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A novel substituted aminoquinoline selectively targets voltage-sensitive sodium channel isoforms and NMDA receptor subtypes and alleviates chronic inflammatory and neuropathic pain

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Conflict of Interest

The other authors declare no competing financial interests related to this work.

Abstract

Recent understanding of the systems that mediate complex disease states, has generated a search for molecules that simultaneously modulate more than one component of a pathologic pathway. Chronic pain syndromes are etiologically connected to functional changes (sensitization) in both peripheral sensory neurons and in the central nervous system (CNS). These functional changes involve modifications of a significant number of components of signal generating, signal transducing and signal propagating pathways. Our analysis of disease-related changes which take place in sensory neurons during sensitization led to the design of a molecule that would simultaneously inhibit peripheral NMDA receptors and voltage sensitive sodium channels. In the current report, we detail the selectivity of N,N-(diphenyl)-4-ureido-5,7-dichloro-2-carboxy-quinoline (DCUKA) for action at NMDA receptors composed of different subunit combinations and voltage sensitive sodium channels having different α subunits. We show that DCUKA is restricted to the periphery after oral administration, and that circulating blood levels are compatible with its necessary concentrations for effects at the peripheral cognate receptors/channels that were assayed in vitro. Our results demonstrate that DCUKA, at concentrations circulating in the blood after oral administration, can modulate systems which are upregulated during peripheral sensitization, and are important for generating and conducting pain information to the CNS. Furthermore, we demonstrate that DCUKA ameliorates the hyperalgesia of chronic pain without affecting normal pain responses in neuropathic and inflammation-induced chronic pain models.

Keywords

Chronic pain; NMDA; GluN2B; Na_v1.8; Na_v1.7; substituted aminoquinoline

1. Introduction

Chronic pain is estimated to affect 1/3 of the U.S. population, and can negatively impact quality of life (Reuben et al., 2015). Although normal pain in response to tissue damage is an important physiologic mechanism for keeping an organism out of harm's way, in some instances, the nervous system can undergo a metamorphosis that makes pain a constant component of life. The pharmacologic treatment of neuropathic, or other chronic pain states, has relied heavily on the use of opiates or their derivatives (Reuben et al., 2015), with the plethora of attendant adverse effects which the patient endures (Chou et al., 2015). More recently, pharmaceuticals, including gabapentin and pregabalin, which target the $\alpha_2\delta$ subunits of P-type and N-type voltage-sensitive calcium channels, and duloxetine, which targets norepinephrine and serotonin uptake, have reached prominence as treatments for chronic pain (Lunn et al., 2014; Moore et al., 2014; Schreiber et al., 2015). However, opiates remain a mainstay for treatment of chronic pain, and a significant effort continues to be made to generate new and better pain medications. Many of these efforts have focused on targeting of single molecular entities such as transient receptor potential vanilloid type 1 (TRPV1), neurokinin 1 (NK1) and cannabinoid type 2 (CB2) receptors (Hill, 2000; Lehto et al., 2008; Rahn and Hohmann, 2009). However, targeting a single receptor or channel or enzyme to control a complex physiologic system has been suggested to result in limited

efficacy (Pang et al., 2012). The systems that subserve chronic pain syndromes are well represented by network models, and it has been posited (Csermely et al., 2005) that a partial inhibition of more than one target within a network can be more efficient than the complete inhibition of a single target.

During the process of sensitization leading to chronic pain syndromes, hyper-responsivity in the peripheral sensory system is followed by sensitization of neurons in the central nervous system (Campbell and Meyer, 2006). There is increasing evidence for the role of peripheral mechanisms in chronic pain syndromes (e.g., Parada et al., 2003; Christoph et al., 2005; Villarreal et al., 2005; Staud, 2010; Ferrari et al., 2014; Ma and Quirion, 2014; Yang et al., 2014; Boada et al., 2015), and some of the most well-established peripheral changes are up-regulation of the NMDA subtype of glutamate receptors (Du et al., 2003; Jang et al., 2004; Childers and Baudy, 2007), and of voltage sensitive sodium channels (Wang et al., 2002; Black et al., 2004; Coggeshall et al., 2004; Lai et al., 2004; Dib-Hajj et al., 2007; Levinson et al., 2012). Such neuroadaptive events provide candidate targets for development of multi-target medications for treatment of chronic pain.

We have previously published on the synthesis of a compound, N,N-(diphenyl)-4-ureido-5,7-dichloro-2-carboxy-quinoline (DCUKA), which simultaneously inhibits NMDA receptor and voltage sensitive sodium channel function (Snell et al., 2000). We now present significantly extended in vitro data on the subtype-specific targets of DCUKA, and proof-of-concept data showing that DCUKA ameliorates chronic inflammatory and neuropathic pain in several animal models.

2. Materials and Methods

2.1 Functional analysis of DCUKA actions: sodium channels

Electrophysiological experiments on three subtypes of sodium channels, i.e., Na_v1.5, Na_v1.7, and Na_v1.8, and on NMDA receptors composed of GluN1-GluN2A, GluN1-GluN2B, GluN1-GluN2C and GluN1-GluN2D subunit combinations were performed. In all cases, a stock solution of DCUKA in dimethylsulfoxide (DMSO) was prepared, and diluted to the desired concentrations for experiments. The final concentration of DMSO was < 0.2%, which had no effect on any of the measured responses.

2.1.1 Na_v1.7 Channel Electrophysiology—The cDNA clone of the pNaEx8 plasmid encoding the Na_v1.7 α subunit (Klugbauer et al., 1995) (gift of Dr. F. Hofmann, Institut für Pharmakologie und Toxikologie, Technischen Universität Munich, Germany) was transiently expressed in Chinese hamster ovary (CHO) cells. CHO cells were maintained in Ham's F12 Medium (Sigma, St. Louis, MO) fortified with 10% fetal bovine serum (Gibco, Grand Island, NY), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco), in a humidified incubator at 37°C and 5% CO₂.

For transfection, CHO cells were plated at 2.5×10^5 cells/35 mm dish for 24 h and then pre-incubated for 30 min in 1 ml Opti-MEM medium (Gibco) containing 0.8 μ g of α subunit plasmid DNA, 0.2 μ g of green fluorescent protein (GFP) plasmid, and 6 μ l of Lipofectamine reagent (Gibco). After incubation for 4 h, the cells were washed 3 times with normal media

and grown for 24 h in standard culture conditions, then plated on glass coverslips for the next day's experiments. Only GFP-expressing cells were selected for electrophysiological recording.

Sodium currents were recorded using the whole-cell patch clamp recording configuration (Hamill et al., 1981) as previously described (Wang et al., 2002). Cultured cells on coverslips were transferred to a handmade recording chamber and continuously perfused at room temperature with extracellular solution (Supplemental File Section I). The recording chamber volume was approximately 0.4 ml and the perfusion flow rate was 0.6 ml/min. Patch pipettes were filled with a filtered internal solution (Supplemental File Section I). The pipettes had input resistance of 0.8–1.4 M Ω . An MP-285 micromanipulator (Sutter Instrument Co.) was used to place the electrode onto the cell.

Recordings were made at room temperature (22° C) with an EPC 9 patch clamp instrument (HEKA Elektronik GmbH, Germany), were filtered at 5 kHz and digitized at 10–25 kHz. Only cells with series resistances of less than 2.5 M Ω and whole cell maximal sodium currents of at least 1 nA were used for drug test experiments. Voltage protocols for determining the interaction of DCUKA with the resting and inactivated states of Na_v1.7 are described in Supplemental File Section I. Leakage currents were subtracted using a P/4 or P/2 protocol. For the determination of tonic inhibition by DCUKA, currents were recorded after 8 min of starting drug perfusion. Pulse software (HEKA Elektronik GmbH) was used to acquire data. The data were analyzed using a combination of PulseFit (HEKA Elektronik GmbH) and SigmaPlot 9.0 (Jandel Scientific, Corte Madera, CA) software.

2.1.2 Na_v1.8 Channel Electrophysiology—Dorsal root ganglion (DRG, L4-L6) neurons were prepared from adult male Sprague-Dawley rats (250–350 g). Animal experiments were approved by the Neurosolutions Institutional Animal Care and Use Committee, and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were killed using CO₂ anesthesia prior to cervical dislocation and the L4-L6 DRG were removed and stored in chilled phosphate buffered saline (PBS). The DRG were trimmed and then digested using 2 mg/ml trypsin (type XII-S) and 2 mg/ml collagenase (type XI), freshly made in PBS, for 45–60 min in a shaking water bath at 37° C. After extensive washing with PBS and then plating medium (50:50 mix of DMEM/Hams F12, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin), the DRG were transferred to a solution of 0.5 mg/ml trypsin inhibitor (type I-S) made up in the plating medium. Careful trituration of the DRG was carried out using sterile glass pipettes of decreasing diameter and the cells were plated on coated glass coverslips (BD Biosciences San Jose, CA, BD Biocoat, poly-D-lysine/laminin), contained in 35 mm tissue culture dishes so that the cells could be easily transferred to a recording chamber for electrophysiological recording. Cells were incubated under standard tissue culture conditions until use.

Whole-cell patch-clamp recordings were conducted as previously described (Rush et al., 1998) at room temperature (~21° C) using an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA) with 1.5–2.5 M Ω electrodes. Data were filtered at 5 kHz and digitized using a sample rate of 20–50 kHz. Extracellular and internal solutions are described in

Supplemental File Section I. The cells were continuously perfused and compounds or control solutions applied using a DAD-16VC fast perfusion system (ALA Scientific Instruments, Farmingdale, NY). Recordings were started 3–5 min after establishing whole-cell configuration. Tetrodotoxin-resistant (TTX-R) currents, that are likely to be carried by $\text{Na}_v1.8$, are regularly found in small cells (15–30 μm) isolated from rat DRG neurons (Kostyuk et al., 1981; Caffrey et al., 1992; Elliott and Elliott, 1993; Rush et al., 1998). Only cells that displayed a robust TTX-R sodium current (over 1 nA) that was stable over time were used.

To examine DCUKA effects on $\text{Na}_v1.8$ channels in the resting state, DRG cells were held at -80 mV (Supplemental File Section I). This holding potential (HP) has the effect of inactivating any TTX-R $\text{Na}_v1.9$ channels that may contaminate recordings, as $\text{Na}_v1.9$ currents have more hyperpolarized voltage-dependent characteristics than $\text{Na}_v1.8$ (Cummins et al., 1999). Voltage protocols for determining the interaction of DCUKA with the resting and inactivated states of pharmacologically and electrophysiologically-isolated TTX-R $\text{Na}_v1.8$ channel sodium currents are described in Supplemental File Section I.

2.1.3 $\text{Na}_v1.5$ Channel Electrophysiology—Adherent cells from a CHO- $\text{Na}_v1.5$ cell line were suspended using Accutase (Innovative Cell Technologies, San Diego, CA). Cells were kept in suspension in CHO-S-SFM II serum-free media (Invitrogen, UK) using the integrated cell stirrer of the QPatch 16 (Sophion Bioscience, Denmark). Cells were centrifuged and resuspended in extracellular recording solution on the QPatch platform and then applied to the QPlate 16 planar electrode plate.

Extracellular and internal solutions are described in Supplemental File Section I. 16 whole cell voltage clamp recordings were performed in parallel at room temperature on QPlate 16. Data were acquired using a Windows-based computer running QPatch Assay Software v3. Series resistance error was compensated by approximately 80–90%. Experimental criteria and voltage protocols are described in Supplemental File Section I.

Recorded currents were analyzed using QPatch Assay Software v3 (Sophion, Denmark). Baseline leak currents, determined at the holding potential of -120 mV, were subtracted from current measurements.

2.2 Functional Analysis of DCUKA Actions: NMDA Receptors

2.2.1 NMDA Receptor (GluN1-GluN2A and GluN1-GluN2B) Electrophysiology—HEK293 cells were obtained from the American Type Culture Collection (ATCC, Bethesda, MD). cDNAs encoding the NMDA receptor 1, 2A, and 2B (GluN1, 2A, 2B) subunits were obtained, packaged in vectors containing a CMV promoter, from Dr. Dolan Pritchett, Philadelphia, PA (Lynch et al., 1994). After transfection (Lovinger, 1995) the HEK293 cells were washed with Dulbecco's phosphate-buffered saline (Sigma Chemical Co, St. Louis, MO) and cultured in minimum essential medium and 10% fetal bovine serum containing the NMDA receptor antagonist, APV (1 mM) (to prevent excitotoxicity) for 48 hours before being used for electrophysiological studies.

Transfected cells were exposed to 3 mM EDTA in PBS for 30 s to 1 min and single cells were plated at limiting dilutions into 35 mm suspension dishes. Cells were allowed to settle for at least 5 min and electrophysiological recording was performed as previously described (Lovinger, 1995). See Supplemental File Section I for details.

Dose-response curves were constructed from I_{peaks} or $I_{\text{steady-state}}$ evoked by 3-s application of varying concentrations of DCUKA and fixed concentrations of NMDA (100 μM) and glycine (10 μM), or 30 μM DCUKA, 100 μM NMDA and varying concentrations of glycine. The relationship between current and agonist or antagonist concentrations was fitted to the logistic equation: $I = I_{\text{max}}/[1 + (\text{EC}_{50}/(\text{agonist})^n)]$. The parameters, I_{max} , EC_{50} , and n were estimated from a best fit generated by a computer-based least-squares fitting program (IGOR, Wavemetrics).

2.2.2 NMDA Receptor (GluN1-GluN2C, GluN1-GluN2D) Electrophysiology—The cDNAs for NMDA receptor subunits were kindly provided by: Dr. S. Heinemann, La Jolla, CA, (GluN1); Dr. R. Sprengel, Heidelberg, Germany (GluN2C); and Dr. P. Seeburg, Heidelberg, Germany (GluN2D). HEK 293 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% fetal calf II serum at 37°C in a 5% CO_2 environment. Twenty-four hours after plating of low-density cultures ($\sim 5 \times 10^4$ cells/ml) in 35 mm dishes, cells were transfected with equal amounts of cDNA (1 μg) using Lipofectamine 2000 reagent (Invitrogen) (Smothers et al., 2001; Smothers and Woodward, 2003). The NMDA receptor antagonists ketamine (500 μM) or APV (200 μM) were added following transfection to prevent excitotoxicity. Cells (after careful washing) were used for electrophysiological recordings 48–72 h following transfection.

Electrophysiological experiments were performed as previously described (Smothers et al., 2001). See Supplemental File Section I for details.

Dose-response curves were constructed from I_{peaks} or $I_{\text{steady-state}}$ evoked by 6-s application of varying concentrations of DCUKA and fixed concentrations of glutamate (10 μM) and glycine (10 μM). Data were fit to the Hill equation to obtain estimates of IC_{50} values.

2.3 Screening for binding to other receptors/transporters/enzymes

Binding studies (Besnard et al., 2012) were performed by PDSP (NIMH Psychoactive Drug Screening Program (<http://pdsp.med.unc.edu>)). The experimental details for all of the binding studies can be obtained by connecting to the PDSP website (<http://pdsp.med.unc.edu>) and clicking on “Assays” (binding or functional) on the menu bar. PDSP initially performed ligand displacement studies at a default concentration of 10 μM DCUKA. For any receptor/transporter at which the compound generated a 50% or greater displacement of the receptor/transporter selective ligand, a secondary binding assay was performed to calculate K_i values after obtaining the best fit IC_{50} values from the radioligand competition binding isotherms. The IC_{50} to K_i conversion was performed using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). If the compound displayed a K_i value $< 10 \mu\text{M}$, functional assays were performed to substantiate activity at the particular receptor. We also performed binding studies in our own laboratories focused on the NMDA receptor and

voltage sensitive sodium channels, and some of these results were previously published (Snell et al., 2000).

2.4 Measurements of DCUKA Activity in Animal Models of Chronic Pain Syndromes

2.4.1 Animals—Experiments conducted at the University of Colorado were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver, and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* as promulgated by the National Institutes of Health. For these experiments (i.e., the Complete Freund's Adjuvant (CFA) and streptozotocin (STZ) models), male Sprague Dawley rats (Taconic, Germantown, New York or Charles River, Raleigh, NC) (200–250 g) were housed in an AAALAC-accredited facility, on a 12-h light/dark cycle (with lights on at 7:00 AM). After 1 week acclimatization, animals were group housed (three to four rats per cage) with *ad libitum* access to food and water. The STZ model was used to test effects of DCUKA on diabetic neuropathy (Tesch and Allen, 2007). After STZ treatment, rats were housed two per cage and bedding was changed daily. Paper bedding was used in STZ experiments to provide better liquid absorbance and to avoid any pressure neuropathies induced by hard bedding. Rats were observed daily and handled/weighed every three days after STZ treatment. All behavioral testing and drug administration were performed during the light cycle (9:00 AM to 6:30 PM). For experiments using the formalin-induced pain model in mice (Tjolsen et al., 1992) and the partial spinal nerve ligation (SNL) model in rats (Kim and Chung, 1992), which were conducted at the National Institute of Neurological Disease and Stroke (NINDS) Anticonvulsant Screening Program, all rats (male Sprague Dawley) and mice (male CF1 strain) were housed, fed and handled in a manner consistent with the recommendations in the NIH *Guide for the Care and Use of Laboratory Animals*.

2.4.2 DCUKA Preparation—For all oral treatments, DCUKA was prepared in 50% gelatin / 50% canola oil emulsion. First, 0.8 g gelatin (Knox, Kraft Foods North America, Tarrytown, NY) and 0.06 g tartaric acid (McCormick & Company, Inc, Hunt Valley, MD) were added to 30 ml of purified water and allowed to stand for a few min before being heated and dissolved. The solution temperature was increased to 98°C and maintained for 20 min. The solution was cooled to 50°C in a water bath and 6.0 ml ethyl alcohol and sufficient water to make 50 ml was added. DCUKA was suspended in 5 ml of canola oil (Safeway INC, Pleasanton CA) by stirring and sonication (VWR Biosonik IV, 70%) for 5 min. The drug/oil suspension was added to 5 ml of the water/ethanol solution of gelatin with stirring and sonication. For dosing rats, the emulsion was diluted appropriately and warmed to 37°C in a water bath. Prior to dosing, the emulsion was vigorously mixed using a vortex mixer. Dosing volume was 5 ml/kg. The final amount of ethanol administered to the animals as part of the vehicle or drug-containing suspension never exceeded 0.24 gm/kg. The vehicle containing no DCUKA, but all other ingredients, was used as control for all behavioral experiments.

For i.p. administration in the SNL and the formalin-induced pain models, DCUKA was prepared fresh daily and dissolved in a vehicle solution consisting of 0.5% Methylcellulose/5% Tween-80 solution/0.9% NaCl (all from Sigma-Aldrich). Again, the vehicle not containing DCUKA was used as control.

2.4.3 In Vivo Pain Models

2.4.3.1 Complete Freund's Adjuvant (CFA) Model (Stein et al., 1988): Following a baseline measurement of the paw withdrawal threshold by the von Frey method (see below), rats were subcutaneously injected with 0.1 ml CFA (Sigma-Aldrich) into the plantar surface of the left hind paw under light isoflurane anesthesia (5% for induction and 2% for maintenance). Rats were left in their home cages for 48 h after CFA administration to allow for the inflammatory pain response to develop in the injected paw. After preliminary experiments demonstrated that the peak DCUKA effect occurred between 60 and 90 min after oral administration, the mechanical pain threshold (see below) was determined 60 min after administration of vehicle or various concentrations of DCUKA.

To evaluate the effect of repeated treatment with DCUKA, following baseline measurement of the paw withdrawal threshold, DCUKA (50 mg/kg) was administered twice daily, at 10-h intervals, for ten days. Rats were then treated with CFA, and the mechanical pain threshold (see below) was assessed two days later. The CFA-treated rats were divided into two groups. One group was tested to measure paw withdrawal threshold at 60 min after oral administration of vehicle (DCUKA/VEH group) and the second group was tested after receiving 50 mg/kg DCUKA (DCUKA/DCUKA group).

2.4.3.2 Streptozotocin (STZ) Model (Tesch and Allen, 2007): Baseline body weights were recorded and blood glucose concentrations were measured by a Glucometer (ASCENSIA CONTOUR Blood Glucose Monitoring System, Bayer, Pittsburgh, USA), and baseline paw withdrawal thresholds were measured by the von Frey method (see below). Rats were then fasted overnight and the next day injected i.p. with vehicle (20 mM sodium citrate buffer solution, pH 4.5, Sigma-Aldrich), or STZ (50 mg/kg, Sigma-Aldrich) dissolved in the same buffer solution. STZ and vehicle were prepared fresh each day and used within 10 min. Food was returned to the rats 30 min after STZ injection. To confirm that animals were hyperglycemic, three days following STZ treatment, blood samples (between 6 µl to 10 µl) were obtained by tail prick and glucose levels were assessed. Only rats with a blood glucose level above 350 mg/dl were included in the experiments. Any rat that lost more than 15% body weight was excluded. The effect of DCUKA was tested 14 days after the STZ treatment. The mechanical pain threshold was determined (see below) at 60 min after vehicle or DCUKA treatment. In these experiments, the effect of DCUKA on the mechanical pain threshold in animals treated with vehicle, instead of STZ ("sham" treatment), was also tested.

2.4.3.3 Partial Spinal Nerve Ligation (SNL) Model (Kim and Chung, 1992): Surgery on rats was performed according to the methods described by Seltzer et al., (1990). The procedure was routinely done on the right side (ipsilateral) while a sham surgery was performed on the left hind leg (contralateral). The rats were closely monitored daily for the development of infection or untoward effects of the surgery in which case the animals were immediately euthanized. After 1 week of post-surgical recovery time, animals were tested to measure the mechanical pain threshold (see below). The effect of DCUKA (75 mg/kg) was determined at 1, 2, 4, 6 and 24 hour after DCUKA administration.

2.4.3.4 Formalin Injection Model (Tjolsen et al., 1992): An injection of 0.5% formalin was made into the plantar region of the right hind paw of a mouse. This elicits a biphasic behavioral profile. Immediately following the injection, the mouse intensely licks the injected paw for approximately 5–10 min. This initial behavior is considered phase 1 (acute), and is followed by a brief latent (usually <5 min) period where there is little or no behavioral activity. A more prolonged (about 20 to 30 min) period of licking then ensues which constitutes phase 2 of the pain response (inflammatory). DCUKA (75 mg/kg) was administered 15 min prior to the formalin injection, and duration of licking for the first 2 min of 5-min time periods was measured thereafter. Areas under the time course curves were calculated for the vehicle and DCUKA groups.

2.4.4 In Vivo Test of Mechanical Pain—Mechanical pain was measured with an Electronic von Frey Anesthesiometer (IITC Life Science, Woodland Hills, CA). Several slightly different protocols have been described for the assessment of mechanical pain (Chaplan et al., 1994; Mogil et al., 2001; Du et al., 2003; Carlson et al., 2007). In each protocol, a paw withdrawal threshold (g) is determined by applying mechanical stimuli (von Frey filaments) of varying force to the hind paw of the rat, until withdrawal of the paw is observed. In the CFA and STZ models, the mechanical pain threshold (shown in Figs. 4 and 5) was defined as the ratio of the paw withdrawal threshold after CFA or STZ treatment to the withdrawal threshold of the same paw at baseline (prior to treatment with CFA or STZ). Thus, each animal was used as its own control. For the SNL model, the paw withdrawal threshold (in g) was used as the outcome measure. In all of these experiments, data points were considered outliers if they were outside two standard deviations of the treatment group mean, and outliers were excluded from analysis. The details of each protocol for measuring mechanical pain threshold are described in Supplemental File Section I.

2.5 Effect of DCUKA on Rat Rotarod Performance

To determine whether DCUKA caused ataxia or incoordination, rotarod performance was assessed after oral doses of up to 1000 mg/kg DCUKA. For training, rats were placed on a rotarod (Stoelting Co., Wood Dale, IL) operating at constant speed (4 rpm) until they remained on the rotarod for 300 s. A total of ten trials was allowed, with a rest period after the first five trials, and rats that did not meet the criterion were not used. For pre-drug, baseline, testing, rats were placed on the rotarod operating at 4 rpm, and the rotarod was accelerated from 4 rpm to 40 rpm over a period of 300 s. This test was repeated 5 times, with a 300-s cutoff. The average of the time that each animal remained on the rotarod, over the last three trials, was considered the baseline value for each animal. For the data shown in Supplemental File, Section IV, DCUKA (1000 mg/kg, p.o., given in three equal doses over one hour) or vehicle was then administered, and at 15, 30, 60 and 90 min after the last dose, rotarod performance was assessed with one 300-s trial. Rotarod performance was expressed as the percent of baseline performance for each rat.

2.6 Measurement of DCUKA Levels in Plasma and Brain after Oral Administration

Male Sprague-Dawley rats (200–250 g) were given oral doses of DCUKA as described for behavioral experiments (32.5, 50 or 100 mg/kg, n = 3 per group). At ninety min after drug administration, rats were anesthetized with isoflurane, and blood (250 µl) was collected by

cardiac puncture, using a heparin-coated syringe. Blood was centrifuged and plasma was aspirated. Plasma samples were frozen at -20°C until use.

DCUKA levels were also assessed in brain tissue. To investigate for possible changes in the blood/brain barrier in animals experiencing chronic pain, brains were taken from rats that had been treated with CFA to induce inflammatory pain, and the pharmacokinetic studies were carried out with DCUKA (32.5, 75 or 125 mg/kg, $n = 6/\text{group}$). At 90 min after DCUKA administration, rats were decapitated, brains were removed, rinsed in saline and frozen on dry ice. Brains were stored at -80°C until analysis.

DCUKA was analyzed by the UCCC Pharmacology Shared Resource at Colorado State University. Plasma (100 μl) or brain homogenate (100 mg/ml in water) was added to 10 μl of acetonitrile containing an internal standard (the methyl ester of DCUKA), an additional volume (300 μl) of acetonitrile plus 2% formic acid was added, the mixture was vortexed for 10 min and then centrifuged at 9,000 g for 10 min and the acetonitrile layer was transferred to HPLC vials and analyzed by mass spectrometry. Positive ion electrospray ionization (ESI) mass spectra were obtained with an MDS Sciex 3200 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems, Inc, Foster City, CA) with a turbo ionspray source interfaced with a Shimadzu HPLC system (Columbia, MD). Samples were chromatographed with a Waters Sunfire C8, 5 μm , 4.6 $\times$ 50 mm column (Waters Corporation, Milford, MA) protected by a C18 guard cartridge (Phenomenex, Torrance, CA). An LC gradient was employed with mobile phase A consisting of 0.1% formic acid and mobile phase B consisting of acetonitrile. Chromatographic resolution was achieved by maintaining mobile phase B for 2.5 min at 50%, increasing to 98% from 2.5–4 min, followed by re-equilibration of the column at 50% mobile phase B from 4–5 min. The LC flow rate was 1.35 ml/min, the sample injection volume was 50 μl , and the analysis run time was 5 min. The optimized mass spectrometer settings for DCUKA are as follows: MRM (m/z — m/z): 452.0 – 168.2; DP: 41; EP: 3.5; CEP: 22.8; CE: 45; CXP: 4. The settings for the internal standard are: MRM (m/z — m/z): 465.9–168.1; DP: 56; EP: 5; CEP 23.19; CE: 51; CXP: 2.5. The source conditions were optimized as follows: turbo ionspray temperature, 550°C ; ion spray voltage, 4500; curtain gas, N_2 (CUR), 35 units; collision gas, N_2 (CAD), 6; nebulizer gas, N_2 , 50 units and auxiliary gas, N_2 , 75 units. Samples were quantified by internal standard reference method in the MRM monitoring ion transitions as indicated. The dwell times for each ion transition were 100 ms. Q_1 and Q_2 were both operated in unit resolution mode. Quantification of DCUKA was based on a standard curve prepared in rat plasma or brain tissue spiked with internal standard, using the ratio of the DCUKA summed peak area to the internal standard peak area and $1/x^2$ weighting of linear regression.

2.7 Design and Analysis

Group sizes in the various experiments were based on preliminary studies and power analysis. Animals were assigned to groups matched by weight, the data acquisition was blinded but the data analysis was not blinded. The data were analyzed by one- or 2-way ANOVA or Student's t-test, as appropriate. Least square means comparisons were used for post-hoc analyses. The software that was used for statistical analyses takes the number of animals per group into account (least square means were used (Charnes et al., 1976) and

corrections were made for unequal variances if they occurred (Satterwaite, 1946; Levene, 1960)). Data are presented as mean $\pm$ S.E.M., and $P < 0.05$ was considered significant.

3. Results

3.1 DCUKA Effect on Na_v1.7 Channels

Following depolarization, roughly 30% of GFP-positive cells displayed fast, transient inward currents in which the maximal peak currents ranged from 0.8 to 12 nA. These currents could be blocked completely by 0.5 μ M tetrodotoxin, confirming their identity as tetrodotoxin-sensitive sodium channels. Tonic inhibition of Na_v1.7 currents by DCUKA was dose-dependent (Fig. 1A). The dose-response curves at different holding potentials fit well to the Hill equation, from which K_d values were calculated. The K_d value for Na_v1.7 at -100 mV was 34.6 μ M, and at -60 mV, the K_d was 20.7 μ M. At -60 mV, most channels are in the inactivated state, and further experiments were performed to estimate the DCUKA affinity of the resting and inactivated states of Na_v1.7. The effect of DCUKA on voltage-dependent availability of Na_v1.7 was determined by measuring the shift in inactivation curves of steady-state Na⁺ currents in the presence of various DCUKA concentrations. Inactivation was determined by holding the cell at -100 mV and applying voltage steps to 0 mV from varying prepulse potentials (-140 mV to -10 mV). Analysis of availability curve shifts (∞), as described by Kuo and Bean (1994), gave an equilibrium dissociation constant for the resting state of 27.9 μ M, and a dissociation constant for the inactivated state of 9.6 μ M, indicating that DCUKA is a more potent inhibitor of the inactivated state in comparison to the resting state for Na_v1.7 channels.

The effect of DCUKA on the current-voltage (I–V) relationship for Na_v 1.7 was determined by holding the cell at -100 mV, stepping to various depolarized potentials, and returning to the holding potential. Fig. 1B shows the I–V curve for a representative cell before and after exposure to 25 μ M DCUKA. Although DCUKA inhibited Na_v 1.7 currents, it did not change the I–V relationship.

The use-dependent inhibition by DCUKA of Na_v1.7 currents was measured at different stimulus frequencies. At a 2 Hz frequency, 25 μ M DCUKA did not induce a significant reduction of Na_v1.7 Na⁺ currents. At higher stimulus frequencies, 5 Hz and 10 Hz with 5-ms pulse durations, 25 μ M DCUKA produced use-dependent inhibition (Fig. 1C).

3.2 DCUKA Effect on Na_v1.8 Channels

The effect of two concentrations (1 and 10 μ M) of DCUKA on resting state currents is shown in Fig. 2A. 1 μ M DCUKA reduced the tetrodotoxin-resistant (TTX-R) currents to $80.8 \pm 6.9\%$ of control values ($n = 3$) and 10 μ M DCUKA caused a reduction to $34.0 \pm 3.8\%$ of control ($n = 3$). The effect of DCUKA on TTX-R currents when channels had been cycled through the inactivated state (see Supplemental File Section I), was examined in a separate set of experiments. Under these conditions, 1 μ M DCUKA reduced the TTX-R currents to $56.6 \pm 10.7\%$ of control values ($n = 3$) and 10 μ M DCUKA caused a reduction to $19.8 \pm 6.2\%$ of control ($n = 3$). Assuming that inhibition is linearly related to the $\log_{10}$

concentration of DCUKA, a regression analysis was used to estimate an IC_{50} value of 4.6 μM for the resting state, and 1.6 μM for the inactivated state of the channel.

The effect of DCUKA on the voltage dependence of activation of $Na_v 1.8$ was determined by holding cells at -80 mV and depolarizing to a range of potentials, returning to the holding potential between depolarizations. Fig. 2B shows the I–V curve before and after exposure to 1 μM DCUKA. There was no apparent shift in the voltage dependence of activation, although DCUKA reduced $Na_v 1.8$ current. This finding was confirmed by plotting the data as conductance curves fitted with the Boltzmann equation, which showed no significant effect of DCUKA on the midpoint of activation (control -19.5 ± 3.0 mV; 1 μM DCUKA, -22.2 ± 2.0 mV, mean $\pm$ S.E.M., $n=5$).

Further experiments examined the possibility of use-dependent block by comparing the rundown of peak activated currents during the train of stimuli with a holding potential of -80 mV. This was completed at frequencies of 1 Hz, 5 Hz and 10 Hz, using 20 ms pulse durations under control conditions or in the presence of 1 μM DCUKA. Fig. 2C shows that at a frequency of 10 Hz, there was very little change in the effect of DCUKA on TTX-R sodium channels, suggesting that $Na_v 1.8$ channels do not show a use-dependent inhibition by DCUKA.

3.3 DCUKA Effect on $Na_v 1.5$ Channels

DCUKA was tested at concentrations ranging from 0.1 μM - 30 μM . DCUKA showed low potency at the human $Na_v 1.5$ channel, with little or no inhibition of activity evident with the highest concentration tested (30 μM) for either the inactivated or resting state (data not shown). No use dependent increases in the potency of DCUKA were found.

3.4 DCUKA Effects on NMDA Receptors (GluN1-GluN2A, GluN1-GluN2B)

DCUKA concentration-dependent inhibition of whole-cell currents in HEK293 cells expressing GluN1-GluN2A or GluN1-GluN2B receptors was measured during superfusion with 100 μM NMDA and 10 μM glycine. Application of DCUKA (1–100 μM) along with application of NMDA and glycine produced a concentration-dependent inhibition of steady-state currents in cells expressing GluN1-GluN2A or GluN1-GluN2B receptors (Fig. 3A). Data were fit to the Hill equation (Hill, 1910), and the resulting estimates of the IC_{50} values for DCUKA inhibition of GluN1-GluN2A and GluN1-GluN2B recombinant receptors were 11 ± 1 and 20 ± 8 μM , respectively. However, GluN2A and GluN2B containing NMDA-receptors differ significantly in their K_d values for the co-agonist glycine. Since we have shown that DCUKA is a competitive antagonist at the strychnine-insensitive glycine binding site on the NMDA receptor (Snell et al., 2000), the K_d value for glycine needed to be considered for assessing potency of DCUKA at NMDA receptors containing different subunits. In cells superfused with 100 μM NMDA, coapplication of glycine produced a glycine concentration-dependent increase in NMDA receptor currents in both GluN1-GluN2A and GluN1-GluN2B expressing cells. The EC_{50} estimates for glycine enhancement of NMDA current were 3.9 ± 0.7 and 0.5 ± 0.1 μM for GluN1-GluN2A and GluN1-GluN2B receptors, respectively. The K_i for DCUKA inhibition of NMDA/glycine-induced currents was then estimated using a modified Cheng-Prusoff equation (Cheng and Prusoff, 1973),

$K_i(\text{DCUKA}) = \text{IC}_{50} / \{1 + ([\text{Gly}]/K_{\text{Gly}}^K)\}$ here IC_{50} is the DCUKA IC_{50} concentration value, $[\text{Gly}]$ is the concentration of glycine under which the DCUKA inhibition curves were performed (10 μM), K_{Gly} is the EC_{50} for glycine potentiation of NMDA induced current, K is the glycine slope function of the glycine concentration-response curve and $K_i(\text{DCUKA})$ is the K_i for DCUKA. The resulting K_i values for DCUKA inhibition of NMDA/glycine-induced currents in GluN1-GluN2A and GluN1-GluN2B receptors were 4.9 μM and 0.5 μM , respectively.

3.5 DCUKA Effects on NMDA Receptors (GluN1-GluN2C, GluN1-GluN2D)

DCUKA concentration-dependent inhibition of whole cell NMDA receptor currents was measured in HEK 293 cells expressing GluN1-GluN2C and GluN1-GluN2D subunits during superfusion with 10 μM glutamate and 10 μM glycine. Application of DCUKA (1–60 μM) before glutamate and glycine produced a concentration-dependent inhibition of steady-state currents in cells expressing GluN1-GluN2C and GluN1-GluN2D subunits (Fig. 3B). A higher DCUKA IC_{50} was observed for receptors containing GluN1-GluN2C (31.5 $\pm$ 6.4 μM) and GluN1-GluN2D (45.1 $\pm$ 3.3 μM), compared to receptors containing GluN1-GluN2A and particularly GluN1-GluN2B.

3.6 DCUKA Effect in CFA Model

The acute effect of DCUKA on CFA-induced pain is shown in Fig. 4. In this experiment, data from two outliers were excluded, one from the vehicle group and one from the group treated with 50 mg/kg DCUKA. The baseline paw withdrawal threshold prior to CFA treatment was 3.5 $\pm$ 0.3 g (n=18). Treatment with CFA reduced the paw withdrawal threshold of the treated paw to 1.2 $\pm$ 0.16 g (n=18), i.e., a significant 66% reduction (t-test). Administration of DCUKA increased the mechanical pain threshold (ratio of paw withdrawal threshold after treatment to baseline paw withdrawal threshold) in a dose-dependent manner, with statistically significant increases observed at 32.5 and 50 mg/kg DCUKA. The DCUKA ED_{50} value was calculated (Cedergreen et al., 2005) to be 27.9 $\pm$ 5.7 mg/kg. The baseline paw withdrawal threshold for the untreated paw was 2.9 $\pm$ 0.3 g. This threshold was not significantly changed by treatment with DCUKA (ratio of paw withdrawal threshold after treatment to baseline paw withdrawal threshold for untreated paw: vehicle, 1.1 $\pm$ 0.1; 17.5 mg/kg DCUKA, 1.1 $\pm$ 0.2; 32.5 mg/kg DCUKA, 1.2 $\pm$ 0.2; 50 mg/kg DCUKA, 1.1 $\pm$ 0.2).

In the experiment in which the effect of repeated exposure to DCUKA was evaluated, data from three outliers were excluded, two from the DCUKA/VEH group, and one from the DCUKA/DCUKA group. Without treatment with CFA, the baseline paw withdrawal threshold was 4.3 $\pm$ 0.6 g (n=10) for the DCUKA/VEH group, and 4.7 $\pm$ 0.4 g (n=11) for the DCUKA/DCUKA group (not significantly different). Treatment with CFA reduced the paw withdrawal threshold to 1.4 $\pm$ 0.4 g (n=10) for the DCUKA/VEH group (significant 53% reduction (t-test), similar to what was seen in native rats (Fig. 4)). Administration of DCUKA increased the mechanical pain threshold to the same degree in rats that had been chronically treated with DCUKA as in rats treated acutely with DCUKA (Fig. 4), i.e., chronic treatment with DCUKA did not alter the allodynic effects of CFA, nor the ability of

DCUKA to reverse the change in mechanical pain threshold produced by CFA (Supplemental File, Section II).

3.7 DCUKA Effect in STZ Model

The effect of DCUKA on STZ-induced pain is shown in Fig. 5. In these experiments, data from two outliers were excluded, one from the group treated with vehicle, and one from the group treated with 40 mg/kg DCUKA. In addition, 7 animals were excluded from the experiment due to weight loss greater than 15%. The baseline paw withdrawal threshold in this experiment was 2.8 ± 0.2 g (n=15). Treatment with STZ reduced the paw withdrawal threshold to 1.6 ± 0.1 g (n=15), a statistically significant 43% reduction (t-test). Administration of DCUKA increased the mechanical pain threshold in a dose-dependent manner, with significant increases seen at 32.5 and 40 mg/kg. The DCUKA ED₅₀ value was calculated (Cedergreen et al., 2005) to be 16.3 ± 6.3 mg/kg.

In two separate studies, DCUKA treatment (50 or 75 mg/kg) did not significantly affect the mechanical pain threshold (1-way ANOVA; see Supplemental File, Section III) in “sham” animals treated with vehicle instead of STZ.

3.8 DCUKA Effect in SNL Model

The outcome measure for this test is the paw withdrawal threshold (g), since baseline paw withdrawal thresholds for the particular animals used for these studies were not available from NINDS, and thus the ratio of post-treatment paw withdrawal threshold to baseline paw withdrawal threshold for each animal could not be calculated. Data from one outlier (0 hour time point) were excluded from the analysis. As shown in Fig. 6, the paw withdrawal threshold determined following spinal nerve ligation was 8.4 ± 1.2 g (n=7). Treatment with DCUKA (75 mg/kg, the lowest dose tested) resulted in a significantly increased paw withdrawal threshold at one and two hours after treatment. There was no longer a significant effect at 4 hours after treatment.

3.9 DCUKA Effect in Formalin Injection Model (Mouse)

Fig. 7 shows the effect of DCUKA (75 mg/kg, the lowest dose tested) on the duration of licking after formalin injection. There was no effect of DCUKA during the acute phase of the response, but DCUKA reduced the response during the inflammatory phase, i.e., the behavioral response was significantly decreased in the DCUKA-treated mice at 20, 25, 30 and 35 min after injection. For this inflammatory phase, DCUKA treatment reduced the area under the curve by $82 \pm 1.0\%$.

3.10 DCUKA Effect on Rat Rotarod Performance

A high dose of DCUKA (1000 mg/kg) did not produce incoordination/ataxia, as measured on the rotarod (repeated measures 2-way ANOVA) (see Supplemental File Section IV).

3.11 Plasma and Brain Levels of DCUKA

The plasma and brain concentrations of DCUKA were measured 90 min after the oral administration of DCUKA. After a dose of 32.5 mg/kg or 50 mg/kg (po) of DCUKA, plasma DCUKA levels were 4.47 ± 0.77 μ M (2020 ± 349 ng/ml) and 15.5 ± 8.9 μ M (7010

± 4023 ng/ml), respectively (n=3 rats/group). Brain concentrations of DCUKA after oral administration were substantially lower than plasma concentrations. After a dose of 32.5 mg/kg (po) of DCUKA, when both plasma and brain DCUKA levels were measured, the brain level was 49 ± 32 ng/g (n=6) (0.15 μ M).

3.12 DCUKA Binding to Other Receptors/Transporters/Enzymes

Table 1 shows the receptors, ion channels and transporters that were tested by PDSP and Lohocla Research Corporation and were not found to be affected by 10 μ M DCUKA. A secondary binding assay indicated a K_i at the delta opiate receptor of 4.5 μ M, but functional assays by PDSP (cAMP levels in response to delta opiate receptor activation) and in our own laboratories (calcium dynamics in CHEM1 cells transfected with delta opiate receptors) demonstrated no significant effect of DCUKA. The methods for and results of these assays are included in Supplemental File Section V. Functional assays of the hERG receptor, performed by PDSP (Huang et al., 2010), demonstrated no effect of DCUKA at concentrations as high as 10 μ M. Electrophysiological studies of the voltage-sensitive L-type calcium channel, $\text{Ca}_v1.2$, with DCUKA (50 μ M), also showed no significant effect of DCUKA.

4. Discussion

Damage to the sensory nervous system, either through inflammation or through physical constriction or lesioning, can result in neuropathic pain, and all of these processes result in a sensitization of sensory transduction, conduction and transmission in the damaged or neighboring peripheral sensory neurons (Abrahamsen et al., 2008; Djouhri et al., 2012). Comparison of plasma and brain levels of DCUKA after administration of a therapeutic dose indicated that DCUKA acts in the periphery, and we therefore focused our studies of DCUKA effects on peripherally localized signal transducers and signal conduction mechanisms that are known to be involved in chronic pain syndromes. One of the most investigated molecular mechanisms leading to neuropathic pain syndromes is an upregulation of the activity of the peripheral voltage-sensitive sodium channels (VSNaCs), including $\text{Na}_v1.7$ and $\text{Na}_v1.8$ (Black et al., 2004; Coggeshall et al., 2004; Lai et al., 2004; Dib-Hajj et al., 2007; Wang et al., 2011; Levinson et al., 2012). $\text{Na}_v1.8$ is localized to small and medium sized dorsal root ganglion (DRG) neurons and their axons (sensory neurons); $\text{Na}_v1.7$ is found in all types of DRG neurons, as well as in sympathetic neurons, Schwann cells and neuroendocrine cells (Catterall et al., 2005). Inflammatory conditions, including diabetic neuropathy, increase the expression of $\text{Na}_v1.7$ channels, which can contribute to increased generation and conduction of action potentials. The contribution of $\text{Na}_v1.7$ to initiation of action potentials is related not only to its own activation characteristics, but also to its ability to amplify generator potentials and promote activation of other sensory neuron VSNaCs such as $\text{Na}_v1.8$ (Dib-Hajj et al., 2007). Numerous studies, including studies of selective channel antagonists, have directly implicated $\text{Na}_v1.7$ and $\text{Na}_v1.8$ in the pathogenesis of pain syndromes (Okuse et al., 1997; Black et al., 2004; Coggeshall et al., 2004; Yeomans et al., 2005; Strickland et al., 2008; Jarvis et al., 2007; Focken et al., 2016). Furthermore, in humans, mutations in the gene that encodes $\text{Na}_v1.7$ are associated with known pain disorders (Cummins et al., 2004; Fertleman et al., 2006), and $\text{Na}_v1.7$ knockout

mice fail to develop hyperalgesia in several inflammatory pain models (Nassar et al., 2004). Similarly, knockdown or knockout of $\text{Na}_v1.8$ results in impaired inflammation-induced pain in mouse models (Akopian et al., 1999; Joshi et al., 2006). There is also evidence that both of these channels are involved in neuropathic pain that develops in diabetic neuropathy and after nerve lesions (Kim et al., 2002; Hong et al., 2004; Chattopadhyay et al., 2008), but universal involvement of these channels in neuropathic and other chronic pain syndromes is still controversial (Wang et al., 2011).

Our results show that DCUKA is an effective inhibitor of the $\text{Na}_v1.8$ channel, with a preference for the inactivated state. Although DCUKA also inhibits the activity of the $\text{Na}_v1.7$ channel, $\text{Na}_v1.8$ has higher affinity for DCUKA than does $\text{Na}_v1.7$. However, $\text{Na}_v1.7$ displays a use dependent sensitivity to DCUKA and thus could be easily inhibited in conditions of significant activation. We have previously shown (Wang et al., 2002), that $\text{Na}_v1.2$, which is restricted to the CNS (Catterall et al., 2005), shows little response to DCUKA (K_d values of 244 and 9.8 μM for the resting and inactivated states, respectively (Wang et al., 2002)).

Other VSNaCs that have been implicated in chronic pain include $\text{Na}_v1.3$ and $\text{Na}_v1.9$. $\text{Na}_v1.3$ is localized to central neurons (Catterall et al., 2005), and, given the limited concentrations of DCUKA in brain after doses that generate a therapeutic effect in the tested animal models of chronic pain, it is unlikely that DCUKA is acting via $\text{Na}_v1.3$ to ameliorate chronic pain. $\text{Na}_v1.9$ is localized on DRG neurons, and recent evidence suggests its role in pain signaling in humans (Dib-Hajj et al., 2015). In our studies of DRG neurons, care was taken to eliminate the contribution of $\text{Na}_v1.9$ to our results. In future analyses, it would be of interest to determine if DCUKA affects the activity of this peripheral VSNaC.

We also investigated the effects of DCUKA on $\text{Na}_v1.5$, which is known primarily as a cardiac sodium channel (Catterall et al., 2005), to assess potential cardiotoxic effects. Our results suggest that DCUKA, at therapeutic doses, would have few, if any, cardiovascular effects due to interaction with $\text{Na}_v1.5$. Similarly, at therapeutic doses, DCUKA would not affect cardiac function by effects on the hERG potassium channel.

The role of glutamate in the physiology of normal pain sensing and transmission, and in chronic pain phenomena, was originally established in the late 1980s (Davies and Lodge, 1987; Dickenson and Sullivan, 1987; Childers and Baudy, 2007). NMDA receptors are intimately involved in both the initiation of a pain sensation and its transmission into the CNS, as well as the phenomenon termed “wind up”, wherein the transmission of signal between primary and second order sensory neurons is amplified in conditions of repetitive sensory input as seen after nerve injury (Davies and Lodge, 1987; South et al., 2003). Changes in the expression levels of NMDA receptor subunit proteins are seen in the peripheral projections of nociceptive neurons, in their soma within the DRG, and in synapses of the primary and second order neurons in the spinal cord in animal models of mechanically-induced sensory nerve damage (Mott et al., 1998; Du et al., 2003; Jang et al., 2004; Bleakman et al., 2006; Childers and Baudy, 2007). Both the peripheral and spinal cord NMDA receptor upregulation is thought to contribute to tactile allodynia and thermal hyperalgesia seen in neuropathic pain syndromes (Petrenko et al., 2003). There is evidence,

including studies of selective inhibitors, that changes in the quantity of GluN2B subunits play the most important role in the hypersensitivity to glutamate in the sensory neurons and dorsal horn neurons (Gaunitz et al., 2002; Karlsson et al., 2002; Wilson et al., 2005; Iwata et al., 2007; Zhang et al., 2009; Wu & Zhuo, 2009).

It is important to emphasize that peripheral nociceptive fibers express GluN2B (and GluN2D) subunits. We consider that it is clinically advantageous to target peripheral receptors for treatment of chronic pain (Ferrari et al., 2014), to reduce CNS mediated adverse consequences of treatment. Our results show that DCUKA has a selective effect on a particular subset of NMDA receptors. When the affinity of the GluN1/GluN2B receptor for glycine is taken into account, the GluN2B-containing NMDA receptor is inhibited by DCUKA with a K_i of approximately 0.5 μ M, which is nearly ten-fold lower than the K_i for GluN1/GluN2A receptors. Similarly, receptors that contain the GluN2C and GluN2D subunits are less affected by DCUKA. Although both peripheral and central GluN2B-containing NMDA receptors can contribute to the development of chronic neuropathic pain, our results demonstrate that, after a therapeutic dose, levels of DCUKA are too low to affect NMDA receptor function in the CNS, indicating that DCUKA is likely to reduce chronic pain through its action on peripheral NMDA receptors.

Fig. 8 illustrates a network supported by the literature for generating and amplifying pain signals in chronic pain syndromes. This fig. also illustrates the selective multitarget action of DCUKA as shown in this manuscript. The actions of DCUKA are focused on the network of molecular entities which lead from the generation of sensory events, involving the NMDA receptor and its interaction with other receptors in the terminals of nociceptive neurons (e.g., TRPV1 or opiate receptors (Fischer and Dykstra, 2006; Lee et al., 2012; Sanchez-Blazquez et al., 2013). DCUKA simultaneously dampens the enhanced propagation of signals to the CNS by upregulated $\text{Na}_v1.7$ and $\text{Na}_v1.8$ channels. As discussed (Csermely et al., 2005; Lu et al., 2012), the selective multisite actions of DCUKA on the peripheral pain initiating and conduction network should produce a valuable approach to the treatment of chronic pain syndromes.

In each model of chronic pain that we evaluated, DCUKA returned the pain threshold back to the baseline level. It is noteworthy that in our studies, DCUKA primarily reduced chronic pain, i.e., acted as an anti-hyperalgesic, rather than as an analgesic to affect acute pain. This can be evidenced in both Figs. 4 and 5 by the fact that a plateau in effect is reached at the normal (uninjured) pain threshold and further increase in dose does not produce an increased threshold for pain. Furthermore, DCUKA treatment did not increase the mechanical pain threshold of the uninjured paw in the CFA model or in “sham” animals in the STZ model. These results are likely due to the fact that DCUKA is acting particularly on elements that are upregulated during the development of chronic pain (i.e., GluN2B, $\text{Na}_v1.7$ and $\text{Na}_v1.8$) and may play a lesser role in normal initiation and conduction of pain.

The restricted entry of DCUKA into brain is advantageous, since not only can central side effects be minimized, but also addiction potential is reduced or absent (Christoph et al., 2005). In fact, our preliminary studies (results not shown) indicate that DCUKA shows no addiction liability when given chronically (conditioned place preference test). Another

positive of peripheral action of DCUKA could be that early blockade of pain signaling produced by sensitized peripheral receptors could prevent CNS sensitization from occurring (Woolf, 2011).

5. Conclusions

In summary, our results demonstrate that DCUKA is effective in ameliorating chronic inflammatory and neuropathic pain. Its effects may primarily be due to its selective inhibitory effects on subtypes of VSNaCs and NMDA receptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. DCUKA Inhibition of $\text{Na}_v1.7$ Channel Activity. CHO cells were transfected with the $\text{Na}_v1.7$ α subunit and sodium currents were recorded using the whole-cell patch clamp recording configuration. A. Superimposed current traces recorded before (Control) and after exposure to the indicated concentrations of DCUKA. The currents were recorded from a single cell and were elicited by a 10 ms pulse to 0 mV from the -100 mV holding potential. B. I-V curve from a representative cell before and after exposure to $25 \mu\text{M}$ DCUKA. C. Use dependence of block by DCUKA: reduction of currents by $25 \mu\text{M}$ DCUKA in cells held at

–100 mV, and stimulated with a train of 5-ms pulses to 0 mV at a frequency of 5 Hz (● control, ○ DCUKA) or 10 Hz (▲ Control, △ DCUKA). Current amplitudes were normalized to the current evoked by the first pulse in the train.

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Fig. 2.

DCUKA Inhibition of $\text{Na}_v1.8$ Channel Activity. Dorsal root ganglion (DRG) neurons were prepared from Sprague-Dawley rats and $\text{Na}_v1.8$ currents were recorded using the whole cell patch clamp configuration. $\text{Na}_v1.8$ currents were isolated by pharmacological and electrophysiological methods (see text and Supplemental File Section I). A. Superimposed current traces recorded before (Control) and after exposure to the indicated concentrations of DCUKA. The currents were recorded from a single cell and were elicited by a 30 ms pulse to -10 mV from a holding potential of -80 mV. B. I-V curve constructed by measuring the

peak current activated at each voltage, in the absence (●) or presence (o) of 1 μ M DCUKA. Values are mean $\pm$ S.E.M. from 5 individual experiments. C. Use-dependence of block by DCUKA: effect of 1 μ M DCUKA on peak currents measured in cells stimulated with a train of 30 (20-ms) depolarizations from the holding potential of -80 mV to -10 mV at a frequency of 10 Hz. Current amplitudes were normalized to the current evoked by the first pulse in the train.

Fig. 3. Inhibition of NMDA Receptor Currents by DCUKA. NMDA receptor subunit heteromers (GluN1/GluN2A; GluN1/GluN2B) (A) or GluN1/GluN2C; GluN1/GluN2D (B) were expressed in HEK 293 cells. Whole cell voltage clamp recordings were carried out in the presence of 100 μM NMDA and 10 μM glycine (A) or in the presence of 10 μM glutamate and 10 μM glycine (B), in the absence or presence of the indicated concentrations of DCUKA. Dose-response curves were constructed from steady-state current amplitudes. Data were fit to the Hill equation to determine IC_{50} values. $N = 3$ cells at each concentration.

Fig. 4.

DCUKA Reverses Pain in the Complete Freund's Adjuvant (CFA) Model of Chronic Inflammatory Pain. Rats were injected into the left hind paw with CFA. Mechanical pain was assessed in the injected paw, using the von Frey method, at baseline (prior to CFA treatment) and at 48 hours after CFA treatment. At the 48-hour timepoint, rats were treated with vehicle or the indicated doses of DCUKA (po), 60 min prior to pain assessment. Data shown are the ratio of the post-CFA paw withdrawal threshold of the CFA-treated paw to the baseline paw withdrawal threshold of the same paw, defined as the mechanical pain

threshold (mean $\pm$ S.E.M., n=6–18/group). The baseline and post-CFA paw withdrawal thresholds (g) are provided in the text. One-way ANOVA (using a Satterthwaite approximation to adjust for unequal variances between treatment groups) indicated a significant effect of DCUKA, and specific group (least square means) comparisons show that the responses to DCUKA doses of 32.5 mg/kg and 50 mg/kg are significantly different (*) from the vehicle response. The number of rats/treatment group is noted at the bottom of each bar.

Fig. 5. DCUKA Reverses Pain in the Streptozotocin (STZ) Model of Chronic Neuropathic Pain. Rats were treated with streptozotocin to induce diabetic neuropathy. Mechanical pain was assessed in both hind paws, using the von Frey method, at baseline (prior to STZ treatment) and at two weeks after STZ treatment. At the 2-week time point, rats were treated with vehicle or the indicated doses of DCUKA (po), 60 min prior to pain assessment. Data shown are the ratio of the post-STZ paw withdrawal threshold to the baseline withdrawal threshold of the same paw (n= 7–15 per group). The mean ratio for both paws is defined as the

mechanical pain threshold. The baseline and post-STZ paw withdrawal thresholds (g) are given in the text. One-way ANOVA indicated a significant effect of DCUKA, and specific group (least square means) comparisons show that the responses to DCUKA doses of 32.5 mg/kg and 40 mg/kg are significantly different (*) from the response of the vehicle group. The number of rats/treatment group is noted at the bottom of each bar. One-sample t-tests comparing the mechanical pain threshold to a null hypothesis of mechanical pain threshold = 1 were performed, and the effects of 32.5 and 40 mg/kg of DCUKA were not significantly different from 1.

Fig. 6.

DCUKA Reverses Pain in the Partial Spinal Nerve Ligation (SNL) Model. One week after surgery, mechanical pain was assessed by the von Frey method. The results are expressed as the paw withdrawal threshold (g) prior to (0 h) and at 1, 2 and 4 hours after administration of DCUKA (75 mg/kg, po). The number of rats per group is noted at the bottom of each bar. One-way ANOVA with repeated measures shows an overall significant effect of time. DCUKA treatment resulted in a significant (*) increase in paw withdrawal threshold at 1 and 2 hours (least square means comparisons).

Fig. 7.

DCUKA Reverses Pain in the Formalin Test. Mice (n=8 per group) were injected with formalin, and the duration of paw licking was measured. Licking was increased at the time of formalin injection, and was reduced at 5–10 min after injection. DCUKA (75 mg/kg, po), administered 15 min prior to formalin injection, had no effect on licking during the acute phase of the response. During the second, inflammatory, phase of the response, licking again increased, and DCUKA significantly (*) reduced the response at 20–35 min after injection (2-way ANOVA with least square means comparisons). The 2-way ANOVA showed a significant time and DCUKA dose (either 0 or 75 mg/kg) interaction effect.

Fig. 8.

Illustration of DCUKA Actions on Peripheral Sensory Systems. The multitarget actions of DCUKA, and interactions of other receptors with the primary targets, are depicted. NMDA receptors containing GluN2B and TRPV1 receptors located on nociceptors initiate signaling to the CNS. DCUKA acts as an NMDA receptor antagonist in the periphery. Functional interactions between NMDA and TRPV1 receptors in sensory neurons (NMDAR + TRPV1) have been shown to contribute to the development of mechanical hyperalgesia (Lee et al., 2012). The NMDA receptor has also been implicated in the antinociceptive effect of morphine, i.e., increased NMDAR activity opposes morphine-induced analgesia, and NMDAR antagonists increase morphine-induced analgesia (NMDAR – MuOR) (Fischer and Dykstra, 2006; Sanchez-Blazquez et al., 2013). Nav1.7 and Nav1.8 mediate conduction of sensory information. DCUKA acts as an inhibitor of Nav1.7 and Nav1.8 channels. Nav1.7 upregulation during inflammation not only increases action potential generation, but also promotes activation of Nav1.8 (Nav1.7 + Nav1.8) (Dib-Hajj et al., 2007), leading to enhanced conduction of sensory signals. NMDA receptors containing GluN2B are also found in cell bodies of sensory neurons in the dorsal root ganglion (DRG), and upregulation of these receptors contributes to hyperalgesia seen in chronic pain syndromes (Petrenko et al., 2003; Ferrari et al., 2014). DCUKA selectively inhibits the function of GluN2B-containing NMDA receptors and in conjunction with its effects on Nav1.7 and Nav1.8, would produce a reduction in hyperalgesia.

Table 1

Receptors, Transporters and Ion Channels Which Were not Affected (Ligand Binding and/or Function) by 10 μ M DCUKA

Serotonin Rs	Dopamine Rs
5-HT1A	D1
5-HT1B	D2
5-HT2A	Opiate Rs
5-HT2B	μ
5-HT3	κ
Adrenergic Rs (alpha adrenergic)	Prostaglandin Rs
α 2 β 2	EP2
α 2 β 4	Muscarinic Cholinergic Rs
α 3 β 2	M1
α 3 β 4	M2
α 4 β 2	Metabotropic Glutamate Rs
α 4 β 2	mGluR5
Adrenergic Rs (beta adrenergic)	Ionotropic Glutamate Rs
β 1	NMDA channel binding site
β 2	Kainate
Voltage sensitive Ca ²⁺ channel (L-type)	Serotonin Transporter
Ca _v 1.2	Vasopressin Rs
	V1A