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sec-Butylpropylacetamide (SPD) has antimigraine properties

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Abstract

Background—Though migraine is disabling and affects 12%–15% of the population, there are few drugs that have been developed specifically for migraine prevention. Valproic acid (VPA) is a broad-spectrum antiepileptic drug (AED) that is also used for migraine prophylaxis, but its clinical use is limited by its side effect profile. *sec*-Butylpropylacetamide (SPD) is a novel VPA derivative, designed to be more potent and tolerable than VPA, that has shown efficacy in animal seizure and pain models.

Methods—We evaluated SPD's antimigraine potential in the cortical spreading depression (CSD) and nitroglycerin (NTG) models of migraine. To evaluate SPD's mechanism of action, we performed whole-cell recordings on cultured cortical neurons and neuroblastoma cells.

Results—In the CSD model, the SPD-treated group showed a significantly lower median number of CSDs compared to controls. In the NTG-induced mechanical allodynia model, SPD dose-dependently reduced mechanical sensitivity compared to controls. SPD showed both a significant potentiation of GABA-mediated currents and a smaller but significant decrease in NMDA currents in cultured cortical neurons. Kainic acid-evoked currents and voltage-dependent sodium channel currents were not changed by SPD.

Conclusions—These results demonstrate SPD's potential as a promising novel antimigraine compound, and suggest a GABAergic mechanism of action.

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Keywords

sec-butylpropylacetamide (SPD); migraine; cortical spreading depression; nitroglycerine test; mechanical allodynia

Introduction

Despite the potential size and unmet need of the migraine treatment market, antimigraine drug development has been limited. Most antimigraine drugs used in the clinic today have been borrowed from other specialties, a prominent example being antiepileptic drugs (AEDs). Migraine and epilepsy are comorbid and are both considered paroxysmal hyperexcitable disorders of the nervous system (1). Certain AEDs, e.g. valproic acid (VPA) and topiramate, are effective in the prevention of migraine (2–4); we hypothesized that novel AEDs might have potential as migraine preventives.

Migraine aura is thought to be caused by cortical spreading depression (CSD), a wavelike depolarization both of neurons and glia that moves across gray matter at the speed of 3 mm/min (5). There is also evidence from rodent models that CSD triggers the pain associated with migraine; CSD increases c-Fos expression in the trigeminal nucleus caudalis (6) and increases trigeminal nociceptor firing rates (7). Finally, migraine prophylactic drugs (including VPA) have been shown to decrease the number of CSDs in animal models (8). Thus CSD has been developed as a tool for drug discovery, particularly for migraine with aura.

Nitroglycerin (NTG) infusion causes a bilateral headache immediately after administration in patients with no prior migraine history (9). In migraineurs, NTG induces delayed headaches that fulfill diagnostic criteria for migraine without aura (10). In rodents, NTG induces c-Fos expression in lamina I and II of the trigeminal nucleus caudalis (suggesting that NTG induces craniofacial pain), elevates plasma calcitonin gene-related peptide (CGRP; similar to what is seen during migraine in humans (11)), and induces peripheral hyperalgesia (12,13) that is reversible with the anti-migraine drug sumatriptan (12). Importantly, NTG infusion does not alter CSD thresholds, suggesting that it affects migraine pain pathways independent of CSD generation (12). NTG infusion has emerged as a complementary technique to CSD in preclinical migraine models (14).

VPA is an older-generation, broad-spectrum AED that is used and approved for migraine prophylaxis. However, VPA's clinical use is limited by its side effects: mainly teratogenicity, but also hepatotoxicity, polycystic ovarian syndrome, tremor, weight gain, and alopecia. This especially limits the use of VPA in women of child-bearing age, the group most affected by migraine. Furthermore, in May 2013, the Food and Drug Administration (FDA) changed VPA from Pregnancy Category "D" (the potential benefit of the drug in pregnant women may be acceptable despite its potential risk) to "X" (the risk of use in pregnant women clearly outweighs any possible benefit of the drug) for migraine prophylaxis. *sec*-Butylpropylacetamide (SPD) is a second-generation VPA amide derivative, developed as a more potent and non-teratogenic second-generation alternative to VPA. The rationale for increased potency is that the use of smaller doses would reduce "off target" effects and thus

side effects. Regarding teratogenicity, in contrast to VPA, SPD was non-teratogenic at a dose 4–13 times higher than its anticonvulsant ED₅₀ values (15). SPD also shows promise both in seizure and neuropathic pain models (16–18), where it was indeed found to be more potent than VPA. Given these promising features, we tested SPD in the CSD and NTG models, and followed with electrophysiological experiments to examine its migraine-relevant mechanism of action.

Materials and methods

All experiments, analysis, and reporting were performed according to Animals in Research: Reporting *In Vivo* Experiments (ARRIVE) criteria (19). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Utah.

Animals

Behavioral and *in vivo* physiological studies were performed on C57Bl/6 male mice (20–35 g; Charles River). Electrophysiological experiments were conducted using cell cultures from CF-1 mice. Animals were housed in clear acrylic cages (12 in × 6 in × 8 in) and were allowed free access to water and food *ad libitum* (Product 8640, Harlan Teklad), in a temperature, humidity, and 12:12-hour (h) light:dark cycle-controlled environment. Every attempt was made to minimize the pain experienced by experimental animals. During behavioral experiments, animals were carefully monitored for signs of distress, which were not evident. During drug testing, animals were also monitored for evidence of sedation (principally reduced spontaneous activity and discoordination; e.g. wobbling stance), as SPD has been previously reported to be sedating at higher doses (17).

Materials

SPD was synthesized according to a previously published procedure (17). SPD was suspended in methyl-cellulose (4000 Centipoise, Sigma-Aldrich) immediately before administration. The control vehicle for SPD testing was methylcellulose. NTG 5.0 mg/ml (in 30% propylene glycol, 30% ethanol, and 40% water) was purchased from American Regent Inc. NTG was diluted in saline in a 1:5 ratio before each injection. The vehicle for NTG testing was 30% propylene glycol, 30% ethanol, and 40% water, diluted in saline at the same 1:5 ratio as NTG.

CSD model

Non-ventilated animals—Eleven animals were randomly divided into control ($n=5$) and drug-treated ($n=6$) groups. These numbers allowed us to detect a difference of 30% (determined from prior experiments) with a power of 80% to reject the null hypothesis. Only male animals were used to avoid the known effects of sex on CSD susceptibility (20). Each animal was anesthetized with isoflurane (5% induction, 1.4%–1.7% maintenance) and mounted on a stereotaxic frame (Kopf Instruments). Throughout the experiment, the animal's vital signs (oxygen saturation, heart rate, respiratory rate, body temperature) were monitored and stabilized using a physiological monitoring apparatus (MouseStat, Kent Scientific). The parietal skull was exposed between bregma and lambda, and the region 1 mm posterolateral to bregma, anterolateral to lambda, and medial to the temporal ridge was

thinned to transparency. A burr hole was created 0.5 mm from the temporal ridge, midway between bregma and lambda for potassium chloride (KCl) solution application (Figure 1(a)). The cortex was illuminated by a white light-emitting diode (LED) (5500K, Phillips Lumileds) and reflected light (optical intrinsic signal; OIS) was collected with a lens system consisting of two f/0.95 lenses connected front-to-front and a high-sensitivity, 8-bit, charge-coupled device camera (MiCAM02, Brain Vision). Images were acquired at 2 Hz for the duration of the experiment. All experiments started with 15 min baseline recording (*pre-CSD baseline*, 15 min) to rule out the occurrence of CSDs as a result of the surgical procedure. CSDs were then induced by a continuous perfusion of 1 M KCl using a syringe pump (1 ml/h) delivered for 15 min *before* drug treatment as an internal control (*KCl baseline*, 15 min). After CSDs were established, vehicle or drug were administered intraperitoneally (i.p.) (in a blinded manner), and CSDs were further induced and recorded for an additional 60 min (*post-treatment*, 60 min) via continuous perfusion of 1 M KCl. Excess KCl was removed by the capillary action of rolled filter paper placed adjacent to the KCl burr hole. A thin (but intact) skull preparation restricted KCl exposure and CSD induction to the burr hole (all CSDs were seen emanating from the burr hole) (Figure 1(c)). The highest dose chosen for SPD was 80 mg/kg, which was lower than the compound's median toxic dose (TD₅₀), previously evaluated in mice (88 mg/kg) (16). The experimental paradigm is depicted in Figure 1(d).

CSD model

Mechanically ventilated animals + LFP recordings—Twenty-five randomly allocated C57Bl/6 male mice were used (14 drug treated and 11 control treated). These numbers allowed us to detect a difference of 30% with a power of 90% to reject the null hypothesis. The CSD protocol was identical to that for the non-ventilated animals with the following exceptions: All animals were mechanically ventilated (TOPO, Kent Scientific) following a tracheotomy procedure. Breath rate was set to 100 breaths/min in all animals tested (control and drug-treated groups). We added LFP recording in these animals to monitor anesthesia levels and correlate electrophysiologically with optically measured CSD. A second burr hole was created 0.2 mm closer to bregma, rostral to the first burr hole for LFP recordings. A glass microelectrode (3 M Ω , saline-filled solution) was advanced to the LFP burr hole. The ground electrode was placed in the cervical muscles. LFPs were recorded using an Axopatch 1D amplifier (Molecular Devices) (0–500 Hz band pass; digitized at 1 kHz) synchronized with imaging data by a LabView Virtual Instrument (National Instruments).

OIS analysis

All analyses were performed blinded as to the treatment group. Ratio images (% $100 \times (R - R_0)/R_0$) were generated from each recording, with an average of the first eight frames serving as R_0 . In each experiment, two circular regions of interest (ROIs, 8×8 pixels) were placed 1 and 2 mm medial to the KCl burr hole, perpendicular to the advancing CSD wavefront, in areas devoid of surface vessels. A plot representing the change in cortical reflectance over time was generated for each ROI.

CSD evaluation

CSDs were identified by multiphasic, concentric changes in OIS. In experiments with LFP recordings, we confirmed that all propagating OIS changes were coincident with a characteristic DC shift in LFP. “*Full*” CSDs were defined as CSDs that propagated concentrically across the whole imaging field, with DC shift amplitude of 5–15 mV and DC shift duration of 125–210 s. “*Total*” CSD count includes full CSDs as well as “*Partial*” CSDs, which did not propagate across the whole imaging field, and had a DC shift amplitude of <5 mV and duration of 80–140 s. Velocity of CSD propagation was calculated only for the full CSDs, using the difference in time between peak OIS reflectance at ROIs 1 and 2. The OIS response was divided into three components as shown in Figure 1(b), and measured before and after administration of drug or vehicle to examine possible drug effects on perfusion.

NTG-induced hind paw mechanical allodynia

Mechanical thresholds were determined with von Frey monofilaments (VFF; eight filaments, range 0.07–2 g, Stoelting Co) using a modified up-and-down method (21). For drug testing, 36 C57Bl/6 male mice (20–30 g) were divided randomly into the following groups: *NTG/control* ($n=18$), *NTG/SPD 60 mg/kg* ($n=10$), *NTG/SPD 80 mg/kg* ($n=8$). On each testing day, a maximum of 10 animals was used, divided equally into drug and control groups. To ascertain that NTG itself was responsible for reducing mechanical threshold (rather than injection, for example), a separate group of mice was randomly divided into NTG and vehicle groups ($n=15$ each) and compared. Mice were confined in clear acrylic cages (8.7 in $\times$ 8.7 in $\times$ 5 in) divided into four chambers, each on a raised wire mesh platform that allowed full access to the tested paws. Mice were acclimated for 2 h, on the day of testing and one day prior. Mechanical thresholds were evaluated before (*baseline*), and 75 and 120 min after i.p. administration of 10 mg/kg NTG (or vehicle), in accordance with NTG’s time to peak effect (TPE) in this model (data not shown). Control, 60, or 80 mg/kg SPD was administered contralaterally to NTG administration 15 minutes before the first post-NTG time point. Filaments were applied in both an ascending (beginning with 0.07 g VFF) and descending (beginning with 2 g VFF) staircase protocol. Each filament was applied perpendicularly to the center of each of the hind paw six times, spaced 1 s apart. In the absence of a response, the next VFF in the series was applied until a response was witnessed. Response to VFF was recorded as an immediate withdrawal of the tested hind paw to the applied stimulus, with or without an observed licking behavior. The withdrawal threshold in each hind paw was quantified as the mean of ascending and descending threshold values. The animal’s mechanical allodynia threshold was the average of both left and right hind paws. The protocol paradigm is depicted in Figure 4(a). The experimenter was blinded to the treatment group.

NTG-induced periorbital allodynia

We attempted to evaluate mechanical allodynia in periorbital regions according to a previously published method (22). To test SPD’s effect on cephalic allodynia, we first needed to establish NTG-induced periorbital allodynia in mice. Forty male C57Bl/6 mice were randomly divided into two groups: control and NTG treated. A week before testing, all

mice were anesthetized with isoflurane (5% induction, 1.7% maintenance), the periorbital area was shaved, and hair was removed using a hair removal cream (Nair, Church & Dwight, Princeton, NJ). The exposed hairless skin was thoroughly washed with saline followed by application of betadine and a triple antibiotic ointment. Animals were handled daily (23), allowing them to walk freely on the handler's gloved hand for six days prior to testing. Two days before testing, animals were allowed to enter the rectangular Plexiglas testing tube (10 cm length $\times$ 3.5 cm inner diameter) uncoaxed and explore it for 1 min. On the day of testing, mice entered freely and acclimated for 5 min prior to testing. Periorbital mechanical allodynia was evaluated before and 120 min after i.p. administration of NTG or control vehicle. We chose 120 min because we observed optimal NTG-induced allodynia at this time point in hind paw allodynia testing; 120 min is also NTG's TPE in rats (24). For mechanical allodynia evaluation, eight VFF (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, and 1 g) were used in an ascending staircase protocol (descending staircase was not possible because of exuberant response to larger VFF). Each filament was applied five times spaced 5 s apart to either the left or right periorbital area until a response was witnessed. A response was defined as a strong head withdrawal followed by repetitive grooming of the stimulated area in three of five stimuli. In case of no response, the next VFF was applied. A pain threshold of 1 g was recorded when the animal failed to respond to the highest VFF. The mechanical allodynia threshold of each periorbital side was defined as the mean of two independent evaluations spaced 5 min apart. The mean of left and right side values was the animal's periorbital allodynia threshold. The experimenter was blinded to the treatment group.

Primary neuronal culture

Primary cultures of dissociated cortical neurons were prepared from embryonic (E-16) CF-1 mice and from NIE-115 neuroblastoma cell lines (25). Gestational day 16 CF-1 mice were anesthetized, and embryonic mice were removed. Cortical hemispheres were dissected and enzymatically dissociated using 0.25% trypsin in Dulbecco's modified Eagle's medium (DMEM). L-glutamine, 2.0 ml of horse serum, and 100 U/ml penicillin-streptomycin were added to the mixture. After trituration, cells were filtered and plated on poly-L-lysine-coated coverslips placed in 35-mm dishes. Cultures were maintained in a humidified incubator at 37°C and 7% CO₂ for 14–21 days before experiments, with medium replaced every three days (25,26).

Effect of SPD on receptor-gated ion channels

Whole-cell voltage clamp recordings were obtained from mouse cortical neurons maintained for three weeks in primary culture (25). Patch pipettes (2–4 M Ω resistance) were pulled (Sutter Instruments) and were filled with internal recording solution (153 mM CsCl₂, 10 mM ethylene glycol tetra acetic acid (EGTA)-CsOH, 10 mM HEPES, 4 mM MgCl₂, 290–310 mOsm, pH=7.3–7.35). The glass coverslips were placed on the recording chamber perfused with Mg²⁺ free external solution (142 mM NaCl, 1.5 mM KCl, 1 mM CaCl₂, 20 mM sucrose, 10 mM glucose, 10 mM HEPES). Voltage-gated sodium channels were blocked with 500 nM tetrodotoxin. A three-barrel borosilicate glass pipette was positioned 200–400 μ m from the cells, and agonist, agonist+SPD (100, 300, and 500 μ M) and wash solution were perfused interchangeably. The concentrations of SPD were chosen based on a previous pharmacokinetic study, and were all at the therapeutic range (16). Perfusion with 5

μM GABA, 10 μM kainate, and 10 μM N-methyl-D-aspartate (NMDA) + 1.0 μM glycine-induced GABA_A, kainate, or NMDA currents, respectively. Agonists and drug were applied for 1–3 s with a 5 s recording followed by a 15–20 s washout period between applications. The evoked current values were averaged for each condition and measured by using Clampfit software v.10 (Axon instruments).

Effect of SPD on voltage-gated sodium channels

The effect of SPD on voltage-gated sodium channels was evaluated using NIE-115 neuroblastoma cells as previously described (27). The internal solution of the micropipette was as above. External solution contained 130 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 5 mM HEPES. Tetraethylammonium chloride (25 mM) and cadmium chloride (0.1 mM) were added to the external solution to block voltage-gated K⁺ and Ca²⁺ channels, respectively. Voltage-gated sodium channels were activated by hyperpolarizing the cell to –90 mV for 90 ms and depolarizing to 0 mV for five trials in control solution followed by the same procedure using a solution containing SPD (100, 300, and 500 μM). A similar procedure was repeated starting from a holding potential of –60 mV.

Data analysis

All analysis was performed in a blinded manner, using GraphPad Prism, v5.03. A $p < 0.05$ was considered significant. All p values are reported as exact values with the exception of the multiple comparison tests. The CSD and electrophysiology results were not normally distributed; they are plotted as box-whisker plots and results are represented as *median (25th percentile, 75th percentile)*; comparisons were made using Mann-Whitney test. Other measures were normally distributed (D'Agostino Pearson test); results are displayed as mean $\pm$ s.e.m, and were compared using either Student's t test or analysis of variance (ANOVA) followed by Tukey test.

Results

SPD reduces CSD number

To evaluate whether SPD affects aura mechanisms, we measured CSD before (*KCl baseline*, 15 min) and after (*post-treatment*, 60 min) administration of SPD and control. Number and velocity of CSDs were similar during *KCl baseline* (Supplementary Figure 1); however, CSD number *post-treatment* was significantly reduced for both *total* (7.5 (6.0, 9.0) vs. 10.0 (9.3, 11.5)) and *full* CSDs (6.0 (5.8, 7.3) vs. 9.5 (8.3, 10)) in SPD-treated animals compared to controls ($p = 0.023$ and $p = 0.017$ for total and full CSDs; Mann-Whitney test, Figure 2). There was no significant difference in CSD velocity between groups (3.1 (2.4, 4.3) vs. 2.4 (1.9, 3.2) mm/min; *full* CSDs). However, under these *non-ventilated* conditions, SPD treatment was associated with a significant reduction in respiratory rate (46 ± 3.8 vs. 86 ± 5.2 per min, $p < 0.001$; in SPD and control groups respectively; Supplementary Table 1).

To test the possibility that reduced respiratory rate influenced CSD susceptibility, we used mechanically ventilated mice. SPD-treated animals continued to show a significant reduction in CSD number vs. controls: 4.0 (2.0, 6.3) vs. 7.0 (5.0, 9.0); $p < 0.001$ (Mann-Whitney test) for *total* CSDs and 2.5 (2.0, 4.0) vs. 6.0 (5.0, 7.0); $p < 0.001$ for *full* CSDs in the SPD and

control groups, respectively (Figure 3). There was no significant difference in CSD velocity between SPD and control groups (3.7 (2.9, 5.5) and 2.1 (1.6, 4.0) mm/min; *full* CSDs). Field potential recordings were used to further monitor anesthesia levels (28) as well as measure CSD effects; there was no significant difference in the inter-burst interval of burst-suppression (range 3–5 s in both groups), nor was there any difference between SPD-treated animals and controls in the DC shift amplitude (data not shown). All systemic parameters were similar both in SPD and control animals for the ventilated experiments (Supplementary Table 1). However, comparing non-ventilated to ventilated experiments, CSD frequency in control groups was significantly different (10.0 (9.3, 11.5) non-ventilated vs. 7.0 (5.0, 9.0) ventilated; $p < 0.05$ for *total* CSDs and 9.5 (8.3, 10) vs. 6.0 (5.0, 7.0); $p < 0.01$ for full CSDs), suggesting physiological differences at the tissue level between the two preparations that were undetected systemically (see Discussion). Nevertheless, both preparations showed a significant effect of SPD on CSD number.

CSD hemodynamic response

No difference in duration or amplitude was noted in the three phases of parenchymal OIS after administration of either vehicle or SPD (Figure 1(b); Supplementary Figure 2), suggesting that SPD does not affect CSD-associated blood volume.

NTG-induced mechanical allodynia model

To determine how SPD affects migraine-associated pain mechanisms, we tested its effect on NTG-induced mechanical allodynia. Baseline mechanical thresholds were similar in all groups (1.4±0.08, 1.2±0.13 and 1.4±0.16 g for *NTG/control*, *NTG/SPD 60 mg/kg* and *NTG/SPD 80 mg/kg*, respectively; $p > 0.05$ ANOVA with post-hoc Tukey test). After NTG injection, thresholds were significantly reduced in the *NTG/control* group (0.6±0.09 and 0.4±0.07 g at the 75 and 120 min time points, respectively; $p < 0.001$ compared to baseline; Figure 4(b)). In contrast, neither the *NTG/SPD 60 mg/kg* nor *NTG/SPD 80 mg/kg* groups showed a significant deviation from baseline, and both were significantly different from *NTG/control* at 75 and 120 min after NTG injection, consistent with reduction of mechanical allodynia (*NTG/SPD 60 mg/kg*: 1.4±0.13 g ($p < 0.001$) at 75 min, 0.9±0.12 ($p < 0.05$) at 120 min; *NTG/SPD 80 mg/kg*: 1.8±0.1 ($p < 0.001$) at 75 min, 1.4±0.12 g ($p < 0.001$) at 120 min; all comparisons to *NTG/control*).

To control for injection effects on pain threshold, NTG was compared to vehicle: NTG but not vehicle significantly reduced mechanical threshold at both time points (0.7±0.1 vs. 1.1±0.1 g ($p < 0.05$); 0.6±0.1 vs. 1.4±0.13 g ($p < 0.001$) for NTG and vehicle at the 75 and 120 min time points, respectively; Figure 4(b) inset). *NTG/SPD 80 mg/kg* was slightly sedating with reduced mobility and a wobbling stance at the 75 min time point, but not the 120 min time point; no sedation was observed in the *NTG/SPD 60 mg/kg* group.

Periorbital allodynia evaluation

We attempted to evaluate periorbital allodynia thresholds in mice before and after administration of NTG vs. vehicle (Figure 5). Baseline thresholds were statistically similar both in NTG and vehicle groups ($p > 0.05$, Student's *t* test) and were all higher than 0.1 g as was shown in previous reports in mice (22). However, NTG-injected mice did not show a

significant difference in periorbital thresholds compared to either vehicle control or to baseline thresholds ($n=20$ NTG-injected, 20 vehicle animals, $p>0.05$, Figure 5). As we were unable to establish NTG-induced periorbital allodynia in mice, we did not test SPD in this model.

Whole-cell recordings

SPD significantly increased GABA_A current amplitudes at concentrations of 300 and 500 μM to 127.8% (117.1, 143.4) and 121.8% (113.7, 122.9) of controls, respectively ($p=0.015$, $p=0.003$ for 300 and 500 μM , respectively; Student's t test; Figure 6). SPD also significantly reduced NMDA current amplitude to 90.5% (85.4, 100.6) and 90.6% (76.3, 96.1) of controls ($p=0.03$ and $p=0.025$ for the 300 and 500 μM , respectively) at 300 and 500 μM , respectively. Kainate-induced current amplitude was not affected by SPD. The lowest concentration of SPD, 100 μM , did not show any significant change in current amplitude compared to controls in any of the tested mechanisms. When evaluated on neuroblastoma cell lines, none of the SPD concentrations tested showed a change in the amplitude of voltage-dependent sodium currents (Figure 6).

Discussion

SPD is a novel amide derivative of VPA that has shown promise in both seizure and neuropathic pain models (16,17). Both epilepsy and migraine are paroxysmal disorders associated with altered network excitability (29), and migraine is clearly a pain disorder. In this work, we evaluated SPD's antimigraine potential and mechanism of action.

Migraine preventives, including SPD's parent compound VPA, decrease CSD number when dosed chronically in the rat (8). We showed that SPD was able to *acutely* decrease the number of CSDs in two separate protocols in mice. Though we cannot rule out species-based differences, the fact that SPD suppressed CSD acutely with a single dose (in contrast to VPA, which did not suppress CSD acutely and required multiple doses (8)) suggests that SPD might be a more potent migraine preventive.

In non-ventilated animals under isoflurane anesthesia, SPD treatment was associated with reduced spontaneous respiratory rate compared to vehicle-injected animals. Decreased respiratory rate could affect CSD threshold by changing pH and oxygenation, and thus might confound a genuine drug effect. The decrease in pH with hypoventilation would be expected to suppress CSD susceptibility (30); hypoxia could increase susceptibility (31,32). We therefore tested the effects of SPD in mechanically ventilated animals and found a similar significant reduction in number of CSDs elicited. This suggests that the alterations in CSD susceptibility we observed were indeed due to drug effects rather than systemic variables. We suspect that the respiratory suppression we observed was a result of synergistic effects (possibly GABAergic; see Figure 6) (33,34) with isoflurane anesthesia, as we never observed respiratory suppression in awake animals at 80 mg/kg SPD ($n=28$ animals, data not shown). However, as with many centrally acting GABAergic compounds (e.g. barbiturates and benzodiazepines), respiratory suppression may need to be considered at higher doses of SPD.

It should also be noted that CSD frequency both for vehicle and SPD treatments was lower in ventilated compared to non-ventilated animals. We cannot be sure of the exact mechanism as we did not sample arterial blood gases. However, we suspect that despite identical systemic variables, in the non-ventilated animals the metabolic challenge of CSD generated hypoxia at the tissue level (28), thus increasing CSD susceptibility and number (35). The important point, however, is that both protocols showed a significant reduction in CSD with SPD, and the ventilated recordings show that this difference was not due to the decreased respiratory rate observed in the non-ventilated recordings.

Changes in OIS during CSD are principally due to changes in blood volume (36,37). No significant changes in CSD-associated OIS were seen before or after administration of either control vehicle or SPD, suggesting that SPD's effects on CSD were due to neural excitability differences rather than differences in CSD perfusion correlates.

NTG causes a delayed migraine indistinguishable from spontaneous migraines (9,38) and like CSD is widely used as a preclinical model of migraine (in the case of NTG, for migraine *without* aura) (12,38–40). We used this model to evaluate NTG-induced extracephalic cutaneous allodynia by measuring hind paw withdrawal threshold. Cutaneous allodynia, both cephalic and extracephalic, is observed in 50%–70% of migraineurs (41–43) and is thought to result from sensitization of higher-order trigeminovascular neurons receiving input from all segments of the spinal cord (44,45). SPD robustly reduced NTG-induced hind-paw cutaneous allodynia in a dose-dependent manner.

We attempted to further evaluate SPD in an NTG-induced cephalic allodynia model in mice, adapting an NTG model in rats (24), as well as a non-NTG cephalic allodynia model in mice (22,46). However, in our hands, we were unable to establish NTG-induced periorbital allodynia in mice. A major factor was significant head movement in mice, both at baseline and in response to VFF. Our observations are consistent with a recent report indicating that evaluation of cephalic mechanical allodynia using VFFs was unreliable in mice because of “excessive head agitation” (47). To our knowledge only two reports successfully evaluated periorbital allodynia in mice—both were performed after traumatic brain injury, and neither used NTG as a stimulus (22,46).

A lack of NTG periorbital allodynia could be inferred to mean that NTG does not generate craniofacial nociception in mice. We do not believe this is the case, as we and others have demonstrated c-Fos expression in trigeminal nucleus caudalis in mice after NTG infusion under identical conditions (12,48). Moreover in rat, similar trigeminal nucleus caudalis c-Fos expression correlates with periorbital allodynia (14,24,49). We suspect the negative findings are due to the insensitivity of the test rather than a lack of a physiological response. Finally, it should be emphasized that extracephalic allodynia, though less common than cephalic allodynia, incontrovertibly occurs in humans with migraine (44,45). Thus we would argue that SPD's reduction of extracephalic allodynia in the mouse, in response to a compound (NTG) known to induce migraine in humans as well as induce mouse trigeminal nucleus caudalis c-Fos expression, is translationally relevant.

Article highlights

To our knowledge this is the first report of SPD's antimigraine potential and mechanism of action. We demonstrate SPD's activity in two translationally relevant migraine models, suggesting SPD has potential as a future antimigraine drug. SPD has been shown to be more potent than VPA also in seizure and pain models (16,17), and our data in CSD models suggest increased potency in this model as well. This is a promising feature as it should allow for a reduced total dose, potentially reducing "off-target" side effects. Another interesting aspect of SPD is that it has a very short half-life: 15–20 min in the mouse (18). This opens up the possibility that SPD could be used in acute settings (e.g. status migrainosus) as well as for preventive purposes. Finally, in stark contrast to VPA, SPD was non-teratogenic in a mouse strain susceptible to VPA-induced teratogenicity, at doses 4–13 times higher than their anticonvulsant-median effective dose (ED₅₀) values (15). Taken together these features of SPD suggest an equally effective but more clinically tolerable drug than its parent compound.

Biological mechanism

We found that SPD's mechanism of action is primarily GABAergic. SPD significantly increased GABA_A-mediated currents, and decreased NMDA mediated currents (though the small size of NMDA changes may not be as physiologically significant). SPD had no effects on kainate-mediated or Na⁺ currents. SPD's ability to reduce neuronal excitability might explain its activity in the CSD model. It also could explain a reduction in the trigeminovascular nociception modeled by NTG testing (50–52).

Strengths and limitations

A strength of this work is identifying the antimigraine potential of a novel second-generation VPA derivative using two unrelated and translationally relevant *in vivo* models. We chose the models for their high face and predictive validity, and their previously reported use for drug screening purposes (8,12,13).

A limitation is that we were not able to evaluate SPD's activity in models of trigeminovascular activation: Our attempts to measure NTG-induced cephalic allodynia in mice were unsuccessful. We hope that reporting our data in full will prompt refinements to mouse trigeminal behavior models that increase their sensitivity. As an alternative, cephalic allodynia can be tested in the rat, where the model is more robust (24). Another alternative is to examine SPD's effects on c-Fos expression after NTG treatment or another trigeminovascular stimulus. Finally, the "gold standard" for evaluating trigeminovascular effects of SPD would be simultaneous treatment and recording from trigeminal ganglion or nucleus caudalis.

A second strength is that we were able to determine SPD's likely mechanism of action. However, this work was performed in cortical neuronal cultures—receptor and ion channel repertoire is expected to be generically relevant to central neurons, but would ideally be examined separately for the trigeminovascular system (e.g. with trigeminal ganglion neurons) and, more important, confirmed *in vivo*. Moreover, it is unclear whether SPD's

GABAergic mechanism of action is pre-synaptic (affecting GABA reuptake or metabolism) or post-synaptic (affecting single-channel conductance or, less likely, number of receptors).

Finally, while SPD's efficacy in acute preparations is encouraging, future work should evaluate SPD in a chronic preparation to confirm its utility for long-term migraine prophylaxis.

Conclusion

The novel VPA amide derivative, SPD, reduced CSD number and NTG-induced mechanical allodynia, likely via modulation of GABAergic transmission. This effect in two very different migraine models—one of which models migraine aura, the other pain pathways in migraine without aura—is promising. Our work supports further development of SPD as a potential anti-migraine drug.

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DK and KCB designed the experiments; DK and GHS performed the experiments; DK and KCB performed analysis and interpretation of the experiments; DK, EAB, HSW, MB, BY, KW and KCB wrote the manuscript.

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Article highlights

- New migraine treatments are scarce despite an overwhelming need.
- *sec*-Butylpropylacetamide (SPD) is effective both in seizure and pain models, suggesting it as a candidate for migraine.
- SPD-treated mice showed a significant reduction in number of elicited cortical spreading depressions (CSDs).
- SPD-treated mice showed a significant reduction in mechanical allodynia induced by nitroglycerin (NTG).
- SPD significantly enhanced gamma-aminobutyric acid (GABA_A) currents, and to a lesser extent suppressed N-methyl-D-aspartate (NMDA) currents, in whole-cell recordings.
- Convergent findings in two very different models suggest SPD is a promising migraine treatment candidate, with a likely GABAergic mechanism of action.

Figure 1.

Optical and electrical detection of CSD. (a) Schematic of CSD experiments, including thin skull preparation between bregma and lambda and locations of KCl syringe and LFP electrodes. (b) Representative traces of CSD recorded as OIS and as LFP. OIS duration and amplitudes were calculated at each of three periods: hypoperfusion (1–2), hypo- to hyper-perfusion (2–3) and return to baseline (3–4). (c) Optical imaging of the thin skull preparation. CSD wave front is shown by contours, labeled by time from initiation of CSD. Asterisks mark the location for bregma and lambda. (d) CSD experimental paradigm. CSD: cortical spreading depression; KCl: potassium chloride; LFP: local field potential; OIS: optical intrinsic signal.

Figure 2.

CSD number in non-ventilated animals. Plots show total and full CSDs (left graph; see text for details) and the velocity of full CSDs (right graph) during post-treatment (60 min) for control (dark gray) and 80 mg/kg SPD (light gray). Both CSD measures were significantly reduced with SPD treatment. No change in velocity was observed. (* $p < 0.05$, ** $p < 0.01$, Mann-Whitney test, $n = 5, 6$ for the control and drug groups, respectively). CSD: cortical spreading depression; SPD: sec-butylpropylacetamide.

Figure 3.

CSD number in mechanically ventilated animals. (a) LFP recordings of control mice (right traces) and mice treated with 80 mg/kg SPD (left traces). Upper traces prior to CSD induction (during pre-CSD baseline, 15 min) show burst-suppression pattern (no significant difference in burst frequency between control and SPD). Lower traces show CSDs during the post-treatment phase (60 min). (b) Quantification of number and velocity of CSDs following treatment with vehicle (dark gray) and 80 mg/kg SPD (light gray). Both numbers of total and full CSDs were reduced following treatment with 80 mg/kg SPD. No change in velocity of CSD was observed between the two groups. (* $p < 0.05$ *** $p < 0.001$, Mann-Whitney test, $n = 11, 14$ for the control and drug groups, respectively). CSD: cortical spreading depression; SPD: sec-butylpropylacetamide; LFP: local field potential.

Figure 4.

NTG-induced mechanical allodynia. (a) Experimental paradigm. Mechanical allodynia was evaluated before (baseline), 75, and 120 min after i.p. injection of vehicle or 10 mg/kg NTG. Contralateral i.p. injection of control or SPD was performed 15 min before the first evaluation of mechanical allodynia. (b) Summary of the effect of 60 (dark gray ovals) and 80 mg/kg SPD (black ovals) vs. control vehicle (light gray rectangles) on NTG-induced mechanical allodynia (mean $\pm$ s.e.m). A significant reduction in mechanical allodynia threshold compared to baseline was seen following injection of control but not 60 or 80 mg/kg SPD. SPD had significantly higher threshold vs. control in both doses at the 75 and 120 min time points. (* p <0.05, *** p <0.001, Tukey multiple comparison test, n =18, 10, 8 for Vehicle/Control, NTG/Control, NTG/SPD 60 mg/kg and NTG/SPD 80 mg/kg groups, respectively). (Inset) Effect of NTG vs. vehicle on mechanical allodynia. NTG but not vehicle significantly decreased mechanical allodynia threshold 75 and 120 min after administration (* p <0.05, *** p <0.001, Tukey multiple comparison test, n =15 per group). NTG: nitroglycerin; SPD: sec-butylpropylacetamide; i.p.: intraperitoneal.

Figure 5.

Periorbital allodynia threshold in mice. Box-whisker plots represent median periorbital allodynia threshold for both left and right sides evaluated in all animals per group. Periorbital allodynia threshold was evaluated before (baseline) and 120 min after administration of vehicle or NTG in accordance with NTG's TPE. No difference was found in periorbital allodynia threshold between groups before or after administration of either vehicle or NTG ($p > 0.05$, Tukey multiple comparison test, $n = 20$ per group). NTG: nitroglycerin; TPE: time to peak effect.

Figure 6.

SPD's effects on whole-cell recordings of ion-gated channels. (a) Representative traces indicate the effect of 300 μM SPD (gray trace) vs. control (black trace) on GABA, kainate, and NMDA-induced currents in cortical neuronal cultures. Box-whisker plots represent the concentration response effect of SPD on all three ligand-gated channels. SPD at 300 and 500 μM was able to significantly reduce the amplitude of NMDA currents and increase the amplitude of GABA_A currents, while having no effect on kainate-induced currents. (* $p < 0.05$, paired Student's t test, 100 μM : $n = 5, 6, 5$; 300 μM : $n = 12, 8, 5$ and 500 μM : $n = 11, 6, 7$ for kainate, GABA and NMDA treatments, respectively). (b) The effect of SPD on whole-cell recordings of voltage-dependent sodium currents recorded from neuroblastoma cells.

Representative traces indicate voltage-dependent sodium currents induced by depolarizing step pulses to 0 mV from a holding potential of either -60 mV (upper trace) or -90 mV (lower traces). Currents were recorded following perfusion with either control (black traces) or SPD (gray traces)-containing solutions. Box-whisker plots represent concentration response of SPD on percentage change (normalized to control) in current amplitude of sodium currents. SPD had no significant effect on voltage-dependent sodium currents evoked from holding potentials of either -60 and -90 mV (paired Student's *t* test, 100 μ M: *n*=6; 300 μ M: *n*=6; 500 μ M: *n*=5 per group for the -60 mV and -90 mV protocols, respectively). SPD: *sec*-butylpropylacetamide; GABA: gamma-aminobutyric acid; NMDA: N-methyl-D-aspartate.