JPET Fast Forward. Published on January 16, 2018 as DOI: 10.1124/jpet.117.245068
This article has not been copyedited and formatted. The final version may differ from this version.

Curcumin acts as a positive allosteric modulator of α_7-nicotinic acetylcholine receptors and reverses nociception in mouse models of inflammatory pain

Eslam Gaber El Nebrisi, Deniz Bagdas, Wisam Toma, Halima Al Samri, Anna Brodzik, Yasmin Alkhlaif, Keun-Hang Susan Yang, Frank Christopher Howarth, Imad M. Damaj, Murat Oz

Departments of Pharmacology (E.G.E.N.; H.A.S.; M.O.) and Physiology (F.C.H.) College of Medicine and Health Sciences, UAE University, Al Ain, UAE. Department of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298-0613, USA (D.B.; W.T.; A.B.; Y.A.; M.I.D.) Experimental Animals Breeding and Research Center, Faculty of Medicine, Uludag University, Bursa 16059, Turkey (D.B.) Department of Biological Sciences, Schmid College of Science and Technology, Chapman University, One University Drive, Orange, CA 92866, USA (K.H.S.Y.). Department of Basic Medical Sciences, College of Medicine, Qatar University, Doha, Qatar (M.O.)

Running title: Effects of curcumin on α_7-nACh receptors

Corresponding author:
Murat Oz, M.D., Ph.D.
Department of Basic Medical Sciences,
College of Medicine, Building H12,
P.O.Box: 2713 Qatar University
Doha, Qatar
Phone: (974)-4403-7851
Fax: (974)-4403-7800
E-mail: murat.oz@qu.edu.qa

Number of text pages: 41

Tables: 1

Figures: 9

Number of references: 57

The number of words in the abstract: 180

The number of words in introduction: 460

The number of words in the discussion: 1427

Non standard abbreviations: ACh, Acetylcholine; ANOVA, analysis of variance; BAPTA, 1,2-bis (*o*-aminophenoxy) ethane-*N*, *N*, *N'*, *N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl) piperazineethane sulfonic acid; MLA, methyllycaconitine; MBS, modified Barth's solution, NEM, *N*-Ethylmaleimide; nACh nicotinic acetylcholine; PAM, positive allosteric modulator; PTX, pertussis toxin.

Recommended section: Neuropharmacology

Abstract

Effects of curcumin, a major ingredient of turmeric, were tested on the function of α_7 subunit of the human nicotinic acetylcholine (α_7 nACh) receptor expressed in *Xenopus* oocytes and on nociception in mouse models of tonic and visceral pain. Curcumin caused a significant potentiation of ACh (100 μM)-induced currents with an EC_{50} value of 0.2 μM. The effect of curcumin was not dependent on the activation of G-proteins and protein kinases and did not involve Ca^{2+}-dependent Cl^- channels expressed endogenously in oocytes. Importantly, extent of curcumin potentiation was enhanced significantly by decreasing ACh concentrations. Curcumin did not alter specific binding of [^{125}I] α-bungarotoxin. In addition, curcumin attenuated nociceptive behavior in both tonic and visceral pain models without effecting motor and locomotor activity and without producing tolerance. Pharmacological and genetic approaches revealed that the antinociceptive effect of curcumin was mediated by α_7 nACh receptors. Curcumin potentiated the antinociceptive effects of the α_7 nACh receptor agonist PNU282987. Collectively, our results indicate that curcumin is a positive allosteric modulator of α_7-nACh receptor and reverses nociception in mouse models of tonic and visceral pain.

Introduction

Turmeric, the rhizome of *Curcuma longa L.* has been used since ancient times as a spice, coloring, flavoring, and traditional medicine. Curcumin, a poly phenolic compound isolated from turmeric, has been shown to exhibit a wide range of pharmacological activities including anti-inflammatory, anti-cancer, anti-oxidant, anti-atherosclerotic, anti-microbial, and wound healing effects (Kunnumakkara et al., 2016; Milani et al., 2016). Notably, in recent years, curcumin has been demonstrated to have beneficial effects in cognitive deficits and neurodegenereative disorders such as Alzheimer and Parkinson diseases (Ji and Shen, 2014; Morales et al., 2014; Spagnuolo et al., 2016; Goozee et al., 2016). Although therapeutic effects of curcumin on wide range of pathological conditions have been reported, the precise mechanisms of these actions are poorly understood.

These diverse pharmacological activities of curcumin are based on its complex molecular structure and chemical features, as well as its ability to interact with multiple signaling molecules (Zhang et al., 2014). Many biological molecules have been identified as targets of curcumin, including transcription factors, growth factors, inflammatory cytokines, protein kinases, enzymes, and ion channels (Zhou et al., 2011; Zhang et al., 2014; Kunnumakkara et al., 2016; Milani et al., 2016).

Nicotinic acetylcholine (nACh) receptors are important members of the ligand-gated ion channel family that includes $GABA_A$ and $5\text{-}HT_3$ receptors. Nicotinic receptors are divided into two groups; muscle nicotinic receptors, which are found at the skeletal muscle junction where they mediate neuromuscular transmission and neuronal nicotinic receptors which are found throughout the peripheral and central nervous systems where they are involved in fast synaptic transmission

and in the modulation of transmitter release (for reviews; Albuquerque et al., 2009; Hogg et al., 2003). The homomeric α_7 nACh receptor subtype is abundantly expressed in the central nervous system (CNS) and periphery (Albuquerque et al., 2009). Importantly, α_7-nACh receptors, which have considerably high permeability to Ca^{2+}, have been shown to be located on both glutamatergic and GABAergic nerve terminals suggesting that both the excitatory and the inhibitory components of synaptic transmission can be modulated by the activity of these receptors (Albuquerque et al., 2009; Hogg et al., 2003). In fact, neuronal α_7-nACh receptors are recognized targets for drug development in several pre-clinical models of cognitive and neuro-degenerative disorders (Thomsen et al., 2010; Hone and McIntosh, 2017) and more recently in early human studies (Gee et al., 2017). Along with their well-documented roles in cognition, activation of α_7-nACh receptors produces analgesic effects in laboratory animal and human studies (Umana et al., 2013; Bagdas et al., 2017; Hone and McIntosh, 2017). Interestingly, curcumin also has been shown to attenuate pain and inflammation in various animal studies (Mittal et al., 2009; Liu et al., 2016). These findings may indirectly suggest the involvement common target between agonists of α_7-nACh receptors and curcumin.

In the present study, we have investigated the effects of curcumin on human α_7-nACh receptors expressed in *Xenopus* oocytes and tested its effects on nociception in mouse models of tonic and visceral pain. Specifically, we provide evidence indicating that curcumin acts as a positive allosteric modulator of α_7-nACh receptors in *in vitro* and *in vivo* conditions.

Materials and Methods

Recordings from oocytes: Mature female *Xenopus laevis* frogs were purchased from Xenopus Express (Haute-Loire, France) and housed in dechlorinated tap water at 19-21 °C with a 12/12-hour light/dark cycle and fed with food pellets supplied by Xenopus Express. The procedures followed in this study were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD) and approved by the Institutional Animal Care and Use Committee at the College of Medicine and Health Sciences, UAEU. Clusters of oocytes were removed surgically under benzocaine (Sigma, St. Louis, MO) local anesthesia (0.03 % w/V) and individual oocytes were dissected away manually in a solution containing (in mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $MgSO_4$, 0.8; HEPES, 10 (pH 7.5) as described earlier (Brauneis et al., 1996; Oz et al., 2004a). Dissected oocytes were then stored for two to seven days in modified Barth's solution (MBS) containing (in mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $CaCl_2$, 2; $MgSO_4$, 0.8; HEPES, 10 (pH 7.5), supplemented with 2 mM, sodium pyruvate, 10,000 IU/L penicillin, 10 mg/L streptomycin, 50 mg/L gentamicin, , and 0.5 mM theophylline. Briefly, oocytes were placed in a 0.2 ml recording chamber and superfused at a rate of 3-4 ml/min. Under these conditions, solution exchange time was less than 100 msec. The bathing solution contained (in mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$,1 and HEPES 5 (pH 7.5). The cells were impaled with two glass microelectrodes filled with a 3 M KCl (0.5- 2 MΩ). The oocytes were routinely voltage clamped at a holding potential of -70 mV using a GeneClamp-500 amplifier (Axon Instruments Inc., Burlingame, CA) and current responses were recorded and stored digitally for further analysis.

Drugs were applied by gravity flow via a micropipette positioned about 2 mm from the

oocyte. Some of the compounds were applied externally by addition to the superfusate (flow rate of 3-4 ml/min). Acetylcholine, GDPβS, methyllycaconitine (MLA), *N*-Ethylmaleimide (NEM), α-bungarotoxin, pertussis toxin (PTX), and all chemicals used were obtained from Sigma (St. Louis, MO). Procedures for the injections of BAPTA (50-100 nl, 100 mM) were performed as described previously (Oz et al., 1998). Stock solutions of curcumin used in this study were prepared in DMSO at a concentration of 100 mM. At the highest final concentrations used (0.1 % v/v), DMSO did not have a significant effect on ACh (100 μM)-induced currents (n=6).

The cDNA clone of human α_7-nACh receptor was kindly provided by Dr. J. Lindstrom (University of Pennsylvania, PA). Capped cRNA transcripts were synthesized *in vitro* using a mMESSAGE mMACHINE kit from Ambion (Austin, TX) and analyzed on 1.2 % formaldehyde agarose gel to check the size and the quality of the transcripts. Approximately 3-5 ng of cRNA was injected into each oocyte. The cDNAs for human α4, β2, α3, and β4 subunits were kindly provided by Dr. Isabel Bermudez. Subunit combinations, α and β subunits were injected at 1:1 ratio.

Radioligand binding studies: Oocytes were injected with 5 ng human α_7-nACh receptor cRNA, and the functional expression of the receptors was tested by electrophysiology on day three. Isolation of oocyte membranes was carried out by modification of a method described earlier (Oz et al., 2004b; Mahgoub et al.,2013). Briefly, oocytes (200-300 oocytes per assay) were suspended (approximately 20 μl/oocyte) in a homogenization buffer containing (in mM) HEPES 10, EDTA 1, PMSF 0.1, and 0.02% NaN_3, 50 μg/mL bacitracin (pH 7.4) at 4 °C on ice and homogenized using a motorized Teflon homogenizer (six strokes, 15 sec each at high speed). The homogenate was centrifuged for 10 min at 800 g. The supernatant was collected and the pellet was resuspended

in homogenization buffer and recentrifuged at 800 g for 10 min. Supernatants were then combined and centrifuged for 1 hour at 36,000 g. The membrane pellet was resuspended in homogenization buffer and used for the binding studies.

Binding assays were performed in 500 μL of 10 mM HEPES (pH 7.4) containing 50 μL of oocyte preparation and 0.1-5 nM [^{125}I] α-bungarotoxin (2200 Ci/mmol; Perkin-Elmer, Inc. Waltham, MA). Nonspecific binding was determined using 10 μM α-bungarotoxin. Oocyte membranes were incubated with [^{125}I] α-bungarotoxin in the absence and presence of drugs, for 1 h at room temperature (22-24 °C). The radioligand was separated by rapid filtration onto GF/C filters presoaked in 0.2% polyethyleneimine. Filters were then washed with two 5 ml washes of ice-cold HEPES buffer, and the radioactivity was determined by counting samples in a Beckman Gamma-300 γ-counter.

In vivo studies: Male ICR adult (8–10 weeks of age) mice were obtained from Harlan Laboratories (Indianapolis, IN). Mice on C57BL/6J background null for the α7 subunits (B6.129S7-Chrna7tm1Bay/J- The Jackson Laboratory, Bar Harbor, ME) and their wild-type (WT) littermates were bred in an animal care facility at Virginia Commonwealth University. For all experiments, mice were backcrossed for ≥ 9–10 generations. Mutant and WT mice were obtained by crossing heterozygote mice. This breeding scheme controlled for any irregularities that might occur with crossing solely mutant animals. Mice were housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care–approved animal care facility. They were housed in groups of four and had free access to food and water. The rooms were on a 12-hour light/dark cycle (lights on at 7:00 AM). All experiments were performed during the light cycle (between 7:00 AM and 7:00 PM), and the study was approved by the Institutional

Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Animals were sacrificed via CO_2 following by cervical dislocation after the experiments finished, unless noted otherwise.

Drugs: Methyllycaconitine citrate (MLA) was purchased from RBI (Natick, MA). PNU282987 [N-(3R)-1-Azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide] was obtained from the National Institute on Drug Abuse (NIDA) supply program (Bethesda, MD). PKC-412, Go-6983, KT-5720, and KN-62 were purchased from Tocris (Minneapolis, MN). They were dissolved in DMSO to 100 mM stock solutions. Curcumin was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). In *in vivo* experiments, curcumin was dissolved in a mixture of 2:2:16 [2 volume ethanol/2 volume Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ)/18 volumes distilled water] and administered intraperitoneally (i.p.) for systemic injections. Other drugs were dissolved in physiologic saline (0.9% sodium chloride) and injected subcutaneous (s.c.) at a total volume of 1 ml/100 g body weight, unless noted otherwise. All doses are expressed as the free base of the drug.

Behavioral assessments

Formalin test: The formalin test was carried out in an open Plexiglas cage (29 x 19 x 13 cm each). Mice were allowed to acclimate for 15 min in the test cage prior to injection. Each animal was injected intraplantarly (i.pl.) with 20 μL of (2.5%) formalin to the right hindpaw. Mice were observed from 0 to 5 min (phase I) and 20 to 45 min (phase II) post-formalin injection. The amount of time spent licking the injected paw was recorded with a digital stopwatch. Paw diameter (see measurement of paw edema) was also measured before and 1 h after formalin injection.

Curcumin (3, 10 and 30 mg/kg) or vehicle (veh) were injected in male ICR mice i.p. 45 min before

formalin injection. For the subchronic curcumin administration study, mice were given curcumin (30 mg/kg i.p.) or vehicle for 6 days once daily and were challenged with curcumin (30 mg/kg i.p.) on day 7 and tested in formalin test 45 min after the injection. A vehicle control group, in which mice were exposed to 7 days of vehicle, was also included.

For the antagonist studies, α_7 nicotinic antagonist MLA (10 mg/kg), or vehicle (saline) was injected s.c. 10 min before the curcumin (30 mg/kg; i.p.) or vehicle administration. In a separate group, curcumin (30 mg/kg, i.p.) effects in the formalin test were measured in α_7 WT and KO mice. To test possible potentiation antinociceptive effects of curcumin on PNU282987 in the formalin test, curcumin (3 mg/kg, i.p.) was injected 45 min before PNU282987 (0.1 mg/kg, s.c.) administration. The formalin test was conducted 10 min after last injection.

Measurement of paw edema: The thickness of the formalin treated paws were measured both before and after injections at the time points indicated above, using a digital caliper (Traceable Calipers, Friendswood, TX). Data were recorded to the nearest ± 0.01 mm and expressed as change in paw thickness (ΔPD = difference in the ipsilateral paw diameter before and after injection paw thickness).

Acetic acid-induced writhing test: For the measurement of acetic acid-induced nociceptive behavior, each mouse was placed in a Plexiglas box and allowed to acclimate for 20 min. Then the mouse was given an i.p. injection of acetic acid (1.2%) or saline and then returned to the box. Counting the number of typical writhing behaviors started immediately after acetic acid administration, and the number of stretches (a stretch was operationally defined as a contraction of the abdomen followed by an extension of the hind limbs) was recorded in 10 min bins for a total of 40 min. Experiments were carried out by injecting the mice with either vehicle or curcumin (30

mg/kg, i.p.) and 45 min later they received acetic acid and tested as described above. For the antagonist studies, α7 nAChR antagonist MLA (10 mg/kg) was injected s.c. 15 min before the curcumin (30 mg/kg, i.p.). 45 min after curcumin, mice received acetic acid or vehicle (saline) injection.

Motor coordination: The effects of drugs on motor coordination were measured using the rotarod test (IITC Inc. Life Science, Woodland Hills, CA) as previously described (Freitas et al., 2013). Mice were pretreated with either i.p. vehicle or curcumin (30 mg/kg, i.p.) 45 min before the test. Percent impairment was calculated as follows: % impairment = (180 - test time) / (180 * 100).

Locomotor Activity Test: Mice were placed into individual Omnitech (Columbus, OH) photocell activity cages (28 x 16.5 cm). Interruptions of the photocell beams (two banks of eight cells each) were recorded for the next 30 min. Mice were pretreated with either i.p. vehicle or curcumin (30 mg/kg, i.p.) 45 min before the test. Data were expressed as the number of photocell interruptions.

Statistical Analysis: In oocyte experiments, average values were calculated as the mean ± standard error means (S.E.M.). Statistical significance was analyzed using Student's *t* test or ANOVA as indicated. Concentration-response curves were obtained by fitting the data to the logistic equation,

$$y = E_{max}/(1+[x/EC_{50}]^{-n}),$$

where x and y are concentration and response, respectively, E_{max} is the maximal response, EC_{50} is the half-maximal concentration, and n is the slope factor (apparent Hill coefficient).

In behavioral studies, the data obtained were analyzed using the GraphPad software, version 6.0 (GraphPad Software, Inc., La Jolla, CA) and expressed as the mean ± S.E.M. Statistical analysis was done using the 1-way or 2-way analysis of variance test (ANOVA),

followed by the post hoc Tukey's test. Unpaired student t test was used for spontaneous activity and motor coordination. The *P* values < 0.05 were considered significant.

Results

Effects of curcumin on α_7-nACh receptors:

Application of 100 μM ACh for 3 to 4 sec activated fast inward currents that desensitized rapidly in oocytes injected with cRNA encoding the α_7-subunit of human nACh receptor (Fig. 1A). In addition, ACh-induced currents were inhibited completely with 100 nM α-bungarotoxin (supplement Figure 1A and 1B), indicating that these currents are mediated by the activation of α_7-nACh receptors. Bath application of curcumin (100 μM) for 5 min did not produce detectable currents in oocytes expressing α_7-nACh receptors (n=8 oocytes).

The effect of curcumin was tested on ACh (100 μM)-induced ion currents. An effect of 10 min curcumin (1 μM) application on α_7-nACh receptor mediated currents is shown in Fig. 1A. Time-courses of effects of curcumin or the vehicle (0.1% DMSO) applications on the maximal amplitudes of ACh-induced currents are presented in Fig. 1B. Curcumin caused a significant potentiation of the current, which was partially reversed during a 10 to 15 min washout. In the absence of curcumin, vehicle (0.1 % DMSO) alone did not alter the amplitude of the ACh-induced current, further suggesting that curcumin acts on nACh receptors (Fig. 1B, controls versus curcumin treatment group at 10 min of exposure, ANOVA, n=5-7; $P<0.05$).

The potentiating effect of curcumin was significantly dependent on the application mode. For example, without preincubation, coapplication of curcumin (1 μM) and ACh (100 μM) did not alter the amplitudes of maximal currents (Fig. 1C). However, when oocytes were preincubated with curcumin, the drug was found to potentiate maximal ACh-induced currents in a time dependent manner reaching a maximal level within 5 min with a half-time ($\tau_{1/2}$) of 1.6 min (Fig. 1C). Since the magnitude of the curcumin effect was time-dependent, 10 min curcumin application

time was used routinely to ensure equilibrium conditions. Curcumin was found to upregulate the function of α_7-nACh receptor in a concentration-dependent manner with an EC_{50} and slope values of 0.21 ± 0.14 μM and 1.6, respectively (Fig. 1D).

As it was shown in Fig. 1B and 1C, regulation of α7-nACh receptor function by curcumin occurs gradually reaching steady-state levels within a few minutes of curcumin application. Therefore, it was possible that activation of second messenger pathways by G-protein-coupled receptors (Liu et al., 2013; Pérez-Lara et al., 2011; Yang et al., 2015) is involved in curcumin regulation of α_7-nACh receptors. Thus, we investigated the effects of pretreatments with *N*-Ethylmaleimide (NEM; 10 mM, 50 nl, 30 min preincubation time), a sulfhydryl-alkylating agent that blocks G protein-effector interactions by alkylating α-subunits of PTX-sensitive GTP binding protein (Oz and Renaud, 2002) and GDPβS (10 mM. 50 nl, 30 min preincubation time) an agent that inhibits binding of GTP to α-subunit of G-proteins (Oz et al., 1998). Treatments with NEM, and GDPβS did not alter the extent of curcumin potentiation of α7-nACh receptor (Fig. 2A). Similarly, pretreatment with pertussis toxin (PTX; 5 μg/ml, 50 nl, 30 min preincubation time) a toxin that inhibits the α-subunit of Gi/o proteins, did not reverse potentiating effect of curcumin (Supplement Figure 2). In addition, extent of curcumin-induced potentiation of currents activated by low (30 μM) concentration of ACh were also not altered in the presence of NEM and GDPβS curcumin (Supplement Figure 2)

Activation of α_7-nACh receptors allows sufficient Ca^{2+} entry to activate endogenous Ca^{2+}-dependent Cl^- channels in *Xenopus* oocytes (Sands et al., 1993; Uteshev, 2012). Therefore, it was important to determine whether the effect of curcumin was exerted on nACh receptor mediated currents or on Cl^- currents induced by Ca^{2+} entry. For this reason, we injected the Ca^{2+}

chelator BAPTA into oocytes and replaced extracellular Ca^{2+} with Ba^{2+} since Ba^{2+} can pass through α_7-nACh receptors but causes less activation of Ca^{2+}-dependent Cl^- channels (Sands et al., 1993). Under these conditions, we tested the effect of curcumin in a solution containing 2 mM Ba^{2+} in BAPTA-injected oocytes. Curcumin (1 μM) produced the same level of potentiation (195 ± 18 in controls versus 210 ± 22 in BAPTA-injected oocytes; ANOVA, $P > 0.05$; n=6-7) on ACh-induced currents in BAPTA-injected oocytes when currents were recorded in Ca^{2+} free solution containing 2 mM Ba^{2+} (Fig. 2B). It is important to mention that in the oocyte expression system, curcumin-induced changes in nicotinic receptor-mediated currents can be attributable to Ca^{2+}-activated Cl^- channels and concomitant alterations in the holding currents. However, in control experiments, curcumin (100 μM for 10 min) did not change the magnitudes of holding-currents in oocytes voltage-clamped at -70 mV (n=7), indicating that intracellular Ca^{2+} levels were not altered by curcumin.

We have also investigated the involvement protein kinases A and C, and Ca^{2+}-calmodulin dependent kinase (CaM-kinase) in curcumin potentiation of α_7-nACh receptors. For this purpose, the effects of curcumin were tested in oocytes pretreated with PKC-412 (nonspecific kinase inhibitor, 10 μM for 30 min. pretreatment), Go-6983 (specific protein kinase C inhibitor, 10 μM for 30 min.), KT-5720 (specific protein kinase A inhibitor, 10 μM for 30 min.), and KN-62 (specific inhibitor of CaM kinase II, 50 μM for 30 min). Curcumin continued to upregulate nicotinic receptor-mediated currents in oocytes pretreated with kinase inhibitors (Fig. 2C and 2D). Similarly, potentiating effects of curcumin remained unaltered by inhibitors of protein kinases A and C, and Ca^{2+}-calmodulin dependent kinase (CaM-kinase) at low (30 μM) ACh concentrations (Supplement Figure 2).

In the next series of experiments, we examined if the extent of curcumin potentiation of the α_7-nACh receptor mediated current is altered by membrane potential. As indicated in Fig. 2E, the potentiation of ACh (30 μM)-induced currents by curcumin (1 μM) does not appear to be voltage-dependent. The extent of curcumin potentiation was similar at all tested membrane potentials from -100 to +40 mV. Evaluation of the current-voltage relationship (Fig. 2F) indicates that the extent of potentiation by curcumin does not change significantly at different test potentials ($P>0.05$, n=7, ANOVA).

In the next series of experiments, we attempted to test the effects of curcumin at different ACh concentrations. Traces of low ACh (10 μM)-induced currents after 10 min treatment with 1 μM curcumin was presented in Fig. 3A. At low ACh (10 μM) concentrations, curcumin caused approximately 11 to 12 fold increase of ACh-induced currents with EC_{50} of 58 nM (Fig. 3B). Further experiments indicated that the extent of curcumin potentiation decreased significantly with increasing concentrations of ACh (Fig. 3C). Concentration-response curves for ACh in the absence and presence of 1 μM curcumin are presented in Fig. 3D. In the presence of 1 μM curcumin, the maximal ACh response increased by 60-70% of controls (n=6-8). In the absence and presence of curcumin, the EC_{50} values for ACh were 107 ± 18 μM and 63 ± 16 μM and slope values were 2.2 ± 0.4 and 1.9 ± 0.3, respectively (n=6-7).

The results of functional studies indicate that curcumin significantly decreases desenzitation of currents mediated by the activation of α_7-nACh receptors. Normalized and superimposed current traces in the absence and presence of 1 μM curcumin were presented in Figure 4A. Summary of the results showing the effect of curcumin (1 μM) on the half decay times of the α_7-nACh receptor-mediated currents were shown in Figure 4B. In the absence and presence

of curcumin, means of half decay times were 208 ± 42 sec and 643 ± 85 sec (paired-t test; n=8; P <0.01), respectively. These findings suggested that curcumin caused a significant decrease of α_7-nACh receptor desensitization. We further investigated whether curcumin can convert α_7-nACh receptors that were already desensitized by a concentration of an agonist back to conducting state. We have tested the effect of bath application of curcumin (10 μM) on the α_7-nACh receptors that was desensitized by 100 μM nicotine application for 25-30 sec (n=6). As illustrated in Figure 4C, subsequent addition of curcumin resulted in activation of a sustained inward current that was reversed during washout.

[^{125}I] α-bungarotoxin competes with ACh, an endogenous activator of α_7-nACh receptors by binding to the ACh binding site on the receptor (Albuquerque et al., 2009). For this reason, the effect of curcumin was investigated on the specific binding of [^{125}I] α-bungarotoxin. Equilibrium curves for the binding of [^{125}I] α-bungarotoxin, in the presence and absence (controls) of curcumin are presented in Fig. 5A. Maximum binding activities (B_{max}) of [^{125}I] α-bungarotoxin were 3.61 ± 0.37 and 3.47 ± 0.41 pM/mg (means ± S.E.M.) for controls and curcumin-treated preparations, respectively. The apparent affinity (K_D) of the receptor for [^{125}I] α-bungarotoxin was 0.87 ± 0.26 and 0.67 ± 0.23 pM for controls and curcumin, respectively. There was no statistically significant difference between controls and curcumin-treated groups with respect to K_D and B_{max} values (P>0.05, ANOVA, n=6-7) suggesting that curcumin does not compete with α-bungarotoxin at the same binding site. Curcumin up to concentration of 100 μM did not cause a significant change on the specific binding of [^{125}I] α-bungarotoxin (Fig. 5B). The effect of curcumin on the functional properties of other neuronal nACh receptor subtypes was also examined. Application of curcumin (10 μM for 10 min.) did not cause alterations of ACh (100 μM)-induced currents mediated by

different subtype combinations of nicotinic receptors expressed in oocytes (Fig. 5C). Similarly, curcumin (10 μM for 10 min.) did not cause significant changes on the amplitudes of currents mediated by 5-HT_{3A} (1 μM 5-HT) subunit and glycine receptors (30 μM glycine; mediated by α1β1, α2β1, and α3β1 subunit combinations) (Fig. 5D).

Effects of curcumin treatment in formalin-induced pain responses

To determine the effect of acute curcumin treatment in the formalin-induced pain model, mice were given an i.p. injection of curcumin (3, 10, 30 mg/kg) or vehicle 45 min before i.pl. formalin injection. Ordinary two-way ANOVA revealed significant effects for phase of formalin test [$F_{phase(1,40)} = 14.54$, $P < 0.001$] and dose of curcumin [$F_{dose(3,40)} = 11.04$, $P < 0.001$]. As seen in Figure 6A, curcumin failed to show significant antinociceptive effect in phase I of the test (Tukey post hoc, $P > 0.05$). However, it dose-dependently attenuated nociceptive behavior in Phase II (Tukey post hoc, $P < 0.001$).

Role of α_7 nACh receptors in the effects of curcumin

We explored the possible role of α_7 nAChRs in the effect of curcumin in the formalin test. We first investigated if the α_7 nAChR antagonist MLA would block curcumin's effects in phase II of the test. Ordinary two-way ANOVA revealed significant effects for phase of formalin test [$F_{phase(1,40)} = 37.61$, $P < 0.001$] and treatment [$F_{dose(3,40)} = 13.29$, $P < 0.001$; Fig 6B]. A post hoc Tukey test showed that while the α_7 nAChR antagonist MLA (10 mg/kg, s.c.) given alone did not alter formalin-induced pain responses ($P < 0.05$), it completely blocked the antinociceptive effect of curcumin ($P < 0.001$). We then tested curcumin in the α_7 WT and KO mice. Interestingly, curcumin significantly reduced the phase I nociceptive responses in α_7 WT mice. Surprisingly, the antinociceptive effect of curcumin was also preserved in transgenic KO mice. On the other hand,

while curcumin reduced formalin-induced paw licking in WT mice, the effect vanished in α_7 KO mice in the phase II of the test [$F_{phase(1,44)} = 215.8$, $P < 0.001$; and genotype [$F_{genotype(3,44)} = 75.87$, $P < 0.001$; Fig 6C].

Sub-chronic curcumin treatment attenuates formalin-induced pain responses, without producing tolerance

In the next experiment, we investigated whether the antinociceptive effects of curcumin in the formalin test would undergo tolerance after subchronic administration. Curcumin produced significant effects in phase I [one-way ANOVA, Tukey post hoc, $F_{(2,15)} = 4.896$; $P < 0.05$] and phase 2 [$F_{(2,15)} = 18.24$; $P < 0.001$; Fig 7A]. Sub-chronic curcumin attenuated pain responses at both phase I and phase II without producing tolerance. Moreover, curcumin also reduced the formalin-induced paw edema without producing tolerance [one-way ANOVA, Tukey post hoc, $F_{(2,15)} = 26.74$; $P < 0.001$; Fig 7B].

Potentiation of the antinociceptive effects of the α_7 nicotinic agonist PNU282987 by curcumin

We determined the effects of curcumin on the PNU282987-evoked antinociceptive effects in the formalin test. Data were analyzed using two-way ordinary ANOVA following by Tukey post-hoc test. Analysis revealed significant effects for phase of formalin test [$F_{phase(1,40)} = 47.55$, $P < 0.001$] and treatment [$F_{dose(3,40)} = 12.51$, $P < 0.001$; Fig 8]. Pretreatment with a low dose of curcumin (3 mg/kg, i.p.) or PNU282987 (0.1 mg/kg, s.c) failed to attenuate formalin-induced pain responses when tested alone ($P > 0.05$). However, combination of curcumin and PNU282987 significantly reversed pain behavior in phase II ($P < 0.001$) but not phase I of the test ($P > 0.05$).

Effects of curcumin treatment in acetic acid-induced stretching

Acetic acid significantly evoked stretching behavior as a nociceptive behavior outcome

[$F_{acetic\ acid}(1,56) = 813.1$, $P < 0.0001$; Fig 9]. Curcumin (30 mg/kg) significantly reduced acetic acid-induced nociceptive stretching behaviors and MLA blocked this antinociceptive effects of curcumin [(3,56) = 20.79, $P < 0.0001$; Fig 9].

Effects of curcumin on motor activity and coordination

As seen in Table 1, curcumin at the dose of 30 mg/kg, (i.p.), the highest active dose used in our study, did not affect (A) motor performance or (B) spontaneous activity of mice after acute injection (t test, t = 0.2988, df=8, $P > 0.05$ and t = 0.5841, df=10, $P > 0.05$; respectively).

Discussion

In the present study, using electrophysiological, biochemical and behavioral methods, we provide evidence that curcumin upregulates the function of human α_7-nACh receptors expressed in *Xenopus* oocytes and reverses nociception in mouse models of tonic and visceral pain through an α_7-nACh receptor mechanism. The enhancement of α_7-nACh receptors function by curcumin is reversible and occurs in a time and concentration dependent manner, but is independent of G-protein activation, protein kinase activity, intracellular Ca^{2+} levels, and membrane potential.

Relatively slow time course of curcumin effect and the results of earlier studies on curcumin modulation of various second messenger pathways and kinases (Mahmmoud, 2007; Takikawa et al., 2013) suggest that activation of G-protein coupled receptors and/or kinase-mediated phosphorylation are involved in curcumin-induced upregulation of α_7-nACh receptors. However, neither treatments with established kinase inhibitors nor pharmacological disruption of G-protein activity did reverse curcumin potentiation of α_7-nACh receptors suggesting that curcumin acts directly on ion channel-receptor complex.

In *Xenopus* oocytes, activation of α_7-nACh receptors, due to their high Ca^{2+} permeability, allows sufficient Ca^{2+} entry to activate endogenous Ca^{2+}-dependent Cl^- channels (Hartzell et al., 2005; Sands et al., 1993). Therefore, the direct actions of curcumin on Ca^{2+}-dependent Cl^- channels may contribute to the observed effects of curcumin on ACh-activated currents in this expression system. In oocytes injected with BAPTA and recorded in a solution containing 2 mM Ba^{2+}, curcumin continued to potentiate α_7-nACh receptor-mediated ion currents, suggesting that Ca^{2+}-dependent Cl^- channels were not involved in curcumin potentiation of nicotinic responses. In addition, the reversal potential in solutions containing Ba^{2+} was not altered in the presence of

curcumin, suggesting that the potentiation by curcumin is not due to alterations in the Ca^{2+} permeability of the α_7-nACh receptor-channel complex.

Curcumin has been reported to alter Ca^{2+} homeostasis in various cell types (Dyer et al., 2002; Ibrahim et al., 2011; Moustapha et al., 2015; Wang et al., 2012). However, in *Xenopus* oocytes, Ca^{2+}-activated Cl^- channels are highly sensitive to intracellular Ca^{2+} levels (K_D of Ca^{2+}-activated Cl^- channels for Ca^{2+} is less than 1 μM; for a review, Hartzell et al., 2005), and alterations in intracellular Ca^{2+} levels would be reflected by changes in the holding current under voltage-clamp conditions. In our experiments, application of curcumin (1-100 μM) did not cause alterations in baseline or holding currents, and curcumin continued to potentiate nicotinic receptors after the chelation of intracellular Ca^{2+} by BAPTA, suggesting that alterations in intracellular Ca^{2+} concentrations does not play a role in curcumin's effect on nicotinic receptors.

Previous studies have demonstrated that curcumin acts on several integral membrane proteins including enzymes, transporters, and ion channels (Li et al., 2016; Zhang et al., 2014). T-type Ca^{2+} channels in bovine adrenal cells (Enyeart et al., 2009, IC_{50} = 10-20 μM), L-type Ca^{2+} channels in hippocampal neurons (Liu et al., 2013; $IC_{50} \approx 10$ μM), TREK-1 K^+ channels (Enyeart et al., 2008; IC_{50} = 0.9 μM) and Kv1.4. K^+ channels (Liu et al., 2006) in bovine adrenal cells, Kv1.4. K^+ channels (Lian et al., 2013; IC_{50} = 4.2 μM) in human T-lymphocytes, ERG K^+ channels (Hu et al., 2012, IC_{50} = 5.5 μM; Choi et al., 2013; IC_{50} = 10.6 μM; Banderali et al., 2011, IC_{50} = 2 μM), K^+ channels in rabbit coronary arterial smooth muscle cells (Hong et al., 2013; IC_{50} = 1.1 μM). In addition to voltage-dependent conductances, curcumin has also been shown to act on transient-receptor potential receptors (Yeon et al., 2010; Zhi et al., 2013).

In this study, curcumin was applied in the concentration range of 1 nM to 100 μM and it

was found that it can enhance the effects of ACh on the function of α_7-nACh receptors in a concentration-dependent manner with EC_{50} values ranging from 58 nM to several µM. The concentration of curcumin in plasma and its ability to pass the blood brain barrier following oral and intravenous administration has been studied previously (Anand et al., 2007). When curcumin is given orally at a dose of 2 g/kg to rats, maximum serum concentration of 1.35 µg/ml (3.5 µM) was attained (Shoba et al., 1998). Since curcumin is a highly lipophilic compound with LogP (octanol–water partition coefficient) value of 3.3 (PubChem, 2017), its membrane concentration is expected to be considerably higher than blood levels. Therefore, the functional modulation of α_7-nACh receptors demonstrated in this study can be pharmacologically relevant.

As it was mentioned earlier, the roles of G-proteins, kinases, and intracellular Ca^{2+} levels in curcumin actions were excluded in our functional and pharmacological studies. Radio-ligand binding experiments suggested that curcumin does not interact with ACh binding to receptors. Further analysis of the curcumin effect indicated that curcumin significantly (more than 3 fold) decreased desensitization of the receptor and converted already desensitized nACh receptor back to conducting state (Fig. 4C). Importantly, binding of α-bungarotoxin, a competitive antagonist of ACh, was not altered in the presence of curcumin suggesting that curcumin does not interact with the ACh binding site in the receptor. It is plausible that curcumin acts as an allosteric modulator for various receptors and ion channels at the lipid membrane accounting for some of its pharmacological actions in animal studies (Zhang et al., 2014). Allosteric modulators alter the functional properties of ligand-gated-ion channels by interacting with site(s) that are topographically distinct from the ligand binding sites (for a review; Onaran and Costa, 2009). Two different types of positive allosteric modulator (PAM) have been postulated (Uteshev, 2014;

Chatzidaki and Millar, 2015). While type I enhances agonist-induced currents without affecting macroscopic current kinetics, type II PAMs also delay desensitization and reactivate desensitized receptors. Results of our experiments indicate that curcumin significantly decrease desensitization (Fig. 4A and 4B) and reactivates completely desensitized nicotinic receptors (Fig. 4C), suggesting that curcumin acts as a type II PAM.

It is likely that curcumin, a highly lipophilic agent, first dissolves into the lipid membrane and then diffuses into a non-annular lipid space to potentiate the function of the ion channel-receptor complex. Consistent with this idea, the effect of curcumin on α_7-nACh receptor reached a maximal level within 5-10 min of application. suggesting that the binding site(s) for these allosteric modifiers is located inside the lipid membrane and require a relatively slow (in minutes) time course to modulate the function of the receptor. It is likely that these hydrophobic agents affect the energy requirements for gating-related conformational changes in ligand-gated ion channels (Spivak et al., 2007).

Our *in vitro* data suggested that curcumin is a selective positive allosteric modulator (PAM) of α_7-nACh receptors. We therefore tested this possibility after systemic *in vivo* administration in mice. α7 nAChR are present in supraspinal and spinal pain-transmission pathways as well as on immune and non-immune cytokine-producing cells such as macrophages and microglia. α7 nAChRs that are expressed on immune cells involve in the initiation, maintenance, resolution of inflammation, and modulate inflammation processes. In addition, α7 nAChRs expressed on microglia regulate inflammatory factors (Bagdas et al. 2017). Since α_7-nACh receptor PAMs have been reported to be active in animal models of tonic and chronic

pain (Munro et al., 2012; Freitas et al., 2013a,b; Bagdas et al., 2015), we evaluated the antinociceptive and anti-inflammatory effects of curcumin in the mouse formalin test, a model of tonic and persistent pain (Hunskaar and Hole, 1987). The formalin test consists of two distinct phases. The first phase (immediately after formalin injection) seems to be caused by the direct effect of formalin on sensory C-fibers. The second phase (starting later after formalin injection), known as the inflammatory phase, is associated with the development of a delayed inflammatory response and spinal dorsal horn sensitization (Abbott et al., 1995; Davidson and Carlton, 1998). In the outbred ICR mice, curcumin attenuated pain behaviors dose-dependently in the late (inflammatory) but not the early phase of the formalin test. Importantly, no changes were seen in motor locomotion or coordination with antinociceptive doses of curcumin in mice (Table I). Using both pharmacological (i.e., the selective α_7-nACh antagonist MLA) and genetic approaches (i.e., α_7 KO mice), we confirmed that curcumin's effect in the late phase of formalin is mediated by α_7-nACh receptors. The effects of curcumin in the early phase of formalin is strain-dependent since in contrast to ICR mice, curcumin at the dose of 30 mg/kg significantly attenuated pain behaviors in phase I in the α_7 WT (C57BL/6J strain) mice. Interestingly, the effects of curcumin in the phase I was not eliminated in the α_7 KO mice, suggesting the involvement of non- α_7-nACh receptor mechanisms in curcumin's effects.

Furthermore, our results show that tolerance did not develop following subchronic exposure to the antinociceptive and anti-inflammatory effects of curcumin. PAMs are compounds that facilitate endogenous neurotransmission and/or enhance the efficacy and potency of exogenous agonists, without directly stimulating the agonist binding sites. Supporting this

possibility, curcumin enhanced the effects of subactive dose of PNU282987, a full α_7-nACh receptor agonist, in the formalin test.

Our vivo data extend curcumin behavioral effects reported in several animal pain models, including acetic acid-induced visceral nociception (Tajik et al., 2008), formalin-induced orofacial pain (Mittal et al., 2009) and suggest a new mechanism for curcumin-induced antinociception. In addition, the results obtained from this study are in agreement with previous studies, which shows anti-inflammatory and antinociceptive actions of PAMs for α_7 nACh receptors in the tested mouse models (Freitas et al., 2013a; Bagdas et al., 2015, 2016). In conclusion, using both *in vitro* and *in vivo* approaches this study establishes that curcumin acts as a PAM of the α7 nACh receptors and provides evidence for a new mechanism for the analgesic-like properties of curcumin.

Acknowledgements

The authors gratefully acknowledge Dr. Jon Lindstrom (University of Pennsylvania, PA, USA) for providing cDNA clones of the human α_7-nACh receptor subunit, Dr. Isabel Bermudez-Diaz (Oxford Brookes University, Oxford, UK) for human α_2, α_3, α_4, β_2, and β_4 subunits.

Authorship Contributions:

Conducted experiments: E.G.E. Nebrisi; D. Bagdas; W. Toma; A. Brodzik; H. Al Samri; Y. Alkhalif.

Participated in research design: H.K.S. Yang; F. C. Howarth; M.I. Damaj; M. Oz.

Performed data analysis: E.G,E Nebrisi; D. Bagdas; M.I. Damaj; M. Oz.

Wrote or contributed to the writing of the manuscript: E.G,E Nebrisi; D. Bagdas; M.I. Damaj; M. Oz.

References

Abbott FV, Franklin KB, and Westbrook RF. (1995) The formalin test: scoring properties of the first and second phases of the pain response in rats. Pain 60: 91-102.

Albuquerque EX, Pereira EF, Alkondon M, and Rogers SW (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev 89: 73-120.

Anand P, Kunnumakkara AB, Newman RA, and Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. Mol Pharm 4: 807-818.

Bagdas D, Targowska-Duda KM, López JJ, Perez EG, Arias HR, and Damaj MI (2015) The Antinociceptive and Antiinflammatory Properties of 3-furan-2-yl-N-p-tolyl-acrylamide, a Positive Allosteric Modulator of α7 Nicotinic Acetylcholine Receptors in Mice. Anesth Analg 121: 1369-1377.

Bagdas D, Wilkerson JL, Kulkarni A, Toma W, AlSharari S, Gul Z, Lichtman AH, Papke RL, Thakur GA, and Damaj MI (2016) The α7 nicotinic receptor dual allosteric agonist and positive allosteric modulator GAT107 reverses nociception in mouse models of inflammatory and neuropathic pain. Br J Pharmacol 173: 2506-2520.

Bagdas D, Gurun MS, Flood P, Papke RL, Damaj MI (2017) New Insights on Neuronal Nicotinic Acetylcholine Receptors as Targets for Pain and Inflammation: A Focus on α7 nAChRs. Curr Neuropharmacol. doi: 10.2174/1570159X15666170818102108.

Banderali U, Belke D, Singh A, Jayanthan A, Giles WR, and Narendran A. (2011) Curcumin blocks Kv11.1 (erg) potassium current and slows proliferation in the infant acute monocytic leukemia cell line THP-1. Cell Physiol Biochem 28: 1169-1180.

Brauneis U, Oz M, Peoples RW, Weight FF, and Zhang L (1996) Differential sensitivity of recombinant N-methyl-D-aspartate receptor subunits to inhibition by dynorphin. J Pharmacol Exp Ther 279: 1063-1068.

Chatzidaki A, and Millar NS. (2015) Allosteric modulation of nicotinic acetylcholine receptors. Biochem Pharmacol. 97: 408-417.

Choi SW, Kim KS, Shin DH, Yoo HY, Choe H, Ko TH, Youm JB, Kim WK, Zhang YH, and Kim SJ (2013) Class 3 inhibition of hERG K+ channel by caffeic acid phenethyl ester (CAPE) and curcumin. Pflugers Arch. 465: 1121-1134.

Davidson EM, and Carlton SM (1998) Intraplantar injection of dextrorphan, ketamine or memantine attenuates formalin-induced behaviors. Brain Res 785: 136-142.

Dyer JL, Khan SZ, Bilmen JG, Hawtin SR, Wheatley M, Javed MU, and Michelangeli F (2002) Curcumin: a new cell-permeant inhibitor of the inositol 1,4,5-trisphosphate receptor. Cell Calcium 31: 45-52.

Enyeart JA, Liu H, and Enyeart JJ (2008) Curcumin inhibits bTREK-1 K+ channels and stimulates cortisol secretion from adrenocortical cells. Biochem Biophys Res Commun 370: 623-628.

Enyeart JA, Liu H, and Enyeart JJ (2009) Curcumin inhibits ACTH- and angiotensin II-stimulated cortisol secretion and Ca(v)3.2 current. J Nat Prod 72: 1533-1537.

Freitas K, Carroll FI, and Damaj MI (2013a) The antinociceptive effects of nicotinic receptors α7-positive allosteric modulators in murine acute and tonic pain models. J Pharmacol Exp Ther 344: 264-275.

Freitas K, Ghosh S, Carroll FI, Lichtman AH, and Damaj MI (2013b). Effects of α7 positive allosteric modulators in murine inflammatory and chronic neuropathic pain models. Neuropharmacology 65: 156-164.

Gee KW, Olincy A, Kanner R, Johnson L, Hogenkamp D, Harris J, Tran M, Edmonds SA, Sauer W, Yoshimura R, Johnstone T, and Freedman R (2017) First in human trial of a type I positive allosteric modulator of alpha7-nicotinic acetylcholine receptors: Pharmacokinetics, safety, and evidence for neurocognitive effect of AVL-3288. J Psychopharmacol 31: 434-441.

Goozee KG, Shah TM, Sohrabi HR, Rainey-Smith SR, Brown B, Verdile G, and Martins RN (2016) Examining the potential clinical value of curcumin in the prevention and diagnosis of Alzheimer's disease. Br J Nutr 115: 449-465.

Hartzell C, Putzier I, and Arreola J (2005) Calcium-activated chloride channels. Annu Rev Physiol 67: 719-758.

Hogg RC, Raggenbass M, and Bertrand D (2003). Nicotinic acetylcholine receptors: from structure to brain function. Rev Physiol Biochem Pharmacol 147: 1-46.

Hone AJ, and McIntosh JM. Nicotinic acetylcholine receptors in neuropathic and inflammatory pain. FEBS Lett. 2017 Oct 14. doi: 10.1002/1873-3468.12884.

Hong DH, Son YK, Choi IW, and Park WS (2013). The inhibitory effect of curcumin on voltage-dependent K^+ channels in rabbit coronary arterial smooth muscle cells. Biochem Biophys Res Commun. 430: 307-312.

Hu CW, Sheng Y, Zhang Q, Liu HB, Xie X, Ma WC, Huo R, and Dong DL (2012) Curcumin inhibits hERG potassium channels in vitro. Toxicol Lett 208: 192-196.

Hunskaar S, and Hole K (1987) The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30: 103-114.

Ibrahim A, El-Meligy A, Lungu G, Fetaih H, Dessouki A, Stoica G, and Barhoumi R (2011) Curcumin induces apoptosis in a murine mammary gland adenocarcinoma cell line through the mitochondrial pathway. Eur J Pharmacol 668: 127-132.

Ji HF, and Shen L (2014) The multiple pharmaceutical potential of curcumin in Parkinson's disease. CNS Neurol. Disord Drug Targets 13: 369-373.

Kunnumakkara AB, Bordoloi D, Padmavathi G, Monisha J, Roy NK, Prasad S, and Aggarwal BB (2017) Curcumin, the golden nutraceutical: multitargeting for multiple chronic diseases. Br J Pharmacol 174: 1325-1348.

Li Y, Revalde J, and Paxton JW (2016) The effects of dietary and herbal phytochemicals on drug transporters. Adv Drug Deliv Rev doi: 10.1016/j.addr.2016.09.004.

Lian YT, Yang XF, Wang ZH, Yang Y, Yang Y, Shu YW, Cheng LX, and Liu K (2013) Curcumin serves as a human kv1.3 blocker to inhibit effector memory T lymphocyte activities. Phytother Res 27: 1321-1327.

Liu H, Danthi SJ, and Enyeart JJ (2006) Curcumin potently blocks Kv1.4 potassium channels. Biochem Biophys Res Commun 344: 1161-1165.

Liu K, Gui B, Sun Y, Shi N, Gu Z, Zhang T, and Sun X (2013) Inhibition of L-type Ca(2+) channels by curcumin requires a novel protein kinase-theta isoform in rat hippocampal neurons. Cell Calcium 53: 195-203.

Liu S, Li Q, Zhang MT, Mao-Ying QL, Hu LY, Wu GC, Mi WL, and Wang YQ. (2016) Curcumin ameliorates neuropathic pain by down-regulating spinal IL-1β via suppressing astroglial NALP1 inflammasome and JAK2-STAT3 signalling. Sci Rep. 6:28956

Mahgoub M, Keun-Hang SY, Sydorenko V, Ashoor A, Kabbani N, Al Kury L, Sadek B, Howarth CF, Isaev D, Galadari S, and Oz M (2013) Effects of cannabidiol on the function of α7-nicotinic acetylcholine receptors. Eur J Pharmacol 720: 310-319.

Mahmmoud YA (2007) Modulation of protein kinase C by curcumin; inhibition and activation switched by calcium ions. Br J Pharmacol 150: 200-208.

Milani A, Basirnejad M, Shahbazi S, and Bolhassani A (2017) Carotenoids: biochemistry, pharmacology and treatment. Br J Pharmacol 174: 1290-1324.

Mittal N, Joshi R, Hota D, and Chakrabarti A (2009) Evaluation of antihyperalgesic effect of curcumin on formalin-induced orofacial pain in rat. Phytother Res 23: 507-512.

Morales I, Guzmán-Martínez L, Cerda-Troncoso C, Farías GA, and Maccioni RB (2014) Neuroinflammation in the pathogenesis of Alzheimer's disease. A rational framework for the search of novel therapeutic approaches. Front Cell Neurosci 8: 112.

Moustapha A, Pérétout PA, Rainey NE, Sureau F, Geze M, Petit JM, Dewailly E, Slomianny C, and Petit PX (2015) Curcumin induces crosstalk between autophagy and apoptosis mediated by calcium release from the endoplasmic reticulum, lysosomal destabilization and mitochondrial events. Cell Death Discov 1:15017. doi: 10.1038/cddiscovery.2015.17.

Munro G, Hansen R, Erichsen H, Timmermann D, Christensen J, and Hansen H (2012) The α7 nicotinic ACh receptor agonist compound B and positive allosteric modulator PNU-120596 both alleviate inflammatory hyperalgesia and cytokine release in the rat. Br J Pharmacol 167: 421-435

Onaran H O, and Costa T (2009) Allosteric coupling and conformational fluctuations in proteins. Curr Protein Pept Sci 10: 110-115.

Oz M, Melia MT, Soldatov N M, Abernethy D R, and Morad M (1998) Functional coupling of human L-type Ca2+ channels and angiotensin AT1A receptors coexpressed in xenopus laevis oocytes: involvement of the carboxyl-terminal Ca2+ sensors. Mol Pharmacol 54: 1106-1112.

Oz M, and Renaud LP (2002) Angiotensin AT(1)-receptors depolarize neonatal spinal motoneurons and other ventral horn neurons via two different conductances. J Neurophysiol 88: 2857-2863.

Oz M, Spivak CE, and Lupica CR (2004a) The solubilizing detergents, Tween 80 and Triton X-100 non-competitively inhibit alpha 7-nicotinic acetylcholine receptor function in Xenopus oocytes. J Neurosci Methods 137: 167-173.

Oz M, Zakharova I, Dinc M, and Shippenberg T (2004b) Cocaine inhibits cromakalim-activated K+ currents in follicle-enclosed Xenopus oocytes. Naunyn Schmiedebergs Arch Pharmacol 369: 252-259.

Pérez-Lara A, Corbalán-García S, and Gómez-Fernández JC (2011) Curcumin modulates PKCα activity by a membrane-dependent effect. Arch Biochem Biophys 513: 36-41.

Pubchem was accessed in May 2017. https://pubchem.ncbi.nlm.nih.gov/compound/curcumin#section=Top

Sands SB, Costa AC, and Patrick JW (1993) Barium permeability of neuronal nicotinic receptor alpha 7 expressed in Xenopus oocytes. Biophys J 65: 2614-2621.

Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, and Srinivas PS (1998) Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med 64: 353-356.

Spagnuolo C, Napolitano M, Tedesco I, Moccia S, Milito A, and Russo GL (2016) Neuroprotective Role of Natural Polyphenols. Curr Top Med Chem 16: 1943-1950.

Spivak CE, Lupica CR, and Oz M (2007) The endocannabinoid anandamide inhibits the function of alpha4beta2 nicotinic acetylcholine receptors. Mol Pharmacol 72: 1024-1032.

Tajik H, Tamaddonfard E, and Hamzeh- Gooshchi N (2008) The effect of curcumin (active substance of turmeric) on the acetic acid-induced visceral nociception in rats. Pak J Biol Sci 11: 312–314.

Takikawa M, Kurimoto Y, and Tsuda T (2013) Curcumin stimulates glucagon-like peptide-1 secretion in GLUTag cells via Ca2+/calmodulin-dependent kinase II activation. Biochem Biophys Res Commun 435: 165-70.

Thomsen MS, Hansen HH, Timmerman DB, and Mikkelsen JD (2010) Cognitive improvement by activation of alpha7 nicotinic acetylcholine receptors: from animal models to human pathophysiology. Curr Pharm Des 16: 323-343.

Umana IC, Daniele CA, and McGehee DS (2013) Neuronal nicotinic receptors as analgesic targets: it's a winding road. Biochem Pharmacol 86: 1208-1214.

Uteshev VV (2012) alpha7 nicotinic ACh receptors as a ligand-gated source of Ca(2+) ions: the search for a Ca(2+) optimum. Adv Exp Med Biol 740: 603-638.

Uteshev VV (2014) The therapeutic promise of positive allosteric modulation of nicotinic receptors. Eur J Pharmacol. 727:181-185.

Wang WH, Chiang IT, Ding K, Chung JG, Lin WJ, Lin SS, and Hwang JJ (2012) Curcumin-induced apoptosis in human hepatocellular carcinoma j5 cells: critical role of ca(+2)-dependent pathway. Evid Based Complement Alternat Med 512907. doi:

10.1155/2012/512907.

Yang Y, Wu X, Wei Z, Dou Y, Zhao D, Wang T, Bian D, Tong B, Xia Y, and Dai Y (2015) Oral curcumin has anti-arthritic efficacy through somatostatin generation via cAMP/PKA and Ca(2+)/CaMKII signaling pathways in the small intestine. Pharmacol Res 96: 71-81

Yeon KY, Kim SA, Kim YH, Lee MK, Ahn DK, Kim HJ, Kim JS, Jung SJ, and Oh SB (2010) Curcumin produces an antihyperalgesic effect via antagonism of TRPV1. J Dent Res 89: 170-174.

Zhang X, Chen Q, Wang Y, Peng W, and Cai H (2014) Effects of curcumin on ion channels and transporters. Front Physiol 5: 94 doi: 10.3389/fphys.2014.00094

Zhi L, Dong L, Kong D, Sun B, Sun Q, Grundy D, Zhang G, and Rong W (2013) Curcumin acts via transient receptor potential vanilloid-1 receptors to inhibit gut nociception and reverses visceral hyperalgesia. Neurogastroenterol Motil 25: e429-440.

Zhou H, Beevers CS, and Huang S (2011) The targets of curcumin. Curr Drug Targets 12: 332-347.

Footnotes:

1) Authors E.G.E. Nebrisi and D. Bagdas) contributed equally to the paper.

The research in this study was supported by grants from National Cancer Institute [CA206028] to MID and from CMHS, UAE University to MO.

Figure legends

Figure 1. Effects of curcumin on α_7-nicotinic acetylcholine receptor. **(A)** Records of currents activated by acetylcholine (ACh, 100 μM) in control conditions (*left*), after 10 min pretreatment with curcumin (1 μM) and co-application of 1 μM curcumin and ACh (*middle*), and 15 min washout (*right*). **(B)** Time-course of the effect of vehicle (0.1% DMSO; *open circles*) and curcumin (1 μM; *filled circles*) on the maximal amplitudes of the ACh-induced currents. Each data point represents the normalized mean ± S.E.M. of 6 to 8 experiments. The horizontal bar in the figure indicates the duration of curcumin or vehicle application. **(C)** Effect of curcumin as a function of curcumin pre-application time. Each data point represents the mean ± S.E.M. of 6 to 7 oocytes. **(D)** Curcumin potentiate α_7-nACh receptor function in a concentration-dependent manner. Each data point represents the mean ± S.E.M. of 6 to 9 oocytes. The curve is the best fit of the data to the logistic equation described in the methods section.

Figure 2. Effects of curcumin on α_7-nACh receptor are not mediated by G-proteins and protein kinases, and not dependent on intracellular Ca^{2+} levels and changes in membrane potential. **(A)** Bar presentation of the effects of 1 μM curcumin application (10 min) on the maximal amplitudes of ACh (100 μM) induced currents in oocytes injected with 50 nl distilled-water, controls (n=16) or *N*-Ethylmaleimide (NEM; 10 mM, 50 nl, n=8) and GDPβS (10 mM. 50 nl, n=7) 30 min before recordings. **(B)** Bar presentation of the effects of 1 μM curcumin application (10 min) on the maximal amplitudes of ACh (100 μM) induced currents in oocytes injected with 50 nl distilled-water, controls (n=5) or BAPTA (200 mM, 50 nl, n=7). **(C)** Bar presentation of the effects of 1 μM curcumin on α_7-nACh receptor-mediated currents in oocytes pretreated with vehicle (0.01 % DMSO, n=5) or PKC-412 (PKC, 10 μM for 30 min. pretreatment, n=7), or Go-6983 (GO, 10

μM, 30 min pretreatment, n=6). **(D)** Bar presentation of the effects of 1 μM curcumin on α_7-nACh receptor-mediated currents in oocytes pretreated with vehicle (0.01 % DMSO, n=7) or KT-5720 (KT, 10 μM, 30 min. pretreatment, n=7), and KN-62 (KN, 50 μM, 30 min. pretreatment, n=6). **(E)** Current-voltage relationships of acetylcholine-activated currents in the absence and presence of curcumin. Normalized currents activated by 30 μM ACh before (control, *filled circles*) and after 10 min treatment with 1 μM curcumin (*open circles*). Each data point presents the normalized means and S.E.M. of 7 experiments. **(F)** Quantitative presentation of the effect of curcumin as percent of controls at different voltages.

Figure 3. Effects of curcumin at different concentrations of acetylcholine. **(A)** Records of currents activated by acetylcholine (ACh, 10 μM) in control conditions (*left*), after 10 min pretreatment with curcumin (1 μM) and co-application of 1 μM curcumin and ACh (*middle*), and 10 min washout (*right*). **(B)** Concentration-dependent effect of curcumin on α_7-nACh receptors activated by low acetylcholine concentration. Each data point represents the mean ± S.E.M. of 6 to 8 oocytes. The curve is the best fit of the data to the logistic equation described in the methods section. **(C)** Bar presentation of the effect of curcumin at different acetylcholine concentrations. Bars represent the means ± S.E.M. of 5 to 8 experiments. **(D)** Effect of curcumin on the acetylcholine (ACh) concentration-response relationship. Oocytes were voltage-clamped at -70 mV and currents were activated by applying ACh (1 μM to 3 mM). Oocytes were exposed to 10 μM curcumin for 10 min and ACh was reapplied. Paired concentration-response curves were constructed and responses normalized to maximal response under control conditions. Data points obtained before (control) and after 10 min treatment with curcumin (10 μM) are indicated by *filled* and *open* circles, respectively. Each data point presents the normalized means and S.E.M. of 7 to

11 experiments.

Figure 4. Effect of curcumin on the desensitization of nicotinic receptors. **(A)** Normalized current traces in control (100 μM ACh) and in the presence 1 μM curcumin. **(B)** Bar presentation of the effect of curcumin (1 μM) on mean desensitization half-times of nicotinic receptors activated by 100 μM ACh. Bars represent the means ± S.E.M. of 8 experiments. **(C)** Effect of curcumin (10 μM) on α_7-nicotinic receptors desensitized by bath application of 100 μM nicotine (n=6).

Figure 5. Effects of curcumin on the specific binding of [^{125}I]α-bungarotoxin and on the currents mediated by different nicotinic receptor subunits. **(A)** The effects of curcumin on the specific binding of [^{125}I] α-bungarotoxin to oocyte membrane preparations. In the presence and absence of curcumin, specific binding as a function of the concentration of [^{125}I] α-bungarotoxin is presented. Data points for controls and curcumin (10 μM) are indicated by *filled* and *open circles,* respectively. Data points are the means of four independent experiments carried out in triplicate. **(B)** The effects of increasing concentrations of curcumin on the specific binding of [^{125}I] α-bungarotoxin. Each data point presents the normalized means and S.E.M. of 5 to 7 experiments. **(C)** Comparison of the effect of 10 μM curcumin on ACh (100 μM)-induced currents mediated by α_7, $\alpha_4\beta_2$, $\alpha_3\beta_4$, $\alpha_3\beta_2$, and $\alpha_4\beta_4$ subunit combinations of nicotinic receptors expressed in oocytes. Bars represent the mean inhibition ± S.E.M. from 6 to 8 experiments. **(D)** Comparison of the effects of 10 μM curcumin on 5-HT$_3$ receptors and $\alpha_1\beta_1$, $\alpha_1\beta_2$,and $\alpha_3\beta_1$ glycine receptor subunits expressed in oocytes. . Bars represent the mean effect ± S.E.M. from 5 to 7 experiments.

Figure 6. The antinociceptive effects of acute curcumin in the formalin test. **(A)** The effect after i.p. administration of curcumin (3, 10 and 30 mg/kg) on formalin-induced pain behavior in ICR mouse. Mice were treated with i.p. curcumin 45 min prior to formalin (2.5%, 20 μl) injection into

the plantar region of the right hind paw. The cumulative pain response of time of licking was measured during the period of 0-5 min (first phase) and 20-45 min (second phase). **(B)** Blockade of the antinociceptive effect of curcumin in the second phase of the formalin test by the α7 antagonist methyllycaconitine citrate (MLA). MLA (10 mg/kg, s.c.) was given 10 min before curcumin (30 mg/kg, i.p.) or vehicle (veh) in ICR mice. After 45 min, formalin test was performed. **(C)** Antinociceptive effects of curcumin (30 mg/kg, i.p.) in the formalin test in the α7 WT and KO mice on C57BL/67 background. Data are given as the mean ± S.E.M. of 6 animals for each group. * $P < 0.05$, significantly different from its vehicle group; $^{\#}$ $P < 0.05$, significantly different from its corresponding control group.

Figure 7. The antinociceptive effects of subchronic curcumin in the formalin test. **(A)** The effect of subchronic curcumin administration on formalin-induced pain behavior was measured. Mice were treated with curcumin (30 mg/kg, i.p.) or vehicle for 6 days once daily and were challenged with curcumin (30 mg/kg, i.p.) on day 7 and tested in formalin (2.5%) test. A vehicle control group, in which mice were exposed to 7 days of vehicle, was also included. **(B)** The anti-edema effect of formalin injection of curcumin, measured by the difference in the ipsilateral paw diameter before and after formalin injection (ΔPD), was assessed 1 hour after formalin injection. Data are given as the mean ± S.E.M. of 6 animals for each group. *$P < 0.05$, significantly different from its vehicle group.

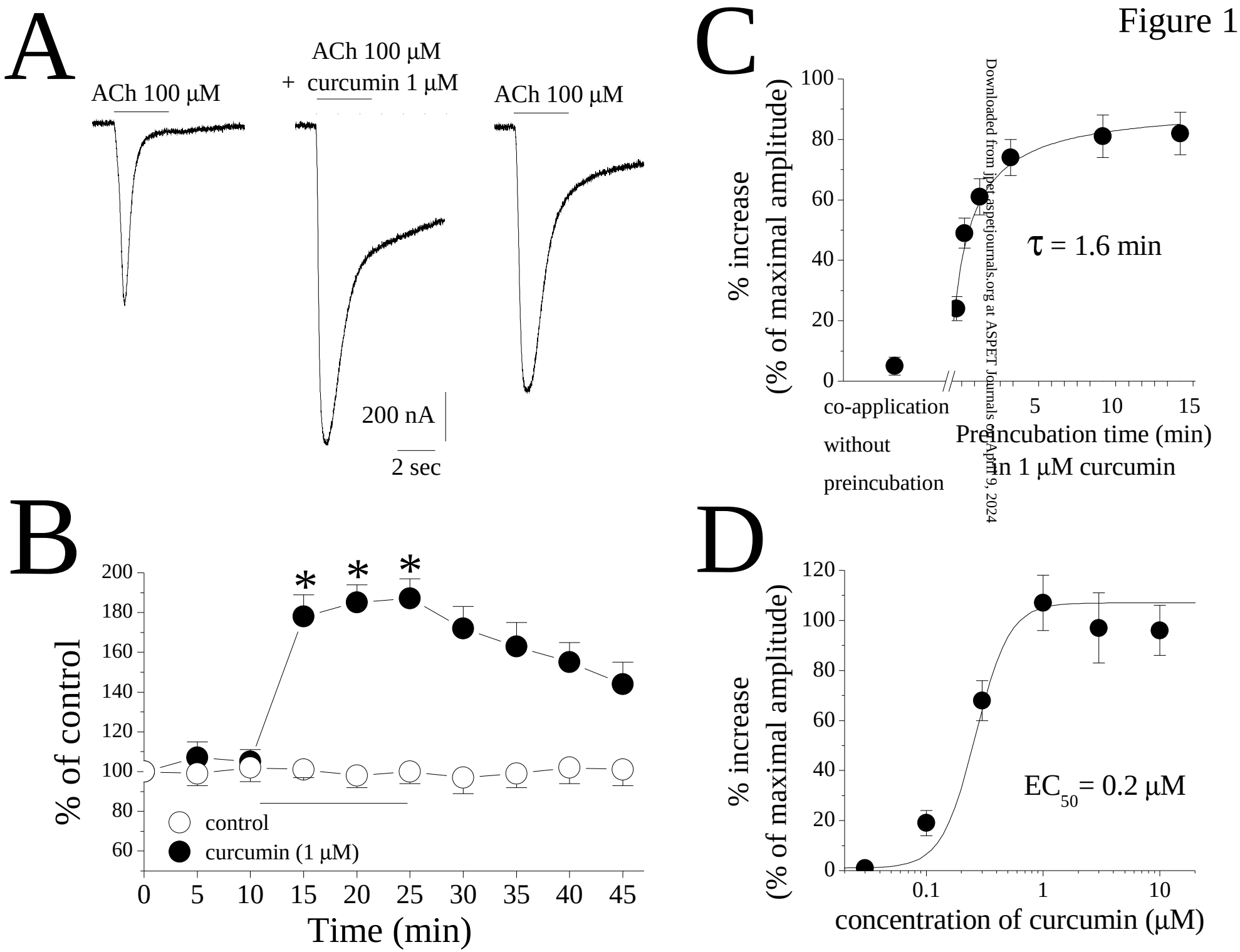
Figure 8. Antinociceptive effects of curcumin plus PNU282987 combination in the formalin test. Curcumin (3 mg/kg, i.p.) was injected 45 min before PNU282987 (0.1 mg/kg, s.c.) injection. The formalin test was conducted 10 min after PNU282987 administration in ICR mice. Data are given as the mean ± S.E.M. of 6 animals *$P < 0.05$, significantly different from vehicle group.

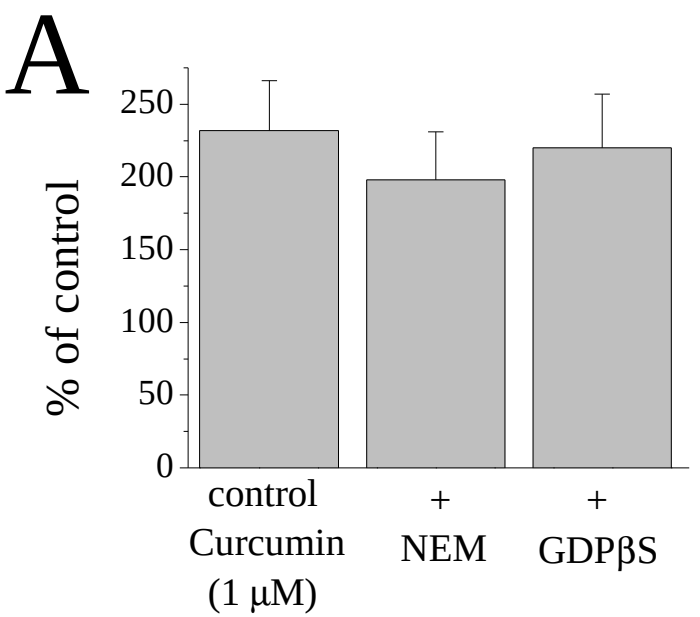
Figure 9. Effects of curcumin on acetic acid-induced writhing. ICR mice were treated with i.p. vehicle or curcumin (10 or 30 mg/kg) 45 min prior to i.p. acetic acid (1.2%) injection. Animals were observed for 40 minutes for the number of typical stretching behaviors. To test blockade of the antinociceptive effect of curcumin in the writhing test, the α7 antagonist methyllycaconitine citrate (MLA, 10 mg/kg, s.c.) was given 10 min before curcumin (30 mg/kg, i.p.) or vehicle. After 45 min, acetic acid writhing test was performed. Data are given as the mean ± S.E.M. of 8 animals for each group. *$P < 0.05$, significantly different from vehicle group; $^{\#}P < 0.05$, significantly different from its acetic acid control group; $^{\wedge}P < 0.05$, significantly different from curcumin (30 mg/kg) group.

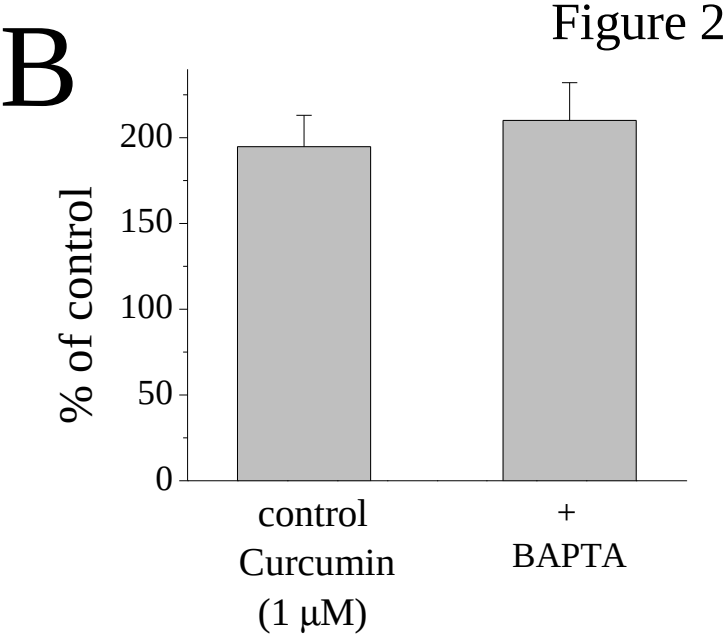
Table 1. Effects of curcumin on motor activity and coordination of mice. ICR mice were placed into photocell activity cages for 30 min or placed on the rotarod for 3 min after 45 min i.p. administration of curcumin (30 mg/kg). Data were presented as mean ± SEM as the number of photocell interruptions and time to fall in % impairment for each group respectively (5-6).

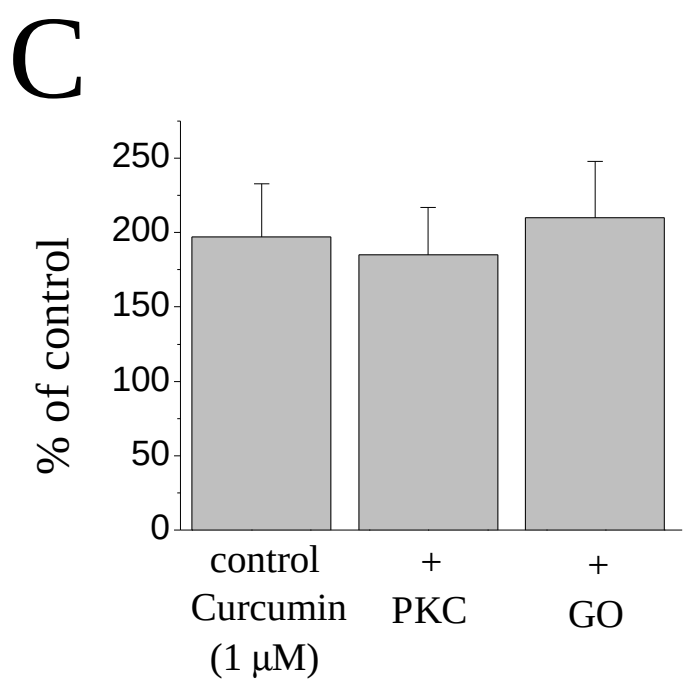
Treatment	**Spontaneous Activity (# Interrupts/30 min)**	**Rotarod Activity (% Impairment)**
Vehicle	1018 ± 146.7	17.3 ± 10.3
Curcumin	1125 ± 111.5	12.5 ± 12.5

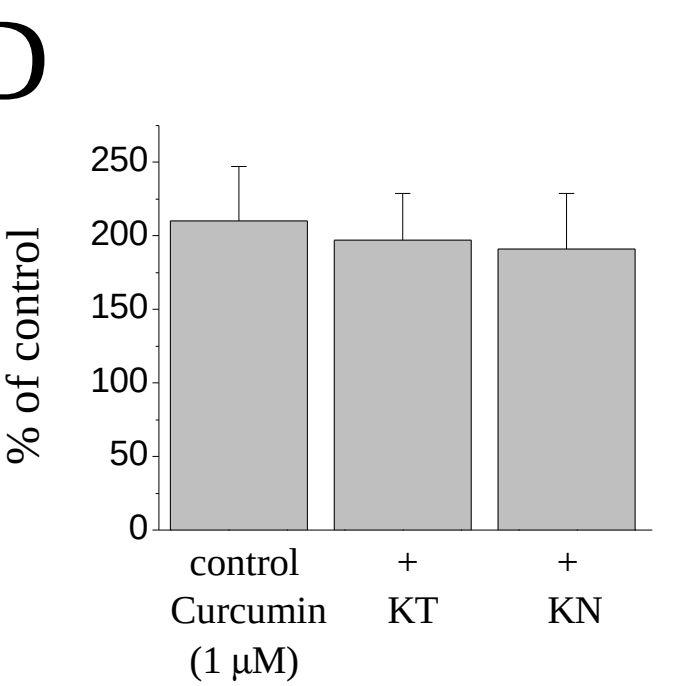
Figure 1

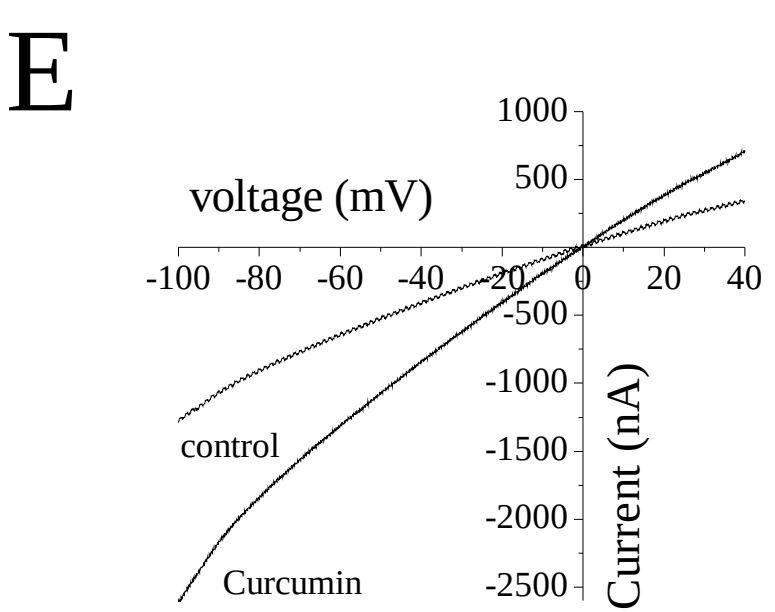



A
% of control
250
200
150
100
50
0
control
Curcumin
(1 μM)
+
NEM
+
GDPβS


B
% of control
200
150
100
50
0
control
Curcumin
(1 μM)
+
BAPTA


C
% of control
250
200
150
100
50
0
control
Curcumin
(1 μM)
+
PKC
+
GO


D
% of control
250
200
150
100
50
0
control
Curcumin
(1 μM)
+
KT
+
KN


E
voltage (mV)
1000
500
-100
-80
-60
-40
-20
0
20
40
-500
-1000
-1500
-2000
-2500
Current (nA)
control
Curcumin

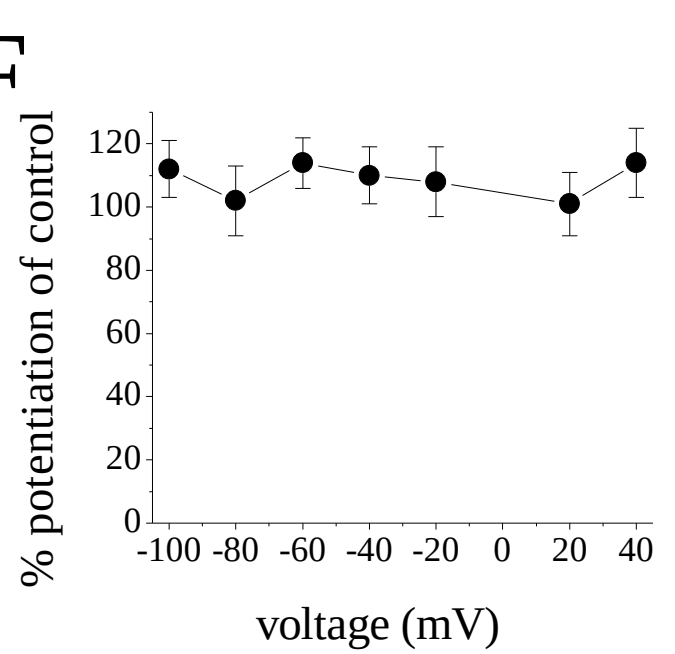
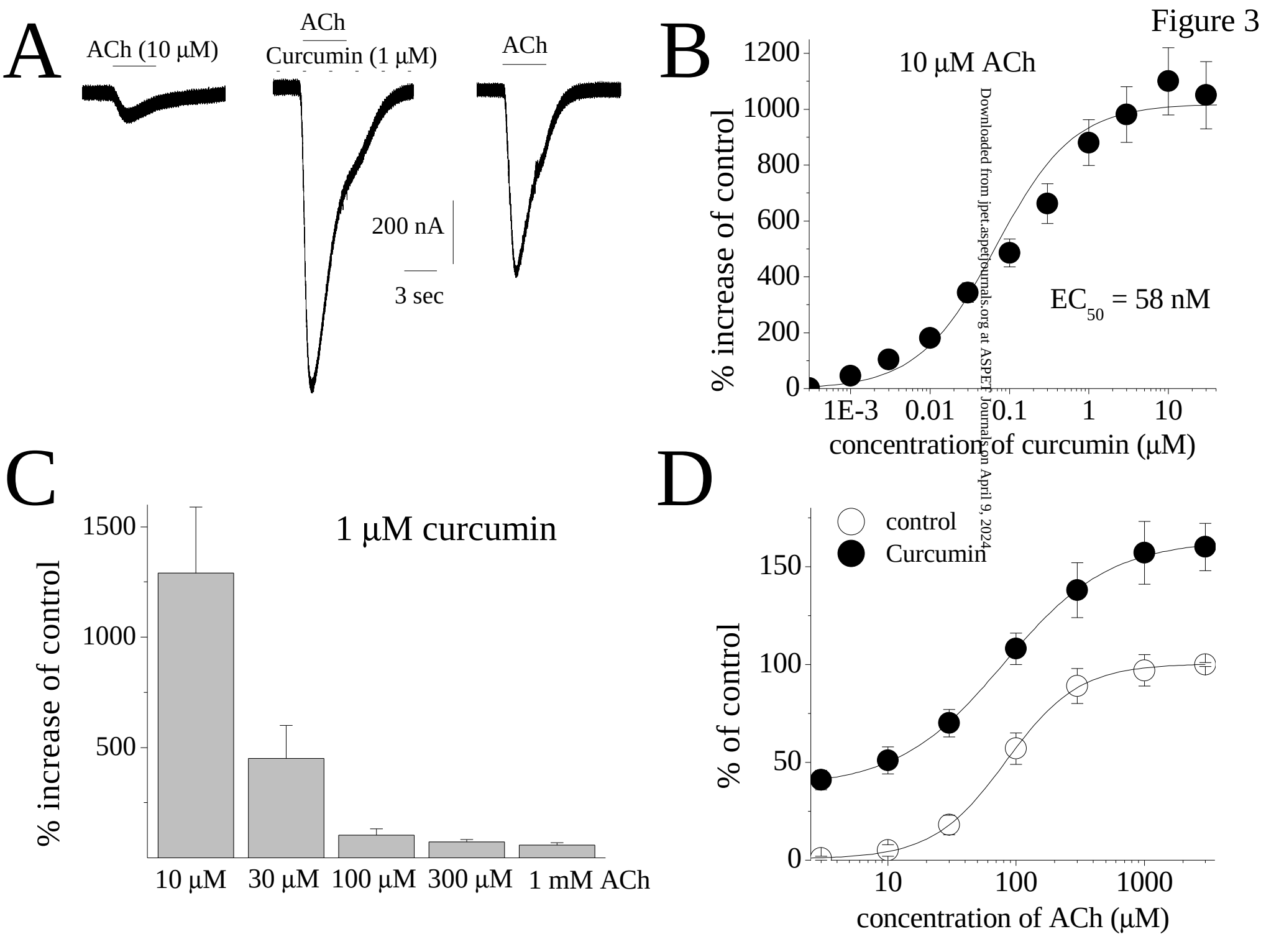

F
% potentiation of control
120
100
80
60
40
20
0
-100
-80
-60
-40
-20
0
20
40
voltage (mV)

Figure 3



JPET Fast Forward. Published on January 16, 2018 as DOI: 10.1124/jpet.117.245068
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4

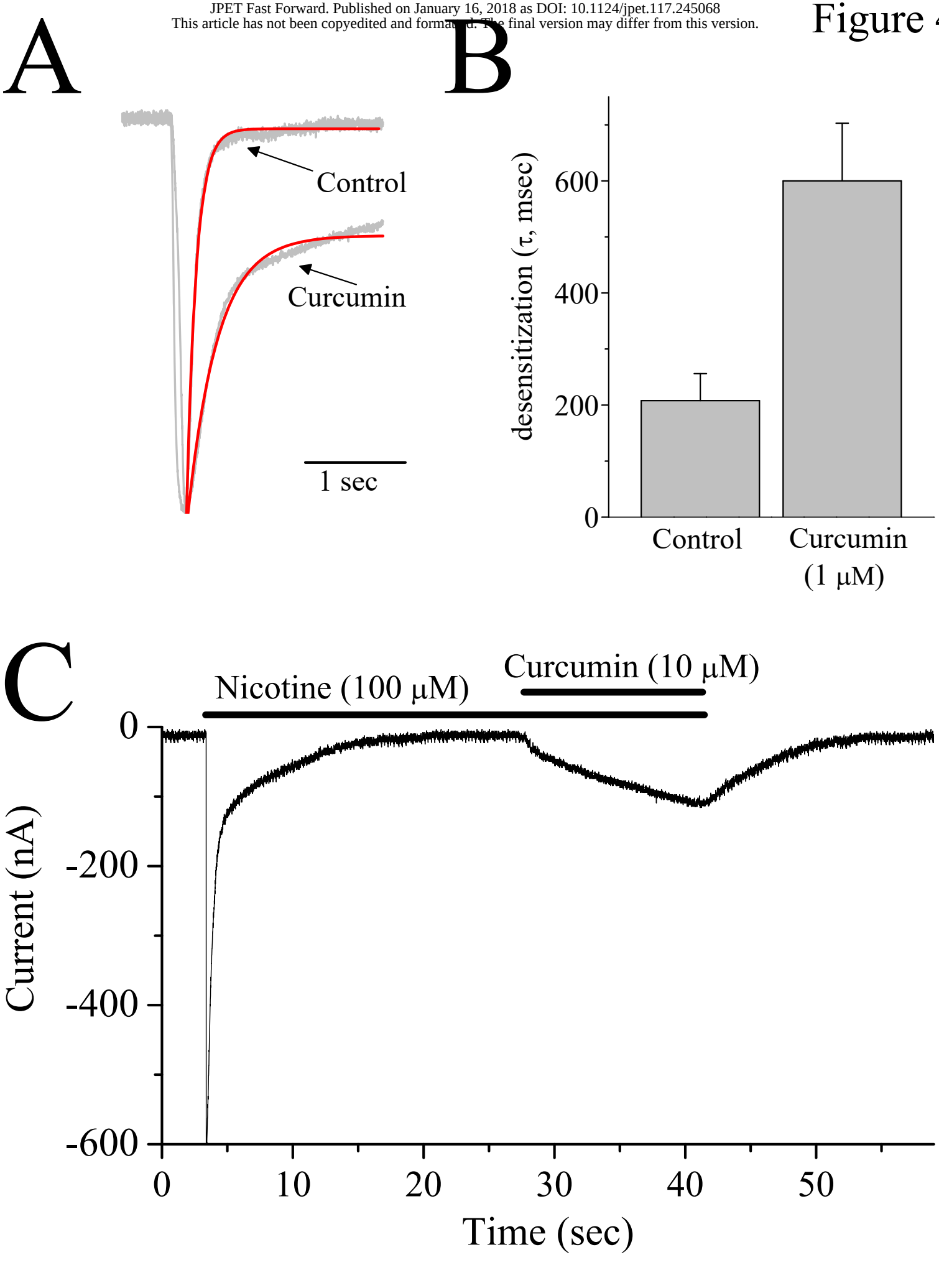
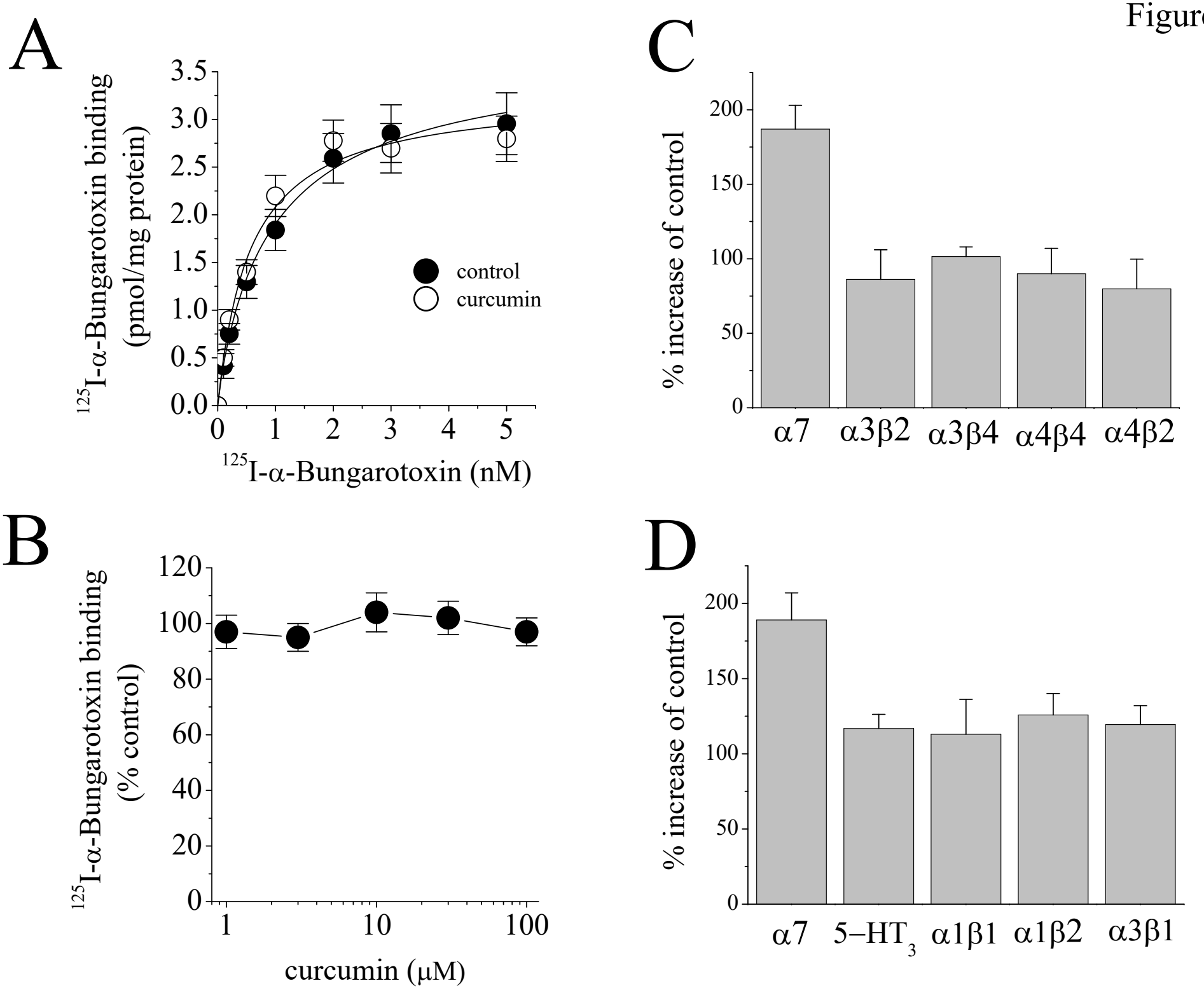


Figure 5



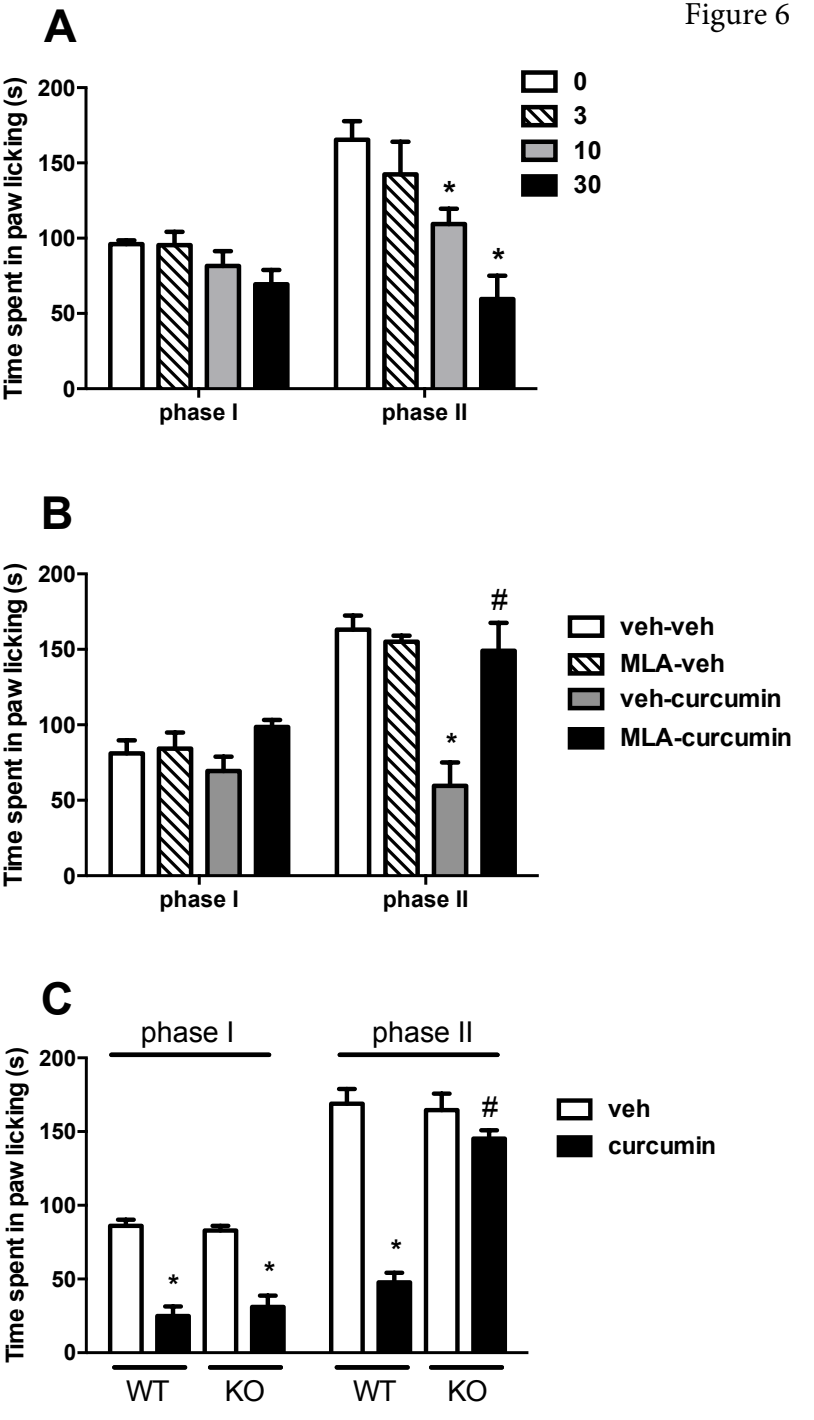

A
Time spent in paw licking (s)
200
150
100
50
0
0
3
10
30
*
*
phase I
phase II
B
Time spent in paw licking (s)
200
150
100
50
0
veh-veh
MLA-veh
veh-curcumin
MLA-curcumin
*
#
phase I
phase II
C
phase I
phase II
Time spent in paw licking (s)
200
150
100
50
0
veh
curcumin
*
*
*
#
WT
KO
WT
KO

Figure 7

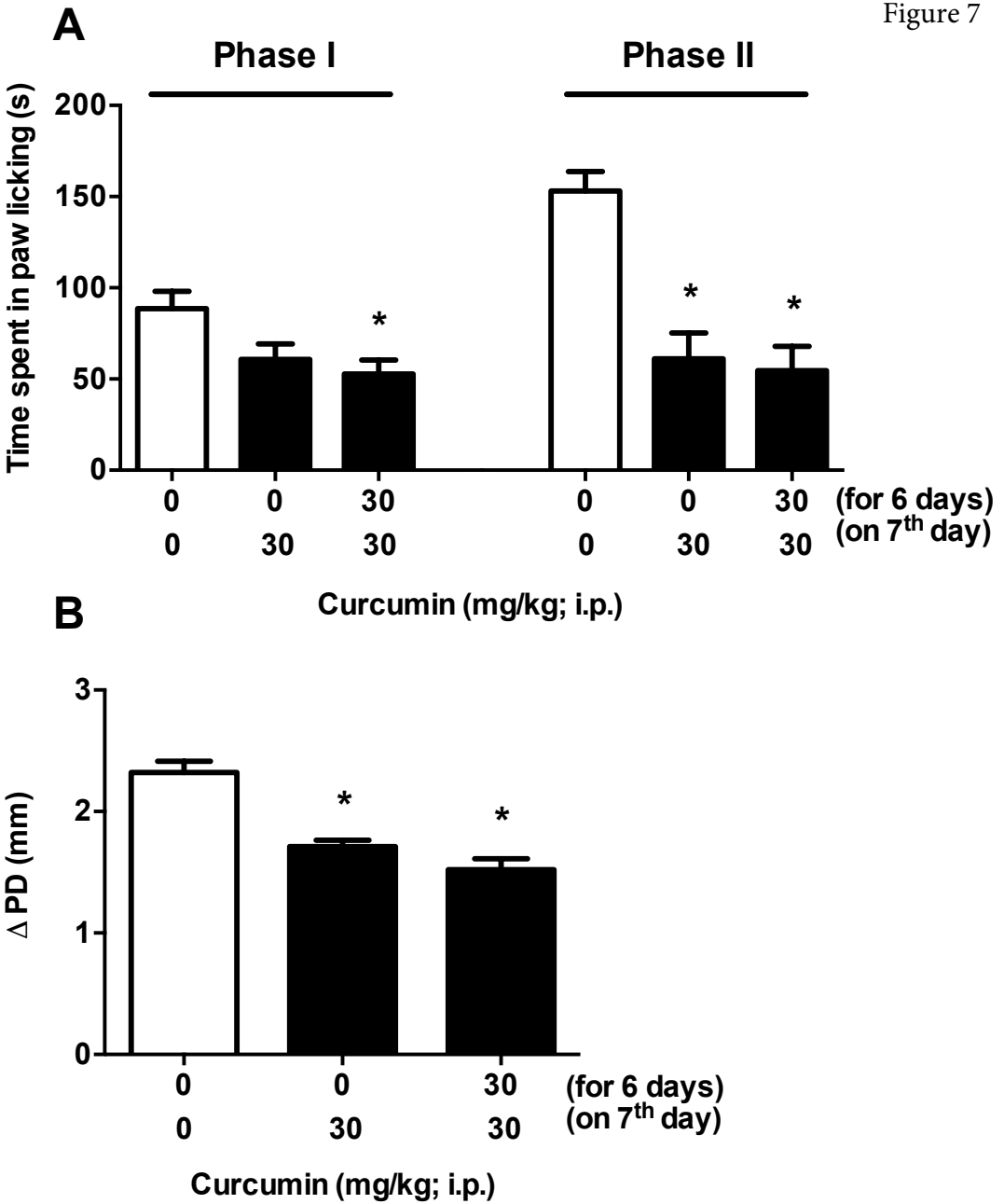

A
Phase I
Phase II
Time spent in paw licking (s)
200
150
100
50
0
*
*
*
0
0
30
0
0
30
(for 6 days)
0
30
30
0
30
30
(on 7th day)
Curcumin (mg/kg; i.p.)
B
ΔPD (mm)
3
2
1
0
*
*
0
0
30
(for 6 days)
0
30
30
(on 7th day)
Curcumin (mg/kg; i.p.)

Figure 8

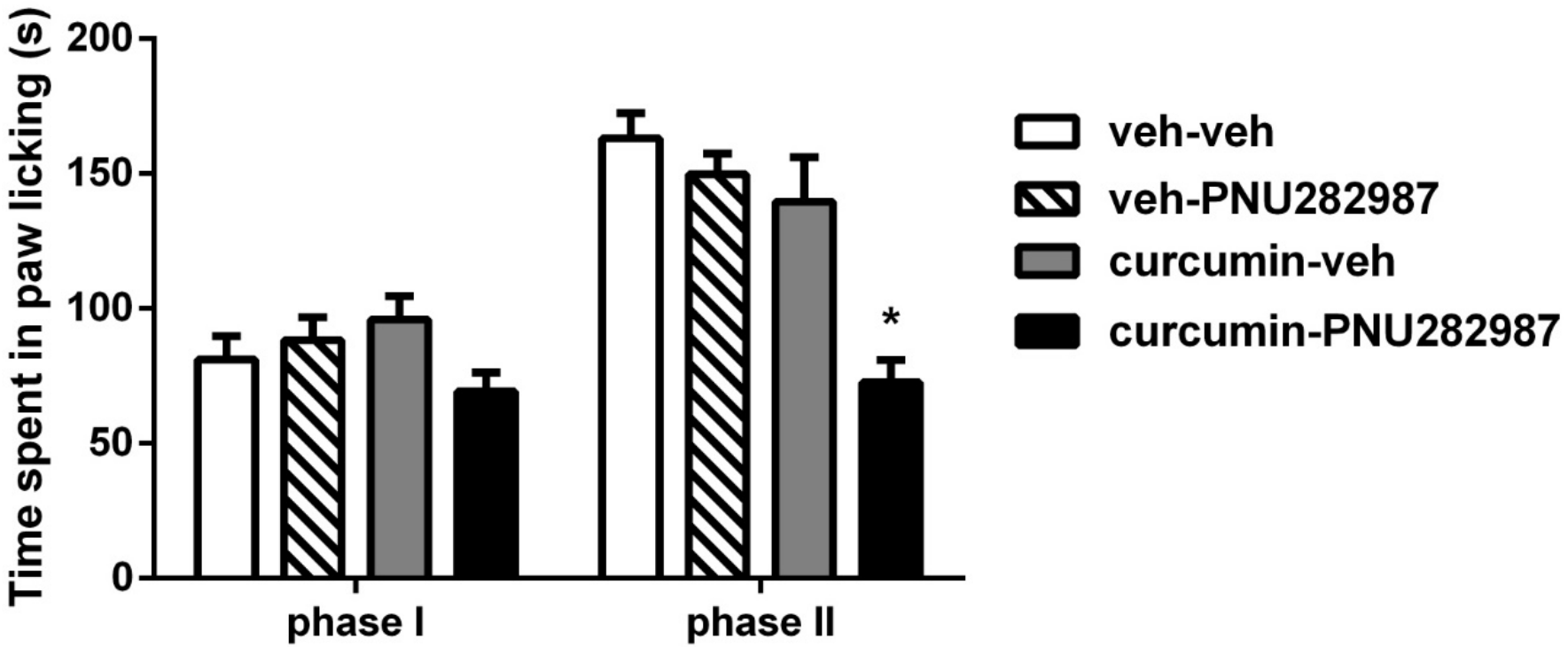


Figure 9