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Analgesic Neuropeptide W Suppresses Seizures in the Brain Revealed by Rational Repositioning and Peptide Engineering

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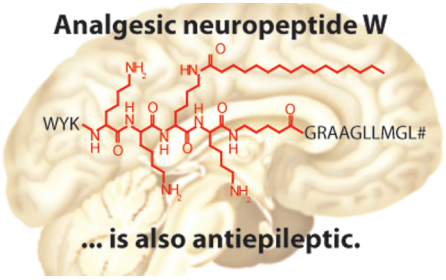
Abstract



Anticonvulsant neuropeptides play an important role in controlling neuronal excitability that leads to pain or seizures. Based on overlapping inhibitory mechanisms, many anticonvulsant compounds have been found to exhibit both analgesic and antiepileptic activities. An analgesic neuropeptide W (NPW) targets recently deorphanized G-protein coupled receptors. Here, we tested the hypothesis that the analgesic activity of NPW may lead to the discovery of its antiepileptic properties. Indeed, direct administration of NPW into the brain potently reduced seizures in mice. To confirm this discovery, we rationally designed, synthesized, and characterized NPW analogues that exhibited anticonvulsant activities following systemic administration. Our results suggest that the combination of neuropeptide repositioning and engineering NPW analogues that penetrate the blood-brain barrier could provide new drug leads, not only for the treatment of epilepsy and pain but also for studying effects of this peptide on regulating feeding and energy metabolism coupled to leptin levels in the brain.

Keywords: Neuropeptide W, neuropeptide repositioning, lipidization-cationization, anticonvulsant, metabolic stability, systemic bioavailability

Neuropathic pain and epileptic seizures both result from an imbalance between excitation and inhibition of neuronal firing. The recent use of antiepileptic compounds for the treatment of pain (repositioning) is largely due to the compounds' ability to inhibit excessive firing in neurons through voltage and ligand-gated ion channel activities (*1*). It has been well documented that several anticonvulsant neuropeptides possess concurrent antiepileptic and analgesic activities (Supporting Information Table S1). For example, galanin (GAL), neuropeptide Y (NPY), and somatostatin (SRIF) have been reported to control seizures in the brain (*2*−*4*) in addition to exerting their analgesic activities (*5*−*7*). Past studies of neurotensin (NT) and enkephalin (Enk) widely focused on their analgesic activities; however, these peptides were recently shown to also act as potent anticonvulsants (*8*−*10*). More studies are needed to describe mechanisms that underlie the mutual relationships between analgesic and anticonvulsant properties of these neuropeptides.

Recently, two highly homologous endogenous neuropeptides, namely, neuropeptide W (NPW) and neuropeptide B (NPB), were identified as high affinity ligands for $NPBW_1$ (GPR7) and/or $NPBW_2$ (GPR8) receptors (Supporting Information Figure S1) (*11*). NPW binds to both $NPBW_1$ and $NPBW_2$ with low nanomolar affinity (K_i = 0.14 and 29 nM, respectively). Early studies showed that NPW played an important role in modulating feeding behaviors (*11*, *12*). Subsequent studies revealed that intrathecal (i.t.) administration of this peptide suppressed inflammatory pain in the mouse formalin test. NPW was also shown to be antiallodynic in the partial sciatic nerve ligation model (*13*−*15*). Of particular interest, Zaratin and coworkers reported increased expression of $NPBW_1$ receptor subtypes in myelin-forming Schwann cells in animal models of acute inflammatory and trauma-induced neuropathic pain, suggesting a central role for this peptide as an analgesic (*16*). Price et al showed that direct injection of NPW-23 into the hypothalamus influenced the excitability of specific groups of neurons in rats (*17*, *18*). In short, NPW has been shown to be a multifunctional endogenous ligand for $NPBW_1$ and $NPBW_2$ where it plays an important role in modulating

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pain pathways and feeding behaviors (*19*). In this work, we tested the hypothesis that, based on its analgesic properties, NPW may also suppress seizures when delivered into the brain. To the best of our knowledge, this is the first example of using neuropeptide repositioning as a strategy to rationally discover new bioactivity of endogenous peptides.

To test the hypothesis that analgesic NPW can possess anticonvulsant properties, this peptide was evaluated in the 6 Hz seizure test in mice. hNPW-23 was injected intracerebroventricularly (i.c.v.) into mice at doses 0.01, 0.1, 1, 2, and 4 nmol, and its activity was evaluated after 15 min post drug administration (experimental details are provided in the Supporting Information). At these doses, 2/8, 4/8, 5/8, 6/8, and 6/8 mice, respectively, were protected from the seizure activity (Figure 1A). At each concentration, 1/8 mice displayed rotorod toxicity. The ED_{50} value was calculated as 0.24 nmol with a 95% confidence interval of 0.003−1.2 nmol ($b = 0.52 \pm 0.22$).

Similarly, the effect of NPB-29 was also evaluated in the same epilepsy model. The time of peak effect was determined to be 15 min following i.c.v. administration. Mice were tested at 0.1, 1.0, 1.5, and 2 nmol doses, resulting in the protection from seizure activities in 1/8, 2/8, 4/8, and 6/8 mice, respectively (Figure 1A). Furthermore, at these doses, 0/8, 3/8, 3/8, and 4/8 mice were found to display motor toxicity, respectively. The ED_{50} was determined to be 1.5 nmol with a 95% confidence interval of 0.4−128 nmol ($b = 1.2 \pm 0.56$). Notably, the ED_{50} value determined for NPB-29 was approximately sixfold higher than that for NPW-23. This is the first report on the anticonvulsant activities of NPW and NPB.

The discovery of the anticonvulsant activity of NPW/NPB prompted us to confirm this finding by generating systemically bioavailable NPW analogues that could penetrate the blood-brain barrier (BBB). To design such NPW-derived compounds, we employed the lipidization−cationization strategy that was successfully applied toward systemically active analogues of GAL, NT, and NPY (GAL-B2, NT-BBB1, and NPY-BBB2, respectively)-(*10*, *20*−*23*). Structures and summary of anticonvulsant and physicochemical properties of GAL-B2, NT-BBB1 and NPY-BBB2 are provided in Figure S2 in the Supporting Information. The penetration of these neuropeptide analogues across the BBB was accomplished by introduction of a lipoamino acid (lysine-palmitoyl) in the context of several Lys residues, yielding the so-called "lipidization−cationization" motif. The BBB-permeable analogues of GAL, NT, and NPY exhibited significantly improved resistance to degradation in the serum stability assay, as well as increased log *D* values.

Design of BBB-permeable NPW analogues was based on previous structure−activity relationship (SAR) findings that the C-terminal fragment NPW^{14-23}

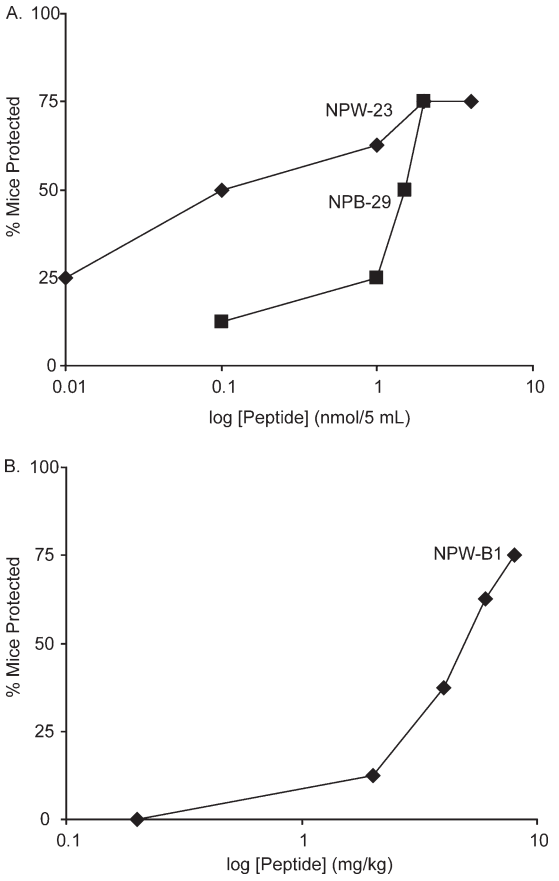

Figure 1. Anticonvulsant activities of NPW-23, NPB-29, and the modified NPW-B1 analogue. (A) Dose response curves for i.c.v. administered NPW-23 and NPB-29. Data represent the number of animals which did not exhibit classical seizure-type behaviors at 0.25 h post drug administration. ED_{50} values were calculated at 0.24 nmol (95% CI = 0.003−1.2) and 1.5 nmol (95% CI = 0.4−128) for hNPW-23 and NPB-29, respectively. (B) Dose response curve for the modified analogue NPW-B1. Peptide was administered i.p. into groups of eight adult male CF-1 mice ($n = 4$ for 0.2 and 8 mg/kg doses). Data represent the percentage of animals which did not exhibit classical seizure-type behaviors at 30 min post administration (TPE). ED_{50} for this analogue was calculated as 4.9 mg/kg (95% CI = 2.9 − 12.2) using Probit software.

and the N-terminal tripeptide of NPW are important for its structural and functional properties (*24*). Furthermore, Kanesaka and co-workers constructed centrally truncated analogues of NPW in which nonessential amino acid residues were replaced by backbone spacer units (5-aminovaleric acid), resulting in potent and selective $NPWB_1$ agonists (*12*). The NPW analogue containing three 5-aminovaleric acid units, namely, NPW Ava-3 shown in Figure 2, exhibited similar $NPBW_1$ binding affinity to that of the full-length peptide (K_i ($NPBW_1$) = 0.40 nM versus 0.14 nM). The design strategy for systemically active NPW analogues was identical to that previously described for NT and NPY analogues (*12*) and is illustrated in Figure 2 and Table 1. The lipidization−cationization motif was inserted between

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the N-terminal "WYK" sequence and the C-terminal active fragment (Figure 3), while we varied the position of Lys-palmitoyl and the number of Lys residues (based on our previous experience with BBB-permeable GAL analogues, the position of the lipoamino acid and number of Lys residues affected their in vivo activities in the epilepsy tests in mice (*20*)). Seven NPW analogues, NPW-B1 to NPW-B7, contained 5-aminovaleric acid as a backbone spacer, similar to that used in the NPW Ava-3 analog (*12*).

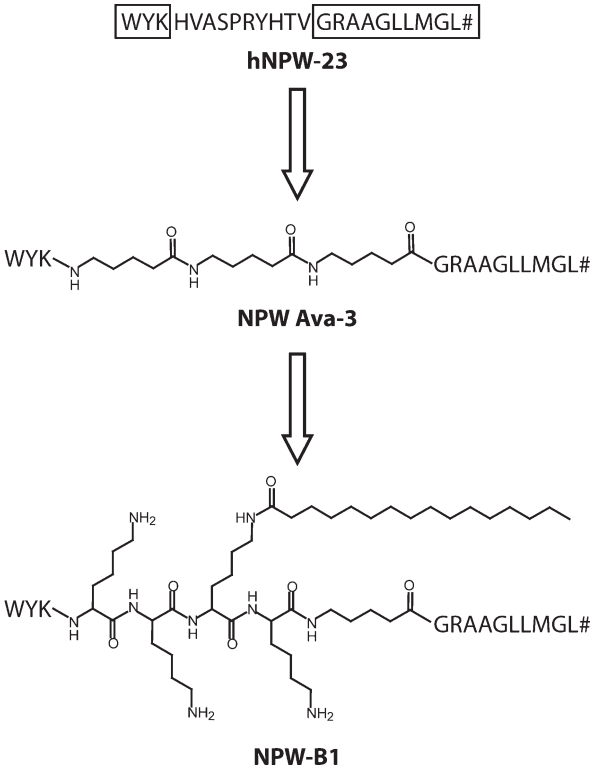

Figure 2. Rational design of systemically active NPW analogues. The primary sequence for hNPW-23 is shown with bioactive fragments highlighted by boxed regions of the peptide. Based on previous SAR data, it was shown that centrally truncated analogues of hNPW-23 (NPW Ava-3) could be constructed that exhibited high affinity binding for $NPBW_1$ (*12*). This work describes the synthesis and characterization of a series of centrally truncated NPW analogues which have been modified with the described lipidization−cationization motifs. Shown here is the analogue NPW-B1 which was chosen as the lead candidate for further characterization.

All analogues were synthesized on solid support using an automated peptide synthesizer and standard Fmoc protocols (synthesis details are provided in the Supporting Information). Peptides were cleaved from the resin by treatment with reagent K and were purified by preparative reversed-phase HPLC. Masses of all compounds were confirmed by MALDI-TOF MS (Supporting Information Table S2). Retention times were calculated from the average of three independent HPLC separations for each analogue and are summarized in Table S2 in the Supporting Information. Log *D* values for NPW analogues were determined using both the shake-flask and HPLC capacity factor methods. Calculated values for partitioning are summarized in Table 1 and Figure 3A. The unmodified peptide hNPW-23 was hydrophilic with a negative log *D* value, whereas the modified analogues showed dramatic increase in their hydrophobicities with log *D* values ranging from 1.6 to 1.8. The lead compound, NPW-B1, possessed a log *D* value of 1.7 (Table 1; Figure 3A).

To determine metabolic stability of NPW analogues, the analogues were incubated in 25% rat blood serum at 37 °C and their degradation was monitored by HPLC. Aliquots were withdrawn after 30 min, 1 h, 2 h, 4 h, and 8 h. The time courses of the disappearance of the intact species were plotted ,and $t_{1/2}$ values were calculated. As summarized in Table 1 and Figure 3B and C, in vitro

Table 1. Summary of Sequences, Physicochemical, and Pharmacological Properties of Neuropeptide W Analogues

		physicochemical properties		in vivo activity (4 mg/kg, i.p.)	
analogue	sequence[a]	log D[b]	$t_{1/2}$ (h)[c]	mice protected at 30 min[d]	motor toxicity
hNPW	WYKHVASPRYHTVGRAAGLLMGL#	−0.13 ± 0.26	0.67 ± 0.05	0/4	0/4
NPW-B1	Ac-WYK**K(K_p)K(Ava)**GRAAGLLMGL#	1.7 ± 0.14	3.6 ± 0.72	2/4	0/4
NPW-B2	Ac-WYK(**K_p)KKK(Ava)**GRAAGLLMGL#	1.8 ± 0.01*	3.7 ± 1.31	2/4	0/4
NPW-B3	Ac-WYK**K(K_p)KK(Ava)**GRAAGLLMGL#	1.6 ± 0.01*	1.5 ± 0.14	2/4	0/4
NPW-B4	Ac-WYK**KK(K_p)K(Ava)**GRAAGLLMGL#	1.6 ± 0.01*	n.d.[e]	1/4	1/4
NPW-B5	Ac-WYK**KKK(K_p)(Ava)**GRAAGLLMGL#	1.7 ± 0.15	1.9 ± 0.24	2/4	0/4
NPW-B6	Ac-WYK**K(K_p)K(Ava)(Ava)**GRAAGLLMGL#	1.7 ± 0.01*	n.d.[e]	3/4	4/4
NPW-B7	Ac-WYK**K(K_p)KK(Ava)(Ava)**GRAAGLLMGL#	1.5 ± 0.01*	n.d.[e]	2/4	0/4

[a] K_p is N^{ε}-palmitoyl-L-lysine, Ava is 5-aminovaleric acid, (#) denotes an amidated C-terminus. [b] Log *D* values determined using the shake-flask method or based off of HPLC retention times. (*) denotes log *D* values determined using the capacity factor (k') which was calculated by average retention HPLC time. [c] Half-life ($t_{1/2}$) values were determined using an in vitro serum stability assay. Peptides were incubated in 25% rat blood serum, and degradation was monitored by analytical HPLC methods. [d] Anticonvulsant activity was assessed in the 6 Hz (32 mA) psychomotor seizure assay. Data represent the number of mice within each group that did not exhibit seizure activity at 30 min post i.p. administration (4 mg/kg, i.p.). Motor toxicity was determined using the rotorod motor impairment test. [e] n.d.: not determined.

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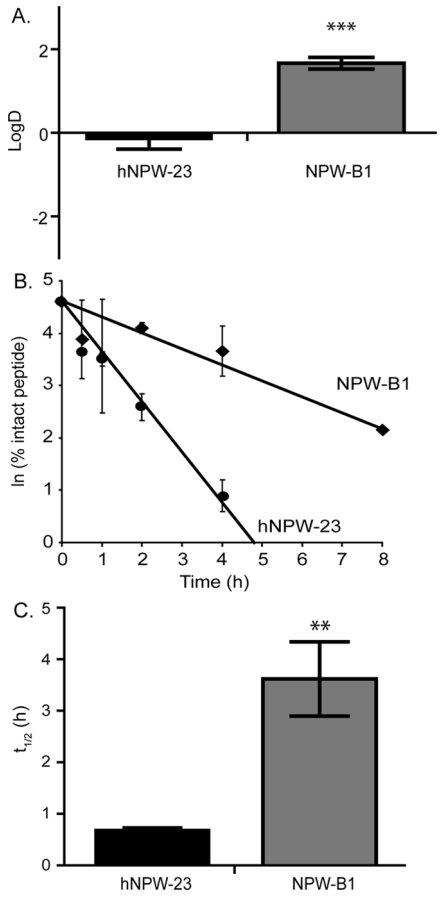

Figure 3. Octanol/water partitioning and serum stability studies for NPW and the lead compound, NPW-B1. (A) Comparison of experimentally determined log *D* values for hNPW-23 and NPW-B1. Values were calculated from the average of three independent experiments via the shake-flask method using a 50:50 ratio of n-octanol/PBS, pH 7.4. Log *D* for the native peptide was calculated as −0.13, whereas NPW-B1 had a log *D* value of 1.7. Statistical comparison between the full-length and modified peptide was performed using the two-tailed *t* test function of GraphPad software. Statistical significance was noted as follows: (***) *P*-value < 0.001. (B) Time-course experiments showing the time-dependence of the disappearance on the intact peptide between 0 and 8 h. Plot compares the degradation of hNPW-23 to that of NPW-B1. Using the slope of the fitted line, half-lives ($t_{1/2}$) were calculated as 0.67 and 3.6 h, respectively. (C) Comparison of the calculated half-lives ($t_{1/2}$) of hNPW-23 and NPW-B1 in the serum stability assay. Results were obtained from the average slopes of three independent time-course experiments. Statistical comparison was performed using the two-tailed *t* test function. (**) *P*-value < 0.01.

metabolic stability was significantly increased ranging from 1.5 − 3.7 h. For the native peptide, the $t_{1/2}$ value was 0.67 h, whereas the NPW-B1 had a $t_{1/2}$ value of 3.6 h. These results are consistent with our previous observations that the lipidization−cationization of GAL, NT, and NPY increased their metabolic stabilities and yielded log *D* values favorable for central nervous system (CNS) drugs (log *D* > 1).

The anticonvulsant activity of the modified NPW analogues was evaluated in the 6 Hz model of epilepsy in mice following bolus intraperitoneal injections. At a dose of 4 mg/kg, protection from seizures was assessed after 30 min post drug administration. As shown in Table 1, NPW-B1, B2, B3, B5, and B7 analogues exhibited 50% protection of the animals tested, whereas the unmodified NPW had no apparent antiseizure activity. NPW-B6 exhibited 75% protection but was associated with concurrent motor impairment toxicity. To confirm the observed antiseizure activities of the analogues, a dose−response study was carried out using NPW-B1 as a lead compound. Figure 1B illustrates the dose−response results that yielded ED_{50} of 4.9 mg/kg (95% CI = 2.9−12.2).

Through neuropeptide repositioning, we discovered the anticonvulsant properties of NPW using both a direct administration of the peptide into the brain and by engineering systemically bioavailable NPW analogue. Recent studies have shown a high level of expression of NPW and NPW-receptors in the hippocampus and amygdala of both rodents and humans, suggesting an important role for this peptide in the CNS (*13*, *16*, *25*). The NPW analogues described in this work likely mediate their anticonvulsant activities through activation of the $NPBW_1$ receptor, as it has been previously shown that rodent models do not possess the $NPBW_2$ subtype (*26*). However, without detailed pharmacological studies including the receptor binding experiments, we cannot definitely exclude a possibility of off-target interactions with other G-protein coupled receptors (GPCRs) (noteworthy, the lipidization−cationization motifs applied for NPW analogues neither significantly affected receptor binding properties of systemically bioavailable galanin, NPY, and NT-based analogues, nor exhibited intrinsic anticonvulsant activity (*23*), suggesting on-target mechanism of suppressing seizures). It has been previously reported that activation of $NPBW_1$ modulates hyperexcitability of neurons in the CNS (*25*). Price and co-workers previously reported that application of NPW to neurons in the paraventricular nucleus (PVN) of the hypothalamus resulted in a large population of hyperpolarized neurons (57%) with smaller mixed populations of depolarized and unchanged neurons (*18*). We hypothesize that the observed anticonvulsant effects exerted by hNPW-23, and the modified NPW analogues, resulted from activation of the $NPBW_1$ receptor which may result in hyperpolarization of the cell membrane. Hyperpolarization of the membrane would then result in decreased excitatory behavior of the neuron, thus modulating seizure activity (*1*, *17*). This hypothesis remains to be tested, and it is acknowledged that further studies into the precise mechanism by which NPW inhibits neuronal hyperexcitability need to be conducted.

This work further extends the strategy of lipidization−cationization to improve CNS bioavailability of anticonvulsant neuropeptides. Upon i.p. administration,

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hNPW-23 did not exhibit anticonvulsant activity, suggesting that the native peptide did not effectively penetrate into the brain. However, all NPW analogues containing the lipidization−cationization motif exhibited comparable anticonvulsant activities. We show in our previous work that the combination of the lipidization and cationization did not affect high affinities of the GAL, NPY, or NT to their respective native receptors -(*10*, *20*−*22*). Thus, based on SAR results for centrally truncated NPW analogues (*12*), NPW-B1 to B7 are expected to retain similar binding affinities toward $NPBW_1$ receptors. We acknowledge here that more pharmacological studies are needed, both in vitro and in vivo, to dissect the exact mechanism of seizure control by BBB-permeable analogues of NPW. We also acknowledge that further optimization of NPW analogues should be undertaken as a part of the lead optimization studies. It is conceivable that through the addition/deletion of 5-Ava spacer units or introduction of other backbone spacers (*9*, *27*), future NPW analogues may exhibit even more potent antiepileptic activities as compared to NPW-B1.

The development of systemically active NPW analogues further increases the number of anticonvulsant neuropeptides that can be explored as CNS neurotherapeutics. In addition to having potential as new antiepileptic drug candidates, NPW analogues that penetrate across the BBB may have broader applications. For example, NPW has garnered recent interest because of its ability to regulate feeding behaviors and influence metabolic function (*28*−*30*). It was previously reported that NPW accomplishes these roles through activation of NPW-receptors in the hypothalamus. It has been hypothesized that NPW acts through a compensatory mechanism to regulate energy homeostasis when leptin levels in the brain are decreased (*29*). Therefore, the modified NPW analogues described here may be useful pharmacological compounds toward the development of therapies for metabolic illnesses leading to obesity. In summary, the combination of rational repositioning and peptide engineering has provided new tools to study a role of NPW in the brain.

Supporting Information Available

Detailed description of experimental procedures. A summary of anticonvulsant and analgesic activities of endogenous neuropeptides is shown in Table S1. Mass spectrometry data and HPLC retention times for NPW analogues are shown in Table S2. Four figures summarize sequences of hNPW and hNPB (Figure S1), structures and properties of systemically active analogues of GAL, NT and NPY (Figure S2), HPLC chromatograms of NPW and its analogues (Figure S3 and S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Abbreviations

BBB, blood-brain barrier; CNS, central nervous system; GAL, galanin; i.c.v., intracerebroventricular; i.t., intrathecal; i.p., intraperitoneal; NPFF, neuropeptide FF; nH_2O, nanopure water; NT, neurotensin; NPW, neuropeptide W; NPY, neuropeptide Y; SAR, structure−activity relationship.

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