). The phosphorylation site was unambiguously assigned to S554 due to mass assignments from beta-eliminated b_3 , and y_{10}^{2+} with neutral loss of phosphoric acid H_3PO_4 , and y_9 , y_9^{2+} and y_{10}^{2+} fragment ions. Representative spectra for all other Nav1.2 phosphorylation sites are shown in Supplemental Figure 1, and the highest Mascot score for each site in Supplemental Figure 2. All 15 identified phosphorylation sites were from tryptic peptides unique to Nav1.2 with the exception of S610, on a tryptic peptide (DSL FVPHR) also present in Nav1.3. However, as no peptides unique to Nav1.3 were recovered from the mAb K69/3 purifications, this phosphorylation site is likely on a peptide derived from Nav1.2. A summary of phosphorylation sites identified in each of sixteen individual purifications with K69/3 is shown in Supplemental Figure 3.

Analysis of Nav α subunits after immunopurification with the pan-Nav mAb K58/35 did not reveal any additional phosphorylation sites on peptides unique to Nav1.2 (Supplemental Figure 3), but did provide corroborative evidence for phosphorylation of 10 of the 15 sites on Nav1.2 (all except pS468, pS484, pS687, pS688, and pS721; Supplemental Figure 3). Together these analyses (Supplemental Figure 3) also led to identification of three Ser phosphorylation sites within peptides unique to Nav1.1 (Figure 4). The three Ser residues could be assigned

unambiguously by manual inspection of the MS/MS spectra of the corresponding tryptic peptide fragments as pS470, pS551, and pS607. Representative spectra for all three Nav1.1 phosphorylation sites are shown in Supplemental Figure 4. All three phosphorylation sites were located within the ID I-II linker. Note that pS607 was unique among all Nav1.1 and Nav1.2 sites in that it was only identified only in Experiment 12, which employed phosphopeptide enrichment (Supplemental Figure 3). Based on alignments of the Nav1.1 and Nav1.2 sequences, and similarity of surrounding amino acids, the positions of each of the Nav1.1 sites correspond to sites identified in the Nav1.2 in the ID I-II linker. They are pS470:pS471, pS551:pS554, and pS607:pS610, in Nav1.1 and Nav1.2, respectively. No phosphorylation sites were identified on the peptides unique to Nav1.6, Nav1.3 or other Nav1 channels from the Nav α subunits purified with mAb K58/35.

Discussion

Abundance of different Nav α subunits in adult rat brain

The rank order of isoform unique peptides purified from detergent extracts of rat brain membranes with mAb K58/35 follows the pattern of isoform expression in mammalian brain obtained from previous mRNA-based studies [Nav1.2 > Nav1.1 > Nav1.6 > Nav1.3; 6]. A previous study of the relative abundance of different Nav α subunit polypeptides in rat brain utilized subtype-specific antibodies against Nav1.1 and Nav1.2 to immunoprecipitate *in vitro* [32]P-labeled Nav channels that had been purified from rat brain by classic chromatographic approaches⁸. In this study, 70% of the Nav α subunits that were available for immunoprecipitation with a “pan-Nav” antibody could be immunoprecipitated with an anti-Nav1.2 antibody, and 26% with an anti-Nav1.1 antibody. This general pattern also corresponds to studies of mRNA expression levels⁶.

Analysis of Nav α subunit phosphorylation sites by mass spectrometry

For investigation of *in vivo* phosphorylation of the Nav α subunits, we undertook a proteomic analysis based on monoclonal antibody-based affinity purification of appropriately solubilized Nav α subunits combined with nano-LC MS/MS analyses. Using this approach, we identified a total of 15 Ser/Thr phospho-sites in Nav1.2 and 3 Ser phosphorylation sites in Nav1.1. Our previous studies on other ion channels immunoaffinity-purified from rat brain, namely Kv2.1²⁷, and BK_{Ca} channels²⁹, yielded a similar unexpected complexity of *in vivo* phosphorylation. Moreover, as we also found here, the pattern of the *in vivo* phosphorylation sites identified in nano-LC MS/MS analyses differed from predictions obtained from widely-used computer algorithms based on consensus sites for specific protein kinases^{27, 29}. These algorithms predicted a large number of phosphorylation sites on the Nav1.2 sequence (Supplemental Figure 5), but the algorithms did not consistently predict the pattern of *in vivo* phosphorylation detected by nano-LC MS/MS analyses of Nav1.2 α subunits purified from rat brain (Figure 5). The prediction algorithms yielded both highly ranked phosphorylation sites not detected in the *in vivo* situation, and poorly ranked sites that were found phosphorylated *in vivo* (Supplemental Figure 5). There are admittedly a number of caveats associated with mass spectrometric analysis of a sample purified from native brain³³, such as inadvertent selection of specific subpopulations of target proteins by extraction or chromatography, *post facto* changes in phosphorylation at specific sites, loss of specific peptides or lack of their detection in the nano-LC MS/MS analyses, etc. The actual number of phosphorylation sites present on Nav α subunits could differ from that reported here, especially if they are phosphorylated in specific brain regions that may be under-represented in our whole brain sample. Nevertheless, we suggest that the proteomic approach used here is advantageous in providing a comprehensive analysis of phosphorylation sites present *in vivo* on rat brain Nav channel α subunits compared to conventional consensus-site based strategies.

With respect to localization of the identified phospho-sites in the Nav1.2 primary structure, the identified pSer/pThr are located in four regions. One site (S4) is located in the cytoplasmic N-terminus. This site was not identified in consensus site prediction programs (Figure 5) and no previous studies have suggested a role for N-terminal Nav channel phosphorylation. Eleven sites are clustered in the interdomain I-II linker (sites pS468–pS721), which is known from previous studies to be the major modulatory domain of Nav channels (reviewed in 14). Within this domain, PKA has been shown to phosphorylate Nav1.2 at four sites (pS573, pS610, pS623, pS687), as determined by backphosphorylation, phosphopeptide mapping and microsequencing of Nav α subunits purified from rat brain and synthetic Nav1.2 peptides 15–34. Sites pS610, pS623, and pS687 also score highly as PKA phosphorylation sites in consensus prediction algorithms (Figure 5). The high density of *in vivo* phosphorylation sites in this intracellular linker is consistent with a prominent role of this domain in phosphorylation-dependent modulation of channel gating as defined in studies on recombinant Nav1.2 α subunits expressed in heterologous cells [e.g. 35–37; reviewed in 14]. A particularly important role was defined for phosphorylation at S573, which was not detected here as an *in vivo* Nav1.2 phosphorylation site 37. No sites were located in the ID II-III linker, which contains three phosphorylation sites (pS1112, pS1124, pS1126) recently suggested to influence the retention of Nav1.2 at the axonal initial segment 20. Unfortunately, we were unable to recover this sequence, presumably due to the lack of tryptic cleavage sites near these sites. In the Nav1.2 C-terminus we found three novel phosphorylation sites, (pS1930, pS1971, pT1966). The C-terminus between a.a. 1890–2005 is known to be involved in the modulation of Nav1.2 function by interaction with G-protein $\beta\gamma$ subunits 38. The novel phosphorylation sites in the Nav1.2 C-terminus could be involved in dynamic regulation of the interaction between the Nav1.2 α subunit and G-protein $\beta\gamma$ subunit. All three of these C-terminal sites are adjacent to prolines (either Ser-Pro or Thr-Pro), raising the potential for coordinate regulation by a MAP kinase or CDK kinase family member, as has been shown for phosphorylation within the I-II linker of Nav1.6 39 and Nav1.8 40; note that analogous ID I-II sites in Nav1.2 (pS554) and Nav1.1 (pS551) are present in SerPro dipeptide motifs.

In the Nav1.1 primary structure three *in vivo* Ser phosphorylation sites were detected in this study, all of which were clustered in the ID I-II linker. Interestingly, all of the *in vivo* Nav1.1 sites identified in the ID I-II linker correspond to sites also present in Nav1.2. In each case the regions immediately adjacent to the phosphorylated residue is highly conserved, but differences within other positions within the tryptic peptides allow for unambiguous assignment of the peptide to that particular Nav α subunit. The correlation between these sites in the two different Nav α subunits may suggest that they are co-regulated by dynamic changes in phosphorylation state at these sites, as previously suggested based on consensus phosphorylation site predictions ¹⁴, and studies of recombinant Nav α subunits expressed in heterologous cells ²².

A large number of mutations in Nav1.1 and Nav1.2 have been found associated with neurological disease, particular infantile epilepsies. Over 150 mutations have been identified in patients with epilepsy ⁴¹. In primary structure, none of these mutations correspond to or are immediately adjacent to the *in vivo* phosphorylation sites described here. However, detailed three-dimensional structural information is not available for Nav α subunits. As such, the spatial relationships of the *in vivo* phosphorylation sites identified here and the locations of these debilitating mutations in the tertiary structure of Nav1.1 and Nav1.2 are not known. Recently, as a first step a solution NMR structure of the C-terminal fragment of Nav1.2 was obtained ⁴². Unfortunately, the Nav1.2 fragment used in these studies (1777–1882) did not contain the *in vivo* Nav1.2 C-terminal phosphorylation sites (pS1930, pS1966 and pS1971) identified here. Future structural studies, as well as detailed information on physiological and pathological regulation of these sites, and the effects of their mutation on expression, localization and function of Nav channels in brain neurons may help elucidate details of the roles of the *in*

vivo phosphorylation sites described here in brain function. The powerful combination of monoclonal antibody-based immunopurification and nano-LC MS/MS approaches used here could also be used to monitor phosphorylation changes in diseased tissues using multiple reaction monitoring methods. For example, the phosphorylation sites identified here could be used in quantitative multiple reaction monitoring studies to screen for phosphopeptide biomarkers to this channel in normal and epileptic brain, where changes in Nav channel function are suggested to underlie altered neuronal excitability⁴³. Such approaches could lead to quantitation of the phosphorylation state of the unique and non-unique phosphopeptide ions in these and other normal and diseased tissue samples.

In conclusion, this work presents the first comprehensive MS-based analysis of phosphorylation sites on Nav α subunits purified from rat brain. Here, we used mAb-based immunoaffinity methods to purify Nav α subunits from rat brain, and identified 15 Ser/Thr *in vivo* phosphorylation sites on Nav1.2, and 3 *in vivo* Ser residues on Nav1.1. Our results demonstrate that the number of phosphorylation sites present on brain Nav1.2 and Nav1.1 *in vivo* greatly exceeds that suggested from previous studies, suggesting that phosphorylation of these Nav channels in rat brain provides a rich template for modifying Nav channel expression, localization and function, in response to altered physiological and pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cartoon of the membrane topology of the Nav1.2 α subunit showing the phosphorylation sites identified in this study. Red dots mark the novel phosphorylation sites identified in this study. Green dots mark the phosphorylation sites identified here that had been identified in previous studies. Antibody symbols show general regions of K58/35 and K69/3 binding sites in the Nav1.2 α subunit.

Figure 2.

A. Immuno-affinity purification of Nav α subunits. Coomassie brilliant blue-stained SDS gel of a large-scale immunopurification showing the yield of Nav α subunits obtained with mAbs K69/3 and K58/35. Left lane contains 500 ng per band of molecular weight standards; size noted to left of lane. Arrows denote positions of Nav α subunits and immunoglobulin heavy chain. B. Representative tandem mass spectrum of Nav1.2 peptide containing pS554. The spectrum of a doubly charged, singly phosphorylated Nav1.2 peptide at m/z 726.36 that was fragmented to produce a tandem mass spectrum with y-ion and b-ion series that described the sequence FSpSPHQSLLSIR (amino acids 552–563). The phosphorylation site was unambiguously assigned to S554 due to mass assignments from beta-eliminated b3, and $y10^{2+}$ with neutral loss of phosphoric acid H_3PO_4 and y9, $y9^{2+}$, and $y10^{2+}$ fragment ions.

1 MARSVLVPPG PDSFRFFTRE SLAAIEQRIA EEKAKRPQE RDEDDENGK KPNSDLEAGK SLPTIYGDIPEMVSSEPLED LDPYYINKKT FIVLNK GKAI
N-TERM

101 SRFSATSALY ILTPFNPIRK LAIKILVHSL FNVLMCTIL TNCVFMTSN PPDWTKNVEY TFTGIYTFES LIKILARGFC LEDFTFLRNP WNWLDFTVIT
N-TERM IS1 IS2 IS3

201 FAYVTEFVNL GNVSAIRTER VLRAKTIISV IPQLKTIIVA LIQSVKLLSD VMILTIVFCLS VFALIGLQLF MGNLRNKCLO WPPDNSTFEI NITSFFNNSL
IS3 IS4 IS5

301 DWNGTAFNR T VNMFWNDYI EDKSHFYFLE QNDALLCGN SSDAGQCPEG YICVKAGRNP NYGYTSFDTF SWAFSLFRL MTQDFWENLY QLTLLAAGKT

401 YMIFFVLVIF LGSFYLINLI LAVVAMAYEE QNQATLEBAE QKEAEFQOML EQLKKQEEA QAAAAASAE SRDFSGAGGI GVFSSESSVA SKLSSKSEKE
IS6 ID I-II

501 LKNRRKKKKQ KEQAGEEKE DAVRKSASED SIRKKGQFS LEGSRLTYEK RFSSPHOSLL SIRGSLFSR RNSRASLNF KGRVKDIGSE NDFADDEHST
ID I-II

601 FEDNDSRRDS LFVPHRHGER RPSNVSQASH ASRGITPLEM NGKMHSVDC NGVSVLVGGP SALTSPVQQL LPEGTTTETE IRKRSSSYH VSMOLLEDPS
ID I-II

701 RQRAMSMASI LTNTHIELEE SRQKCPPCWY KFNANCLLWD CCKPWLK VVNLVVMDF VDLAITICIV LNTLFMAHEH YPMTEQSSV LSVGNLVFTG
IS1 IS2

801 IFTAEMLKI IAMDPIYYFQ EGNIFDGF I VLSLMELGL ANVEGLSVLR SFRLLRVFKL AKSWPTLNML IKIIGNSVGA LGNLTVLAI IVFIFAVVGM
IS2 IS3 IS4 IS5

901 QLPCKSYKEC VCKISNDCEL PRWHMHFFH SFLIVFRVLC GEWIETMWD MEVAGQTMCL TVFMMVMVIG NLVVLNLFLA LLLSSPSSDN LAATDDDNEM
IS6

1001 NNLQIAVGMR QKGIDFVKR IREFIQKAFV RKQKALDEIK PLEDLNNKKD SCISNHTTIE IGKDLNLYKDG NGGTTSGIGS SVEKYVVDDES DYMSFINNPS
ID II-III

1101 LTVTVPIALG ESDFNENLNT EFSSESDEE SKEKLNATSS SEGSTVDIGA PAEGEQPEAE PEESLEPEAC FTEDCVKRFK CQISIEEGK GKLWNLKRT
ID II-III

1201 CYKIVEHNWF ETFIVFMILL SSGALAFEDI YIEQRKTIKT MLEYADK VFT YIFILEMLLK WYAYGFQMYF TNAMCWLDFL IVDVSLVSLT ANALGYSELG
IIIS1 IIIS2 IIIS3

1301 AIKSLRTRA LRPLRALSRF EGMRVVNNAL LGAIPSIMNV LLVCLIFWLI FSIMGVNLFA GKFYHCINYT TGMFVDVSVV NNYSECQALI ESNQTARWKN
IIIS4 IIIS5

1401 VKVNFNDVGL GYLSLLQVAT FKGWMDIMYA AVDSRNVELQ PRYEDNLYMY LYFVIFIIFG SFTTLNLFIG VIIDNPNQK KFGGODIFM TEEQKKYNA
IIIS6 ID III-IV

1501 MKKLGSKKPK KPIPRPANKF QGMVDFVTK QVFDISIMIL ICLNMVTMV ETDDQSQEMT NILYWINLVF IVLFTGECVL KILSLRHYYF TIGWNIFDV
ID III-IV IVS1 IVS2 IVS3

1601 VVILSIVGMF LAELIEKYFV SPTLFRVIRL ARIGRILRLI KGAGGIRTL FALMMSLPAL FNIGLLFLV MFIYAIFGMS NFAYVKREVG IDDMFNFTF
IVS3 IVS4 IVS5

1701 GNSMICLFQI TTSAGWDGLL APILNSGPPD CDPEKDHPGS SVKGDGCPNS VGIFFFVSYI IISFLVVNM YIAVILENFS VATEESAEP SEDDFEMFYE
IVS6

1801 VWEKFDPPAT QFIEFCKLSD FAALDPPLL IAKPNKVQLI AMDLPMVSGD RIHCLDILFA FTKRVLGESG EMDALRIQME ERFMASNPSK VSYEPITTL
C-TERM

1901 KRKQEEVSAI VIQRAYRRYL LKQKVKVSS IYKDKGKED EGTPIKEDII TDKLNENSTP EKTDTVPTST SPPSYDSVTK PEKEKFEKOK SEKEDKGKDI
C-TERM

2001 RESKK

Figure 3.

In vivo phosphorylation sites and alternative peptides of rat brain Nav1.2 identified by nano-LC MS/MS analyses. A. Amino acid sequence of the canonical form of Nav1.2 (Swiss-Prot: P04775.1; NCBI Reference Sequence: NP_036779.1). Coverage obtained is highlighted in yellow, *in vivo* phosphorylation sites are in red, positions differing in an alternative Nav1.2 sequence (IPI00476429.2) are shown in blue. Underlined sequences represent the proposed transmembrane segments S1-S6 in each domain I-IV.

1 MEQTVLVPPG PDSFNFFTRE SLAAIERRIA EEKAKNPKPD KKDDDENGPK PNSDLEAGKN LPFIYGDIPP EMVSEPLEDL DPYYINKKTF IVLNKGKAIF
 N-TERM
 101 RFSATSALYI LTPFNPLRKI AIKILVHSLF SMLIMCTILT NCVFMTMSNP PDWTKNVEYT FTGIYTFESL IKIARGFCL EDFTFLRDPW NWLDFTVITF
 N-TERM IS1 IS2 IS3
 201 AYVTEFVDLG NVSALRTFRV LRALKTISVI PGLKTIVGAL IQSVKKLSDV MILTVFCLSV FALIGLQLFM GNLRNKCQVW PPTNASLEEH SIEKNVTTDY
 IS3 IS4 IS5
 301 NGTLVNETVF EFDWKSYIQD SRYHYFLEGV LDALLCGNSS DAGQCPEGYM CVKAGRPNPY GYTSFDTFSW AFLSLFRLMT QDFWENLYQL TLRAAGKTYM
 401 IFFVLVIFLG SFYLINLILA VVAMAYEEQN QATLEEAQK EAEFQQMLEQ LKKQQEAAQ AAAATASEHS REPSAAGRLS DSSSEASKLS SKSAKERNR
 IS6 ID I-II
 501 RKKRKQKEQS GGEKDDDEF HKSESEDSIR RKGFRFSIEG NRLTYEKRY SPSHQSLLSIR GSLFSRPRNS RSLFSFRGR AKDVGSENDP ADDEHSTFED
 ID I-II
 601 NESRRDSLFLV PRRHGERRNS NLSQTSRSSR MLAGLPANGK MHSTVDCNG VSLVGGPSVP TSPVGQLLPE VIIDKPATDD NGTTTETEMR KRRSSSFHVS
 ID I-II
 701 MDFLEDPSQR QRAMSIASIL TNTVEELEES RQKCPPCWYK FSNIFLIWDC SPYWLKVKHI VNLVVMDFPV DLAITICIVL NTLFMAMEHY PMTEHFNHVL
 IS1
 801 TVGNLVFTGI FTAEMFLKII AMDPYYYFQE GWNIFDGFIV TSLSLVELGLA NVEGLSVLRS FRLLRVFKLA KSWPTLNMLI KIIGNSVGAL GNLTLVLAI
 IS2 IS3 IS4 IS5
 901 VFIFAVVGMQ LFGKSYKDCV CKIATDCKLP RWHMNDFFHS FLIVFRVLCG EWETMWDCM EVAGQAMCLT VFMMVMVIRN LVVLNLFAL LLSSFADNL
 IS6
 1001 AATDDDNEMN NLQIAVDRMH KGVAVVKRKI YEFIQQSFVR KQKILDEIKP LDDLNNRKN CTSNHTTEIG KDLDCLKDVN GTTSGIGTGS SVEKYIIDES
 ID II-III
 1101 DYMSFINNPS LTVTVPIAVG ESDFENLNT ESDFENLNT DFSSSEDL ESKELNESSS SSEGSTVDIG APAEEQPVME PEETLEPEAC FTEGCVQRFK CCQISVEEGR
 ID II-III
 1201 GKQWNLRRRT CFRIVEHWNF ETFIVFMILL SSGALAFEDI YIDQRKTIKT MLEYADKVFT YIFILEMLLK WVAIGYQTYF TNAWCWLDL IVDVSLVSLT
 IIIS1 IIIS2 IIIS3
 1301 ANALGYSELG AIKSLRTLRA LRPLRALS RF EGMRVVVNAL LGAIPSIMNV LLVCLIFWLI FSIMGVNLFA GKFYHCNTT TGDTEITEV NNHSDCLKI
 IIIS4 IIIS5
 1401 ERNETARWKN VKVNFNDVGF GYLSLLQVAT FKGWMDIMYA AVDSRNVELQ PKYEEESLYM LYFVIFIIFG SFFTLNLFIG VIIDNFNQOK KFGGQDIFM
 IIIS6 ID III-IV
 1501 TEEQKKYNA MKKLGSKKPQ KPIPRPGNKF QGMVDFVTR QVFDISIMIL ICLNMVTMMV ETDDQSDYVT SILSRINLVF IVLFTGECVL KLISLRHYFF
 ID III-IV IVS1 IVS2
 1601 TIGWNIFDFV VVILSIVGMF LAELIEKYFV SPTLFRVIRL ARIGRIIRLI KGAKGIRTL FALMMSLPAL FNIGLLFLV MFIYAIFGMS NFAYVKREV
 IVS3 IVS4 IVS5
 1701 IDDMFNFETF GNSMICLFQI TTSAGWDGLL APILNSKPPD CDPKNVNP GS SVKGDCGNPS VGIFFFVSYI IISFLVVNM YIAVILENFS VATEESAEP
 IVS6
 1801 SEDDFEMFYE VWEKFPDPAT QFMEFEKLSQ FAAALEPPLN LPQPNKLQLI AMDLPMVSGD RIHCLDILFA FTKRVLGESG EMDALRIQME ERFMASNP
 C-TERM
 1901 VSYQPIITTL KRKQEEVSAV IIRAYRRHL LKRTVKQAS TYNKNLKG ANLLVKEDMI IDRINENSIT EKTDLTMSTA ACPPSYDRVT KPIVEKHEQE
 C-TERM
 2001 GKDEKAKGK

Figure 4.
In vivo phosphorylation sites on rat brain Nav1.1 identified by nano-LC MS/MS analyses. A.
 Amino acid sequence of Nav1.1 (Swiss-Prot: P04774.1; NCBI Reference Sequence:
 NP_110502). Color scheme as in Figure 3.

Figure 5.

Cartoon of the membrane topology of the Nav1.2 α subunit showing a comparison of *in vivo* phosphorylation sites *versus* predictions. The *in vivo* Nav1.2 phosphorylation sites described here are color coded to show their frequency of representation in the top 50 phosphorylation sites among four prediction algorithms (NetphosK, Netphos2, ScanSite and Disphos).

Consensus phosphorylation sites where at least one of the protein kinase-specific prediction programs (NetPhosK, ScanSite) identified a specific protein kinase are given. Note that S579 (asterisk) is found only in the alternative IPI00476429.2 sequence.

Table 1

Nav isoform	Total matches obtained	Unique Peptides Obtained	Percent of Total Unique Peptides Obtained
		K69/3-Based Purifications	
Nav1.2	2259	892	96.0
Nav1.6	358	20	2.1
Nav1.1	701	6	0.6
Nav1.3	785	5	0.5
Nav1.5	93	4	0.4
Nav1.7	525	2	0.2
Nav1.4	139	0	0
		K58/35-Based Purifications	
Nav1.2	2176	797	53.7
Nav1.1	1327	338	22.8
Nav1.6	727	217	14.6
Nav1.3	975	104	7.0
Nav1.7	563	20	1.3
Nav1.5	229	6	0.4
Nav1.4	61	1	0.07