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CENTRAL SENSITIZATION OF NOCICEPTIVE NEURONS IN RAT MEDULLARY DORSAL HORN INVOLVES PURINERGIC P2X7 RECEPTORS

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Abstract—Central sensitization is a crucial process underlying the increased neuronal excitability of nociceptive pathways following peripheral tissue injury and inflammation. Our previous findings have suggested that extracellular adenosine 5'-triphosphate (ATP) molecules acting at purinergic receptors located on presynaptic terminals (e.g., P2X2/3, P2X3 subunits) and glial cells are involved in the glutamatergic-dependent central sensitization induced in medullary dorsal horn (MDH) nociceptive neurons by application to the tooth pulp of the inflammatory irritant mustard oil (MO). Since growing evidence indicates that activation of P2X7 receptors located on glia is involved in chronic inflammatory and neuropathic pain, the aim of the present study was to test *in vivo* for P2X7 receptor involvement in this acute inflammatory pain model. Experiments were carried out in anesthetized Sprague–Dawley male rats. Single unit recordings were made in MDH functionally identified nociceptive neurons for which mechanoreceptive field, mechanical activation threshold and responses to noxious stimuli were tested. We found that continuous intrathecal (i.t.) superfusion over MDH of the potent P2X7 receptor antagonists brilliant blue G and periodated oxidized ATP could each significantly attenuate the MO-induced MDH central sensitization. MDH central sensitization could also be produced by i.t. superfusion of ATP and even more effectively by the P2X7 receptor agonist benzoylbenzoyl ATP. Superfusion of the microglial blocker minocycline abolished the MO-induced MDH central sensitization, consistent with reports that dorsal horn P2X7 receptors are mostly expressed on microglia. In control experiments, superfusion over MDH of vehicle did not produce any significant changes. These novel findings suggest that activation of P2X7 receptors *in vivo* may be involved in the development of central sensitization in an acute inflammatory pain model.

Key words: ATP, benzoylbenzoyl ATP, brilliant blue G, minocycline, mustard oil, periodated oxidized ATP.

Central sensitization is a crucial process underlying the increased neuronal excitability of nociceptive pathways. It is reflected in increases in nociceptive neuronal spontaneous activity, mechanoreceptive field (RF) size and responses to noxious mechanical stimuli and a decrease in mechanical activation threshold following peripheral tissue injury and inflammation, and has been implicated in the development and maintenance of persistent pain (for review, see Dubner and Basbaum, 1994; Sessle, 2005; Woolf and Salter, 2006; Ren and Dubner, 2008; Chiang et al., 2011). We have developed an acute inflammatory pain model (Chiang et al., 1998) whereby a glutamatergic-dependent central sensitization (lasting for >40 min) can be induced in brainstem nociceptive neurons of trigeminal subnucleus caudalis (also termed the medullary dorsal horn, MDH) by application to a molar tooth pulp of the inflammatory irritant and small-fibre excitant mustard oil (MO), a transient receptor potential ankyrin 1 (TRPA1) agonist. In this acute pulpitis pain model, we have also recently provided evidence indicating that glial cells as well as extracellular adenosine 5'-triphosphate (ATP) molecules acting at several purinergic receptor subunits may be involved in MDH central sensitization (Chiang et al., 2005, 2007, 2008, 2010; Xie et al., 2007). Growing evidence indicates that extracellular ATP molecules and glia-neuron interactions are involved in mechanisms underlying the development of chronic inflammatory and neuropathic pain (for review, see Ji et al., 2009; McMahon and Malcongio, 2009; Milligan and Watkins, 2009; Chiang et al., 2011). There are seven P2X receptor subunits in the spinal cord (North, 2002; Burnstock, 2008) and numerous studies have shown the involvement in chronic inflammatory and neuropathic pain models of especially P2X7 receptors and P2X4 receptors which are primarily located in microglia (Chessell et al., 2005; Tsuda et al., 2009; for review, see Carroll et al., 2009; Inoue and Tsuda, 2009; Surprenant and North, 2009), but less clear is whether they are involved in acute inflammatory pain (Watkins et al., 2001; Hua et al., 2005; Ledeboer et al., 2005; Qin et al., 2006; Hughes et al., 2007).

In our previous studies using the acute pulpitis pain model, we have shown that the broad spectrum P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2,4-

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Abbreviations: ANOVA, analysis of variances; BBG, brilliant blue G; BzATP, 2′3′-o-(4-benzoylbenzoyl) adenosine 5′-triphosphate (ATP); DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; MDH, medullary dorsal horn; MO, mustard oil; NS, nociceptive-specific; oATP, periodate oxidized ATP; PBS, phosphate-buffered saline; PPADS, pyridoxal-phosphate-6-azophenyl-2,4-disulfonic acid; RF, mechanoreceptive field; RM ANOVA, repeated measures analysis of variances; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)-ATP; TRPA1, transient receptor potential ankyrin 1.

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disulfonic acid (PPADS), a non-selective antagonist of purinergic receptors including P2Y2 and P2Y4 receptor subunits and most P2X receptor subunits (Ralevic and Burnstock, 1998), can completely block the MO-induced central sensitization in MDH. In contrast, the high affinity, selective P2X receptor antagonist 2′,3′-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP), which antagonizes P2X1, P2X3 and heteromeric P2X2/3 subunits with IC_{50} values of 6, 0.9 and 7 nM respectively and has a 1000-fold selectivity over P2X2, P2X4 and P2X7 subunits (Lewis et al., 1998; Virginio et al., 1998; North and Surprenant, 2000), only partially blocks the MDH central sensitization (Chiang et al., 2005). Furthermore, our recent studies have revealed that suramin alone only attenuates the MO-induced MDH central sensitization, whereas its co-application with the P2X7 receptor antagonist brilliant blue G (BBG) can completely block the central sensitization (Chiang et al., 2009). Suramin is a non-selective P2 purinergic receptor antagonist of all P2X receptor subunits as well as most P2Y receptor subunits, and also blocks calmodulin binding to recognition sites and G protein coupling to G protein-coupled receptors, but is less potent at P2X4 and P2X7 receptor subunits (Beindl et al., 1996; Klinger et al., 2001; Soto et al., 1996; for review, see North and Surprenant, 2000; von Kügelgen, 2006; Wu et al., 2004); and BBG is a potent P2X7 receptor antagonist (Chu et al., 2010; Fulgenzi et al., 2008; Gunosewoyo et al., 2007; Jarvis and Khakh, 2009). These findings raise the possibility that P2X7 receptors may also be involved in central sensitization in acute inflammatory pain states, and thus this electrophysiological study was designed to test *in vivo* for the P2X7 receptor involvement in this acute inflammatory pain model.

Data have been partly reported in abstract form (Li et al., 2008; Chiang et al., 2009, 2010; Itoh et al., 2009).

EXPERIMENTAL PROCEDURES

Detailed descriptions of most of the methods have been previously reported (Chiang et al., 1998, 2007; Xie et al., 2007), so the following focuses on methodologies that we have not previously described.

Animals

Male adult rats (275–420 g) were anesthetized by i.p. α-chloralose (50 mg/kg; Fisher Scientific Co., Toronto, ON, Canada)/urethane (1 g/kg; Sigma-Aldrich, Toronto, ON, Canada). The right maxillary first molar pulp was exposed and covered with a saline-soaked cotton pellet, and the dorsal surface of the caudal medulla was surgically exposed. The rat then received a continuous i.v. infusion of a mixture of 70% urethane solution (0.2 g/ml) and 30% pancuronium solution (2 mg/ml) at a rate of 0.3–0.4 ml/h and was artificially ventilated throughout the whole experimental period. Heart rate, percentage expired CO_2, and rectal temperature were continuously monitored and maintained at physiological levels of 333–430 beats/min, 3.5–4.5%, and 37–37.5 °C, respectively. All surgeries and procedures were approved by the University of Toronto Animal Care Committee in accordance with the regulations of the Ontario Animal Research Act (Canada).

Electrophysiological recordings and stimulation procedures

The activity of single neurons was recorded by a tungsten microelectrode (5–15 MΩ) in histologically verified sites in MDH (Lateral: 1.5–2.0 mm; Posterior: 1.5–2.0 mm referred to the obex). Responses to stimulation of the orofacial region were amplified and displayed on oscilloscopes and also led to an analogue-to-digital converter (CED 1401 plus; Cambridge Electronic Design, Cambridge, Cambridgeshire, UK) connected to a personal computer. Data were analyzed off-line with Spike 2 software (Cambridge Electronic Design, Cambridge, Cambridgeshire, UK).

Mechanical (brush, pressure and pinch) and noxious thermal (radiant heat, 51–53 °C) stimuli were applied to classify nociceptive-specific (NS) neurons in the deep laminae of MDH that specifically responded to strong mechanical (e.g., pressure or pinch) and/or thermal (radiant heat) stimuli but not to a brush stimulus applied to the neuronal RF (Chiang et al., 1998, 2005, 2007; Xie et al., 2007). Neurons classified as wide dynamic range or low threshold mechanoreceptive were not included in this study. The neuron's spontaneous activity was determined over an initial 1-min recording period, and its cutaneous orofacial RF was determined with non-serrated forceps. Its activation threshold to a mechanical stimulus applied to its RF was assessed by force-monitoring forceps or an electronic von Frey monofilament, and its responses to graded heavy pressure or pinch were determined (25 g, 50 g, 75 g, 100 g, and sometimes 200 g, applied in ascending order, each for 5 s at an interval of $>$45 s). The pressure- or pinch-evoked responses were assessed by summing the number of spikes evoked by each of these graded stimuli.

Superfusion of chemicals

Adenosine 5′-triphosphate (ATP, 30–300 μM), 2′3′-o-(4-benzoylbenzoyl) adenosine 5′-triphosphate (BzATP, 30–300 μM), brilliant blue G (BBG, 1 μM), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 20 μM), [4*S*-(4α,4aα,5aα,12aα)]-4,7-*Bis*(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,10,12,12a, tetrahydroxy-1,11-dioxo-2-naphthacenecarboxamide (minocycline, 500 μM), periodate oxidized ATP (oATP, 100 μM), and 8,8′-{Carbonyl*bis*[imino-3,1-phenylene-carbonylimino(4-methyl-3,1-phenylene)carbonylimino]}bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt (suramin, 50 μM) were purchased from Sigma-Aldrich Canada Ltd. All chemicals except DPCPX were freshly dissolved in phosphate-buffered saline (PBS, at pH 7.4; Sigma-Aldrich Ltd., Toronto, ON, Canada) before i.t. superfusion. DPCPX was first dissolved in DMSO at 1: 10 as stock solution, and then diluted with PBS to a 20 μM solution for use. All chemicals were taken from stock solutions and freshly dissolved in PBS (vehicle) and continuously superfused onto MDH by means of a micropump (Harvard apparatus Inc, South Natick, MA, USA) at a speed of 0.6 ml/h. The dose of each chemical was chosen based on available related literature and also on our preliminary experiments, in which this dose showed no significant disturbances on baseline values of the neuronal RF and response properties.

Experimental paradigm

To test the effect of P2X7 receptor antagonists, three experimental groups were tested with MO application to the tooth pulp: BBG group, oATP group, and PBS group (as control); each group was comprised of six rats. In all experiments, continuous i.t. superfusion over the exposed ipsilateral medulla of either oATP or BBG or PBS started soon after surgery and was maintained throughout the observation period. Following isolation and identification of a stable NS neuron in MDH 2 h after surgery, two assessments of neuronal properties were carried out for the identified NS neuron at an interval of 10 min and were taken as baseline measures. Thereafter, the saline-soaked cotton pellet which covered the

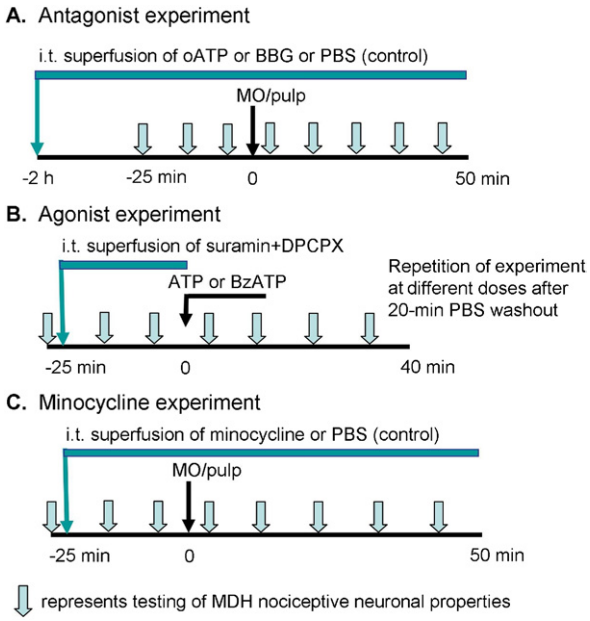

Fig. 1. (A–C) Diagram of experimental paradigms. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

exposed pulp was quickly replaced by a segment of a dental absorbent point (Lux & Zwingenberger Ltd., North York, ON, Canada) soaked with MO (0.2 μl, allyl isothiocynate, 95%, Aldrich Chemical Co., Milwaukee, WI, USA). The exposed pulp was then promptly sealed with CAVIT (3M ESPE AG, St. Paul, MN, USA). Then, 3 min after MO application, neuronal properties were repeatedly assessed at 10-min intervals over the next 50-min (Fig. 1A). A control experiment with vehicle (mineral oil) application to the tooth pulp was intentionally omitted, because mineral oil application does not affect nociceptive neuronal properties in MDH, trigeminal subnucleus oralis or the thalamic ventroposterior-medial nucleus, as reported in our previous studies (Chiang et al., 1998; Park et al., 2001; Zhang et al., 2006).

Another two additional groups were used to test whether activation of P2X7 receptors alone can produce neuronal central sensitization-like responses: the potent P2X7 receptor agonist BzATP or general P2X receptor agonist ATP at different doses was superfused i.t. in an additional 16 rats. In our preliminary experiments (n=4), local application of high doses (500 μM–1 mM) of BzATP produced an initial excitation of NS neurons followed by long-term depression after pretreatment for 25 min with suramin (50 μM; a non-selective P2 receptor antagonist that blocks all P2X receptor subunits as well as most P2Y receptor subunits); higher doses produced earlier onset and longer duration of the depression. The depression observed in these experiments was probably due to release of adenosine because BzATP is readily degraded by ecto-nucleotidase which is ubiquitous in the central nervous system and the end-product adenosine is a potent inhibitor of synaptic transmission (Horiuchi et al., 2010; for review, see Sawynok, 2007). Therefore, after the baseline values of neuronal properties had been assessed, the 25-min i.t. superfusion at 0.6 ml/h of suramin (50 μM) pretreatment was combined with DPCPX (20 μM), an adenosine A1 receptor antagonist, to prevent adenosine producing potent inhibition of synaptic transmission. During this pretreatment period, two other assessments of neuronal properties were performed at 10-min intervals. Then, the BzATP or ATP at a dose of 30, 100, or 300 μM was continuously applied i.t. at 0.6 ml/h for a period of 15 min (n=5–6). Five minutes after starting the ATP or BzATP application, neuronal properties were repeatedly assessed at 10-min intervals over the next 40-min period (Fig. 1B). In each experiment, two or three different concentrations of ATP or BzATP were applied following a 20-min period of PBS washout.

Since P2X7 subunits are especially prominent on dorsal horn microglia (Yu et al., 2008; Chu et al., 2010), a sixth group of rats was used to test whether the microglial blocker minocycline can affect the MO-induced MDH central sensitization (n=6). The same paradigm as used in the BBG/oATP experiments was used, except that the minocycline pretreatment (starting after the baseline values being obtained) started 25 min prior to MO application to the tooth pulp (Fig. 1C).

Histological and statistical analyses

Recording sites were marked electrolytically by an anodal current of 6 μA for 15 s, and verified histologically in Haematoxylin-Eosin stained sections. Statistical analyses were based on normalized data (percentage). Differences between baseline values and values at different time points after MO, minocycline, ATP, BzATP or PBS application for each of the groups were treated by 1-way repeated measures analysis of variances (RM ANOVA) or ANOVA on ranks, followed by Dunnett's test. Because the baseline values of neuronal properties were determined after 2 h superfusion of BBG and oATP in the BBG and oATP experiments (see above), it was impractical to directly test the effects of these chemicals on neuronal properties. Instead, comparison between the (baseline) values of neuronal properties 2 h after BBG or oATP superfusion and those of 2 h after PBS superfusion (as control) was made with Student t-test (see Results). Differences between any two groups were treated by 2-way ANOVA followed by Dunnett's test. The Student t-test and the non-parametric Fisher Exact test were also used in specifically assigned data. The level of significance was set at $P<0.05$. All values are presented as mean±SEM.

RESULTS

A total of 40 functionally identified NS neurons was tested in the five groups. The recording sites of all NS neurons were histologically verified and were located in the deep laminae of MDH (see Fig. D in Figs. 2 and 5). Only two of the 40 NS neurons had baseline activity. After MO application to the pulp, no spontaneous or evoked activity appeared in any neurons in the chemical treatment groups, except for three of the neurons in the PBS control group (n=6) that were excited (for 3–5 min) following MO application.

PBS superfusion did not affect the MO-induced central sensitization

Baseline values of neuronal RF size, activation threshold and responses to mechanical stimuli in the PBS group (n=6) were 2.0±0.6 cm^2, 99±39 g and 74±17 spikes, respectively; baseline values in the other groups were similar and not significantly different from these values in the PBS groups ($P>0.1$–0.7, t-test, n=6; Table 1). Central sensitization was readily induced by MO application to the pulp during PBS (vehicle control) superfusion over MDH: MO application to the pulp significantly increased the RF size, decreased the mechanical activation threshold and increased the pressure- or pinch-evoked responses for 40 min or more ($P<0.05$–0.001, 1-way RM ANOVA; Fig. 2A–C); in addition, a transient novel tactile RF appeared in three of

Table 1. Comparison of baseline values of NS neurons between PBS group and other groups

Group	# of rats	Pinch RF size (cm^2)	Mechanical activation threshold (g)	Responses to mechanical stimulus (# of spikes)	*P*-values (RF; Thr; Resp)
PBS	6	2.0±0.6	99±39	74±17	
oATP	6	2.0±0.2	80±11	93±19	>0.9; >0.6; >0.2
BBG	6	2.4±0.5	59±10	113±32	>0.5; >0.3; >0.1
Suramin+DPCPX	16	2.7±0.3	110±21	80±15	>0.2; >0.5; >0.3
Minocycline	6	2.4±0.4	64±26	109±31	>0.5; >0.4; >0.1

Differences in baseline values between PBS group and other groups were treated by Student's *t*-test.

the six neurons following MO application in this group (data not shown). The pinch RF size was significantly different from the baseline value at all post-MO time-points as was the activation threshold at 8, 18 and 28 min and the responses to the mechanical stimuli at 8, and 18 min after MO application ($P<0.05$, Dunnett's test).

BBG and oATP superfusion attenuated the MO-induced central sensitization

The nociceptive neuronal properties (pinch RF size, activation threshold, and responses to mechanical stimuli) after continuous i.t. superfusion of BBG (1 μM) and oATP (100 μM) over MDH for 2 h were comparable to the base-

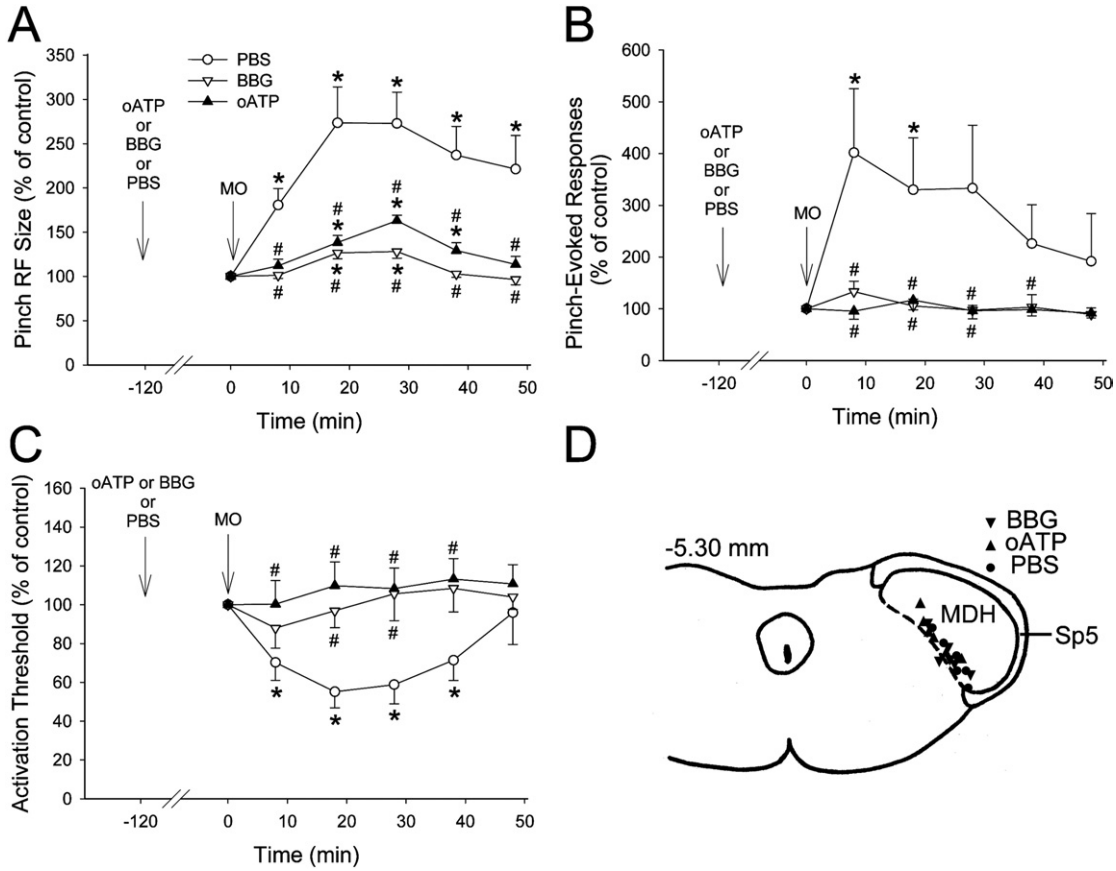

Fig. 2. Effects of continuous i.t. superfusion of oATP, BBG and PBS on the neuroplastic changes in MDH induced by MO application to the tooth pulp. (A) Changes in NS neuronal RF size. Note that during PBS superfusion, MO application produced significant increases in pinch RF size throughout the 50-min observation period ($P<0.001$, RM ANOVA; * $P<0.05$, Dunnett's test; $n=6$), and that superfusion of oATP and BBG only partially but still significantly attenuated the MO-induced increases in neuronal RF size ($P<0.001$, RM ANOVA, both $n=6$). (B) Changes in neuronal responses to graded mechanical stimuli. (C) Changes in mechanical activation threshold of NS neurons. Note that during PBS superfusion, MO application produced significant increases in responses and decreases in threshold ($P<0.001$ and $P<0.05$, respectively, RM ANOVA; * $P<0.05$, Dunnett's test), and that superfusion of oATP and BBG significantly blocked the MO-induced increases in responses and decrease in threshold (both $P>0.5$, RM ANOVA). All these MO-induced neuroplastic changes in the oATP and BBG groups were significantly less than those in the PBS group (all $P<0.001$, 2-way ANOVA). Post hoc analysis indicated that there were significant differences in values at most post-MO time-points between the PBS and these two groups ($^{\#}$ $P<0.05$, Dunnett test). (D) The recording sites of the NS neurons in PBS, oATP and BBG groups were histologically retrieved in the deep laminae of MDH.

line values of the PBS group ($P>0.1–0.9$, t-test, $n=6$; Table 1). In contrast, BBG and oATP superfusion blocked the MO-induced neuronal changes in mechanical activation threshold and responses to pinch stimuli ($P>0.5–0.7$ in BBG group and $P>0.3–0.4$ in oATP group) and attenuated the magnitude of the MO-induced increases in neuronal RF size although the RF size was still significantly increased compared to baseline ($P<0.001$ in both groups; 1-way RM ANOVA, $n=6$) (Fig. 2A–C). Differences in the MO-induced effects on all three parameters between the PBS group and BBG or oATP groups were significant (all $P<0.001$, 2-way ANOVA). Post hoc analysis indi-

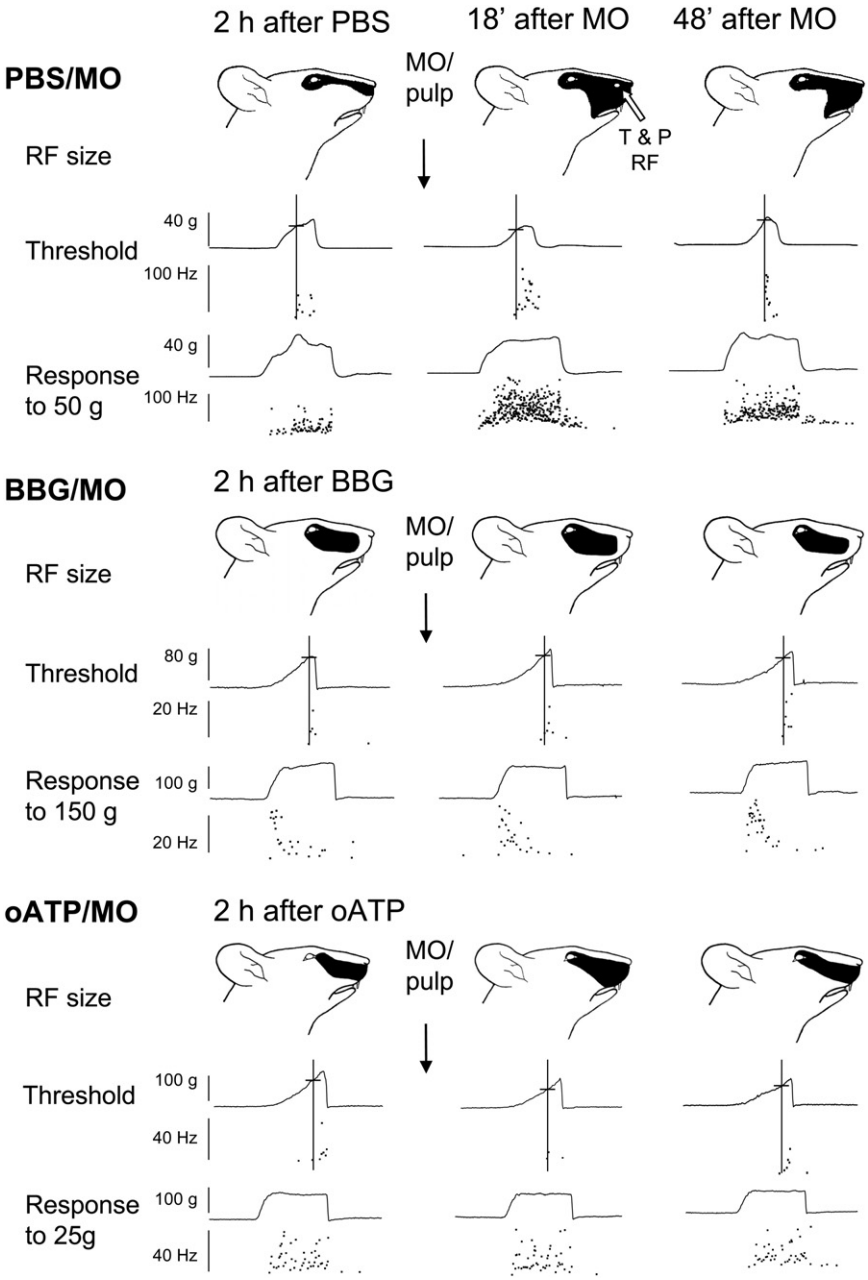

Fig. 3. Examples showing MO-induced neuroplastic changes (central sensitization) in NS neurons after continuous i.t. superfusion of PBS, BBG and oATP over MDH. For each example, the pinch RF (upper panel), activation threshold (middle panel) and responses to pinch/pressure stimuli (lower panel) are shown. Data at 2 h after PBS or BBG or oATP superfusion (baseline, i.e., prior to MO application), 18 min and 48 min after MO application of each NS neuron are arranged in columns from left to right. Note that the NS neurons illustrated from the PBS group showed MO-induced neuroplastic changes, including an appearance of a novel tactile RF at 18 min after MO application, while no marked changes occurred following MO application in the NS neuron of the BBG and oATP group. The neuronal discharges are displayed in an inter-spike instantaneous frequency distribution. The activation threshold of a given neuron is marked by a short horizontal bar at the intersection of the cut-off (vertical) line and the ascending force curve. The unit responses to pinch/pressure stimuli (1–2 times threshold intensity) are also shown in the instantaneous frequency distribution during the 5-s stimulation period. Black area indicates the pinch/pressure RF. T & P RF refers to that part of the RF from which the neuron could be activated by tactile as well as pinch/pressure stimulation.

cated that there were significant differences in values at most post-MO time-points between these two groups (see Fig. 2A–C). Examples of effects of BBG and oATP on MO-induced changes in neuronal properties are shown in Fig. 3.

BzATP and ATP superfusion produced central sensitization-like effects

The baseline values of neuronal properties remained stable during the 25-min pretreatment with suramin+DPCPX ($P>0.3$–0.9, paired *t*-test, $n=16$; Table 2). The i.t. superfusion of BzATP (300 μM) for 15 min produced significant increases in NS neuronal RF size and responses to mechanical stimuli and decreases in activation threshold (all $P<0.01$–0.001, RM ANOVA, $n=6$; Fig. 4A1–3). These BzATP-produced changes in neuronal properties were dose-dependent (Fig. 4A1–3). Similar i.t. superfusion of ATP (300 μM) for 15 min also produced a significant increase in pinch RF size and responses to pinch stimuli (both $P<0.05$, $n=5$; Fig. 4B2, B4, respectively) and also a decrease in activation threshold (Fig. 4B3). The effects of ATP were also dose-dependent but much weaker than those of BzATP (Fig. 4B2–4).

Other notable differences between ATP and BzATP effects were that the novel tactile RF appeared in all five NS neurons tested with 100 μM ATP but not in all six NS neurons tested with 100 μM BzATP (Fig. 4B1), significant pinch RF size increases occurred only at 300 μM of ATP whereas BzATP produced significant increases from 30 to 300 μM (Fig. 4B2), significant increase in pinch-evoked responses to ATP occurred only at 300 μM whereas BzATP produced significant increases from 100 μM (Fig. 4B4), and activation threshold was attenuated at the 100 and 300 μM concentrations of ATP, whereas BzATP significantly reduced it (Fig. 4B3). These results indicate a three times stronger effectiveness of BzATP than ATP in producing significant changes in NS neuronal properties in MDH ($^{\#}P<0.05$, *t*-test, $n=5$–6; Fig. 4B1–4).

Minocycline superfusion attenuated the MO-induced central sensitization

Continuous i.t. superfusion of minocycline (500 μM) over MDH for 25 min prior to MO application did not affect baseline nociceptive neuronal properties ($P>0.2$–0.3, paired *t*-test, $n=6$; Table 2). Minocycline superfusion did completely block the MO-induced neuronal changes in RF size, mechanical activation threshold and responses to mechanical stimuli ($P>0.1$–0.3, 1-way RM ANOVA or ANOVA on Ranks, $n=6$) (Fig. 5A–C). The MO-induced effects were significantly different between PBS and minocycline groups for all three parameters (all $P<0.001$, 2-way ANOVA). Post hoc analysis indicated that there were significant differences between these two groups at most post-MO time-points (see Fig. 5A–C).

DISCUSSION

P2X receptors have seven subunits, of which P2X7 receptor subunits are distributed widely in the body and are predominantly found in immune cells as well as in cells in the central nervous system, that is, microglia and oligodendrocytes (Yu et al., 2008; Chu et al., 2010). Recent studies have suggested that P2X7 receptors in microglia are critically involved in mediating chronic inflammatory and neuropathic pain (Chessell et al., 2005; Burnstock, 2008; Sperlágh and Illes, 2007; Carroll et al., 2009; Clark et al., 2010). The potent P2X7 receptor antagonists oATP and BBG have been used in recent studies to examine P2X7 mechanisms. Studies of Fulgenzi and colleagues demonstrated that local or systemic administration of oATP significantly reduces hyperalgesia and cytokine release in rat and mouse arthritic, neuropathic, visceral as well as carrageenan-induced pain models and concluded that this effect was likely due to suppression of P2X7 receptor activation (Fulgenzi et al., 2005, 2008). Other studies have documented the inflammation-blocking effects of BBG and related these effects to P2X7 receptor blockade (Jiang et al., 2000; Gunosewoyo et al., 2007; Peng et al., 2009; Chu et al., 2010). In the present study, superfusion of BBG or oATP had significant blocking actions on MDH central sensitization parameters, that is, the MO-induced increase in nociceptive responses and decrease in activation threshold of MDH nociceptive neurons, consistent with a recent report that oATP and BBG can attenuate long-term potentiation induced by the tetanic stimulation of the sciatic nerve both *in vivo* and in spinal cord slices (Chu et al., 2010), and also in line with findings that carrageenan-produced hyperalgesia can be blocked by the P2X7 receptor antagonist A-740003 (Honore et al., 2006). Chu et al. (2010) have further demonstrated that pre-administration of BBG inhibits increased expression of the dorsal horn microglial marker Iba-1, phosphorylated p38, interleukin 1β and GluR1 following tetanic stimulation of the sciatic nerve. All these suppressive effects may be explained by P2X7 receptor blockade, because peripheral inflammation (or tetanic stimulation)-induced activation of P2X7 receptors can result in Ca^{2+} influx and release of

Table 2. Effects of different chemical pretreatments on baseline values of NS neurons in MDH

Group	# of rats	Baseline & 25 min after pretreatment	Pinch RF size (cm^2)	Mechanical activation threshold (g)	Responses to mechanical stimulus (# of spikes)	*P*-values (RFp; Thr; Resp)
Suramin+DPCPX	16	Baseline	2.7±0.3	110±21	80±15	
		25′ after pretreatment	2.7±0.3	111±22	85±19	>0.3; >0.9; >0.5
Minocycline	6	Baseline	2.4±0.4	64±26	109±31	
		25′ after pretreatment	2.3±0.4	67±28	93±31	>0.2; >0.2; >0.3

Differences in values between baseline and 25′ after pretreatment were treated by Student's paired *t*-test.

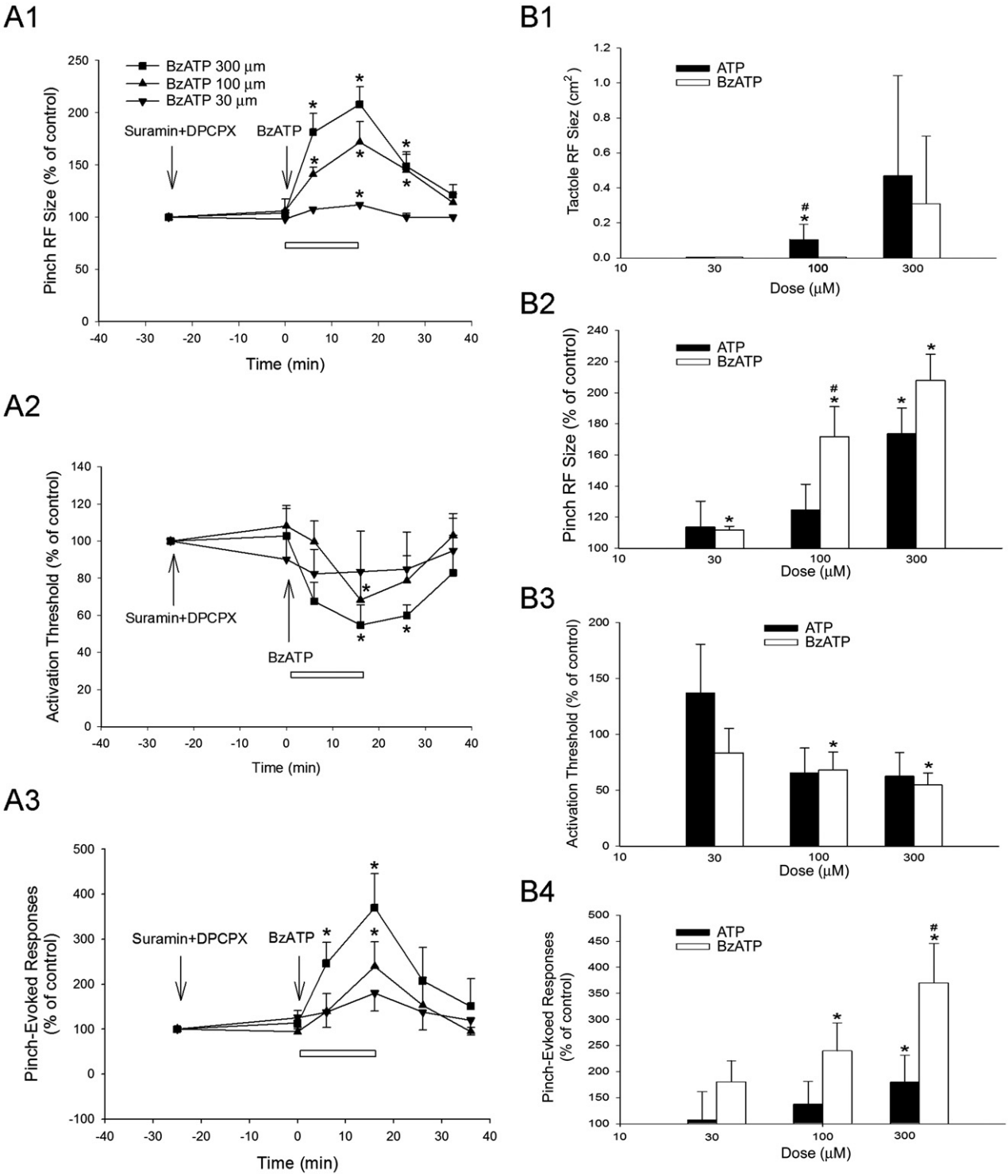

Fig. 4. (A) Dose-dependent responses of i.t. superfusion of BzATP that compare with the MO-induced central sensitization in MDH. After the pretreatment of a mixture of suramin and DPCPX for 25 min, BzATP (30, 100, or 300 μM) was superfused on the medulla for 15 min. BzATP produced dose-dependently central sensitization-like responses (slow onset and gradual build-up), reflected as increases in pinch RF size expansion (A1), decrease in activation threshold (A2), and increases in responses to mechanical stimuli (A3). (B) Comparison between superfusion of BzATP and ATP (at same doses) of chemical-evoked changes in RF size, activation threshold and pinch responses in MDH. Note that at the same 100 μM concentration, ATP evoked novel tactile RF in NS neurons which have been defined as lack of tactile RF in normal conditions, while BzATP did not evoke it ($^{\#}$ $P<0.05$, Fisher test, $n=5$; B1) and was more effective also in reducing activation threshold (B3); in contrast, BzATP produced significantly stronger expansion in pinch RF size than that produced by ATP ($^{\#}$ $P<0.05$, t-test, $n=5$; B2). Regarding pinch-evoked responses, BzATP also was much more potent than ATP ($^{\#}$ $P<0.05$, t-test, $n=5$; B4). For all figures in (A) and (B), * $P<0.05$ represents significant difference between the value of post-BzATP or post-ATP time-points and the baseline values (1-way RM ANOVA or ANOVA on Ranks, $n=5$–6). $^{\#}$ $P<0.05$ represents significant difference between BzATP and ATP at the same dose (t-test, $n=5$–6).

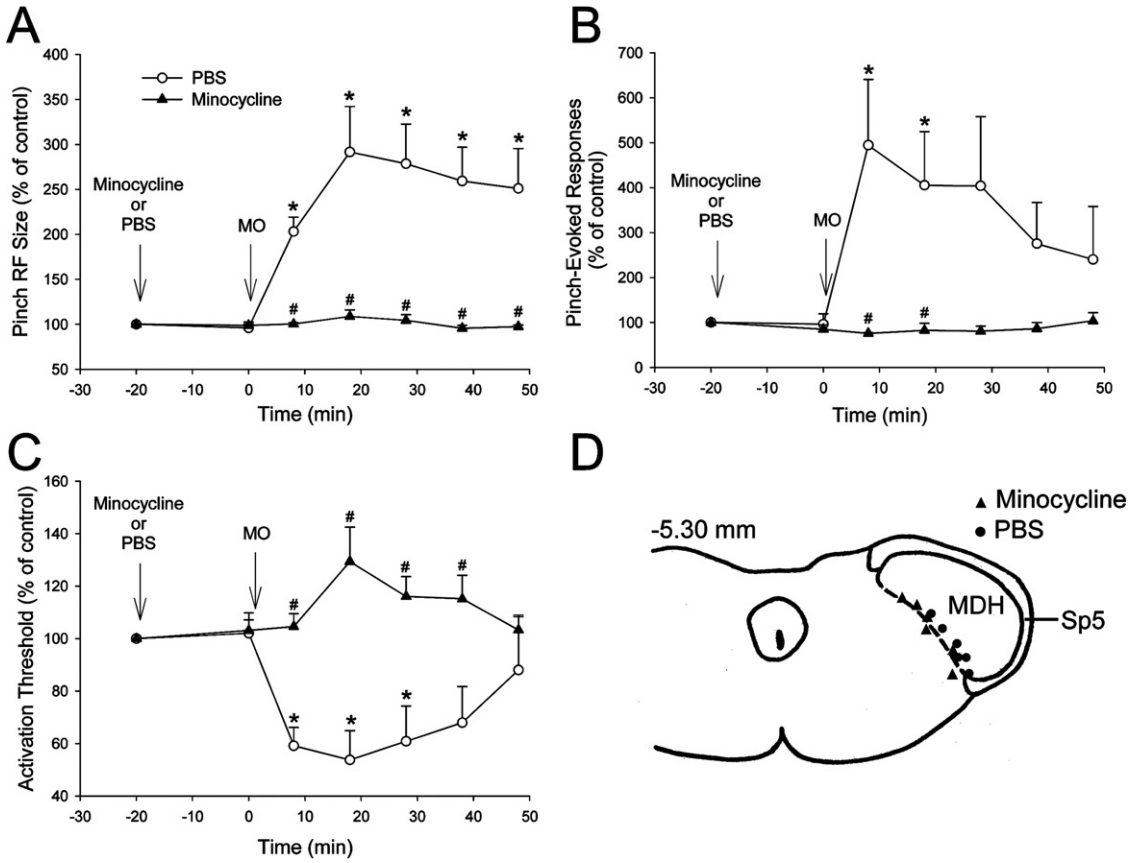

Fig. 5. Effects of continuous i.t. superfusion of minocycline and PBS on the neuroplastic changes in MDH induced by MO application to the tooth pulp. The MO-induced changes in NS neuronal RF size (A), responses to graded mechanical stimuli (B) and mechanical activation threshold (C). Note that during PBS superfusion, MO application produced significant increases in pinch RF size, responses to graded stimuli, and decreases in threshold ($P<0.05$–0.001, RM ANOVA; * $P<0.05$, Dunnett's test; $n=6$) throughout the 50-min observation period, and that minocycline superfusion significantly blocked the MO-induced changes in neuronal properties ($P>0.1$–0.3, RM ANOVA or ANOVA on Ranks, $n=6$). All these MO-induced neuroplastic changes in the minocycline group were significantly less than those in the PBS group (all $P<0.001$, 2-way ANOVA). Post hoc analysis indicated that there were significant differences in values at most post-MO time-points between these two groups ($^{\#}$ $P<0.05$, Dunnett test). (D) The recording sites of the NS neurons in PBS and minocycline groups were histologically retrieved in the deep laminae of MDH.

proinflammatory cytokines from microglia which readily enhance synaptic transmission (Fields and Burnstock, 2006; Sperlágh and Illes, 2007; Surprenant and North, 2009; Clark et al., 2010). One caveat regarding these studies is that there are no completely selective P2X7 receptor antagonists available which is why we have utilized several different approaches in our study in order to minimize the likelihood that observed effects could be due to an action of these drugs on other receptors (Beigi et al., 2003; Di Virgilio, 2003; Burnstock, 2007; Gunosewoyo et al., 2007; Donnelly-Roberts et al., 2009; Jarvis and Khakh, 2009).

The synthetic non-selective P2X7 receptor agonist BzATP is effective at concentrations of 3 μM and thus considerably more potent than ATP whose EC_{50} is only 100 μM (North and Surprenant, 2000). However, BzATP also activates P2Y receptors (Boyer and Harden, 1989). Therefore, to provide additional support for P2X7 receptor involvement in central sensitization, we compared the effects of the P2X7 receptor agonist BzATP with those of ATP. In these experiments, we used a pretreatment with suramin and DPCPX in order to block all P2X receptor subunits, except P2X4 and P2X7 subunits, as well as most P2Y receptor subunits and to prevent adenosine (the end-product of BzATP degradation) producing inhibition of synaptic transmission, respectively. The baseline values of neuronal properties remained stable during the 25-min pretreatment with suramin+DPCPX (see Table 2), while the subsequent application of BzATP dose-dependently produced central sensitization in MDH; the effective doses of BzATP and ATP were 30 μM and 100 μM, respectively (Fig. 4B1, B2). Interestingly, 30 μM BzATP has been recently shown *in vitro* to be sufficient to initiate IL-1β release via P2X7 receptor activation (Hughes et al., 2007), and 100 μM ATP corresponds to the EC_{50} of ATP tested in cloned P2X7 receptors (North and Surprenant, 2000). Therefore, based on these findings, we may speculate that the P2X7 receptor channel can be activated by extracellular ATP at a moderate (around 100 μM) concentration rather than at mM levels as previously found in conditions without co-application of chemicals for preventing adenosine-induced synaptic inhibition *in situ* (Wang et al., 2004; Clark et al., 2010). This moderate concentration in the local environment may be attained by the initial surge of ATP released from central terminals of afferent fibers in asso-

ciation with subsequent ATP release from activated astroglia and microglia (Burnstock, 2008; Zhang et al., 2007; Cotrina and Nedergaard, 2009) during acute inflammation evoked by formalin, MO, carrageenan, capsaicin and afferent C-fiber stimulation (Watkins et al., 2001; Chiang et al., 2005; Hua et al., 2005; Honore et al., 2006; Qin et al., 2006; McGaraughty et al., 2007; Chen et al., 2009; Chu et al., 2010).

It is noteworthy that not all MO-induced central sensitization parameters were completely blocked by BBG or oATP, suggesting that other P2 receptors may also have contributed to some aspects of the MDH central sensitization observed in the present study. In particular, ATP application to the medulla produced MDH central sensitization, including the appearance of a novel tactile RF, at a concentration of 100 μM which corresponds to the EC_{50} of ATP tested in cloned P2X4 receptors (North and Surprenant, 2000) and this same 100 μM concentration also enhances specifically P2X4 receptor responses in murine microglia (Raouf et al., 2007). In addition, allodynia in neuropathic pain models has been documented to be caused by upregulation of P2X4 receptors in microglia (Tsuda et al., 2003, 2009). The possibility of P2Y receptor involvement in central sensitization also cannot be excluded in our acute pulpitis pain model, particularly in view of recent evidence that the P2Y12 receptor subunit may be involved in neuropathic pain by mediating mitogen-activated protein kinase (MAPK) activation in spinal dorsal horn microglia (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008).

Superfusion of minocycline, a potent microglia blocker, completely blocked the MO-induced central sensitization in the present study, consistent with our previous studies using another microglial blocker SB250380 (Xie et al., 2007). Although we cannot rule out a contribution of other glial cells since in a brain injury model, minocycline inhibition of astroglial activation in the hippocampus has been recently reported (Cai et al., 2010), our finding is also consistent with findings in several other inflammatory pain models where minocycline and SB250380 were shown to block p38 MAPK activation which is the essential link in the translational signaling pathway involved in microglial activation (Hua et al., 2005; Piao et al., 2006; Chang and Waxman, 2010; for review, see Ji et al., 2009; Surprenant and North, 2009). In addition, an upregulation of the P2X7 receptors co-localized in spinal microglia (P2X7/OX-42 staining) occurs at 7 days after sciatic nerve tetanic stimulation (Chu et al., 2010). These findings, together with the present results, suggest that activation of P2X7 receptors primarily on microglia may be an essential step in the MO-induced central sensitization.

CONCLUSION

The present study has provided the first documentation that the potent P2X7 receptor antagonists BBG and oATP can significantly reduce the central sensitization induced in functionally identified dorsal horn nociceptive neurons in an acute inflammatory pain model. In addition, application to the MDH of the potent P2X7 receptor agonist BzATP was also able to induce central sensitization of MDH nociceptive neurons and was three times more effective than ATP in producing these changes. Furthermore, we found that superfusion of the microglial blocker minocycline could completely block MDH central sensitization, consistent with findings that P2X7 receptors are primarily expressed in microglia. All these findings suggest that P2X7 receptors primarily on microglia *in vivo* may play a major role in the development of the central sensitization in an acute inflammatory pain model.

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