

Diverse effects of Brilliant Blue G administration in models of trigeminal activation in the rat

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Abstract Activation of the trigeminal system plays an important role in the pathomechanism of headaches. A better understanding of trigeminal pain processing is expected to provide information helping to unravel the background of these diseases. ATP, a key modulator of nociceptive processing, acts on ligand-gated P2X receptors. Antagonists of the P2X7 receptors, such as Brilliant Blue G (BBG), have proved effective in several models of pain. We have investigated the effects of BBG after electrical stimulation of the trigeminal ganglion and in the orofacial formalin test in the rat. The right trigeminal ganglion of male rats was stimulated either with 5 Hz, 0.5 mA pulses for 5 min (mild procedure) or with 10 Hz, 0.5 mA pulses for 30 min (robust procedure), preceded by 50 mg/kg i.v. BBG. The animals were processed for c-Fos and calcitonin gene-related peptide (CGRP) immunohistochemistry. In the orofacial formalin test, 50 µL of 1.5 % formalin was injected into the right whisker pad of awake rats, following the pre-treatment with BBG. Behaviour was monitored for

45 min, and c-Fos and CGRP immunohistochemistry was performed. BBG attenuated the increase in c-Fos-positive cells in the caudal trigeminal nucleus (TNC) after robust stimulation, but not after mild stimulation. No alterations in CGRP levels were found with either methodology. BBG did not mitigate either the behaviour or the increase in c-Fos-positive cells in the TNC during the orofacial formalin test. These results indicate that P2X7 receptors may have a role in the modulation of nociception in the trigeminal system.

Keywords Trigeminal system · P2X7 receptor · Trigeminal ganglion stimulation · Formalin test

Introduction

Primary headaches are very common, but underdiagnosed and undertreated neurological conditions. Even following the correct diagnosis, the available therapeutic options often do not provide complete resolution of the pain, and recurrence of the headache after treatment is also a common complaint. Research is, therefore, currently focused on gaining an understanding of the causes of different headache disorders and developing new therapeutic options.

A common mechanism in primary headaches involves activation and sensitization of the trigeminal system, but the exact mechanism of these phenomena remains to be discovered (Tajti et al. 2011).

Recent results suggest that the purine molecule ATP has an important role in the regulation of nociceptive transmission (Burnstock 2013). Molecules targeting the specific receptors (Rs) for ATP (P2X and P2Y-Rs) have been proven to be effective in modulating different pain

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conditions, e.g., neuropathic and inflammatory pain (Ando et al. 2010). Among the ligand-gated P2X-Rs, the P2X7 receptor (P2X7-R) has been intensively studied in different pain states. The P2X7-R is a non-selective cation channel, unique among the P2X-Rs by virtue of its long C terminal domain, and its ability to open a pore permeable to molecules up to 900 Da (Surprenant et al. 1996). Experiments with knock-out mice have revealed that the absence of the P2X7-R leads to the disappearance of mechanical and thermal hypersensitivity in models of neuropathic and inflammatory pain, whereas normal nociceptive processing is retained (Chessell et al. 2005). P2X7-R antagonists have been examined in similar models, and the results underline the importance of the P2X7-R in chronic pain conditions (Honore et al. 2006; McGaraughty et al. 2007). Results from an acute inflammatory pain model suggest that the P2X7-R may participate in the development of central sensitization (Itoh et al. 2011), a common feature in the trigeminal system during migraine attacks manifested by the presence of allodynia (Burstein et al. 2000).

However, little information is available as concerns the role of P2X7-Rs in the trigeminal system. In a model of orofacial pain, the chronic constriction injury model, inhibition of the P2X7-R led to a decrease in tactile allodynia through a p38 MAPK-dependent mechanism (Ito et al. 2013). Goloncser and Sperlagh recently reported that blockade of P2X7-Rs in mice by Brilliant Blue G (BBG) [a selective, non-competitive P2X7-R antagonist, with good blood–brain barrier permeability (Jiang et al. 2000)] reduced thermal hyperalgesia after systemic nitroglycerin administration (Goloncser and Sperlagh 2014). These results lend support to the theory that P2X7-Rs may play a crucial part in the development of headache disorders.

Calcitonin gene-related peptide (CGRP) has an essential role in trigeminal nociceptive processing. CGRP infusion causes migraine-like headache in migraineurs (Hansen et al. 2010; Lassen et al. 2002), and the levels of CGRP are higher in migraine patients than in healthy controls (Friberg et al. 1994).

Electrical stimulation of the trigeminal ganglion, an animal model of trigeminal activation, causes plasma protein extravasation (Markowitz et al. 1987), which can be attenuated by drugs effective in migraine therapy (Limmroth et al. 2001). The stimulation results in alterations in the dura mater (Knyihar-Csillik et al. 1995), in the trigeminal ganglion and in the caudal part of the spinal trigeminal nucleus (TNC) (Knyihar-Csillik et al. 1998). In previous investigations, various stimulation frequencies, intensities and durations were applied. Results related to peptide release and c-Fos expression in the TNC after the procedure suggest that both higher frequency and higher intensity lead to an increased stimulation of the trigeminal ganglion cells (Samsam et al. 1999; Takemura et al. 2000).

Diverse periods of stimulation caused different morphological alterations in the CGRP-immunoreactive nerve terminals of the dura mater, suggesting the release of CGRP after prolonged stimulation (Knyihar-Csillik et al. 1995). Based on these previous results, we decided to perform the electrical stimulation in two different setups: a mild and a robust one.

Formalin applied to the whisker pad of rats (another model of trigeminal activation) causes a biphasic behavioural effect, the first phase being caused by the direct activation of A δ and C fibres, while the second phase reflects the process of inflammation (Porro and Cavazzuti 1993). Activation of the trigeminal system has been demonstrated by c-Fos immunohistochemistry to be present at the level of the TNC after the injection of formalin (Wang et al. 1994). Both phenomena are suitable for assessments of the influence of substances on inflammation.

The aim of the present study was to examine the effects of a P2X7-R antagonist, BBG, in the electrical stimulation and orofacial formalin models of acute trigeminal activation.

Materials and methods

Ethical approval

The procedures used in this study followed the guidelines of the 8th Edition of the Guide for the Care and Use of Laboratory Animals and the Use of Animals in Research of the International Association for the Study of Pain and the directive of the European Economic Community (86/609/ECC). The experiments were approved by the Committee of Animal Research at the University of Szeged (I-74-12/2012) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XXIV/352/2012). Male Sprague–Dawley rats were housed under standard laboratory conditions, on a 12-h light–dark cycle, with tap water and rat chow available *ad libitum*. The suffering of the animals and the number of animals used were kept at a minimum.

Mild stimulation procedure

Twenty-four animals (250–300 g) were used. Half of the animals received an intravenous (i.v.) injection of 50 mg/kg BBG, while the other half were injected with the vehicle of BBG, physiological saline. Two h after the BBG or saline injection, the animals were deeply anaesthetised with chloral hydrate (400 mg/kg) and the head was secured in a stereotaxic apparatus (Stoelting Co., Wood Dale, USA). A hole, approximately 3 mm in diameter, was drilled into the

right side of the skull with a dental drill, and a concentric bipolar electrode (FHC Inc., Bowdoin, USA, CBBRE75) was lowered to the right trigeminal ganglion. Half of the animals from the saline-treated group (5SStim) and half of the animals from the BBG group (5BStim) were electrically stimulated for 5 min with 5 Hz, 0.5 mA, 0.5 ms delay twin pulses generated by an Electrostimulator ST3 (Medicor Hungary). The other animals from both groups were used as sham animals: the electrode was lowered to the right trigeminal ganglion for 5 min, but no stimulation was performed (5SSham and 5BSham groups). After both procedures, the animals were returned to their home cages and maintained under deep anaesthesia covered by a warming blanket for 2 h.

Robust stimulation procedure

Twenty-one animals (250–300 g) were used; the treatment and surgical procedures were identical to the previous ones, except that the stimulation parameters of 10 Hz, 0.5 mA, 0.5 ms delay twin pulses were applied for 30 min, and the animals were maintained under deep anaesthesia, covered by a warming blanket, in their home cages for 4 h from the beginning of the stimulation. In both the mild and the robust paradigm, the jaw of the animal was twitching during the electrical stimulation, indicating the correct placement of the electrode.

Overview of the robust stimulation groups:

- Saline + 30-min sham: 30SSham ($n = 6$).
- Saline + 30-min stimulation: 30SStim ($n = 5$).
- BBG + 30-min sham: 30BSham ($n = 4$).
- BBG + 30-min stimulation: 30BStim ($n = 6$).

Orofacial formalin test

Behavioural tests

Rats ($n = 52$, 200–240 g) were injected i.v. either with 50 mg/kg BBG or with physiological saline. One hour and fifty minutes later, the animals were placed in a 30 × 30 × 30 cm box, with mirrored walls for the monitoring of behavioural activity. After 10 min of habituation, the animals were taken out of the box and under minimal restraint were injected subcutaneously with 50 µL of either physiological saline (SSal and BSal groups) or 1.5 % formalin (SForm and BForm groups) to the right whisker pad. After the injection, they were returned immediately to the box and their behaviour was monitored for 45 min under video surveillance. The injection of formalin causes a behavioural response, which consists in rubbing and scratching of the injected whisker pad with the ipsilateral fore- or hindpaw. The rate of this behaviour correlates with

the pain sensation caused by formalin (Clavelou et al. 1989). The 45-min period was divided into 15 × 3-min blocks, and the total time spent rubbing the injected whisker pad, measured in seconds, was taken as the nociceptive score in the given block. The normal grooming activity of the saline-treated animals was measured as control. After the monitoring period, the animals were returned into their home cages and maintained under standard laboratory conditions until perfusion, which was performed under deep chloral hydrate anaesthesia 4 h after the whisker pad injections.

Immunohistochemistry

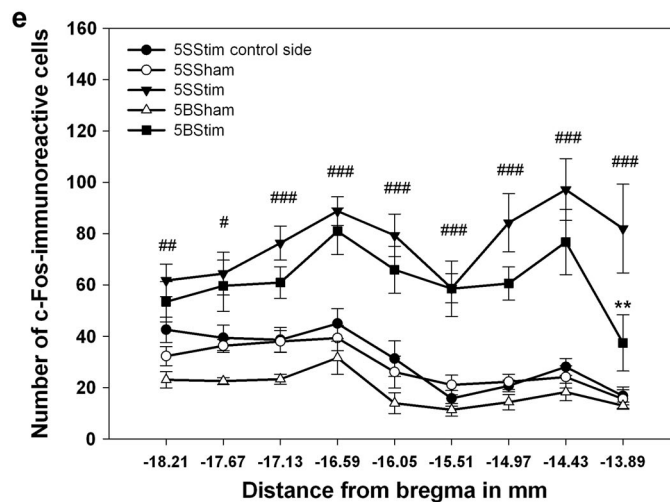
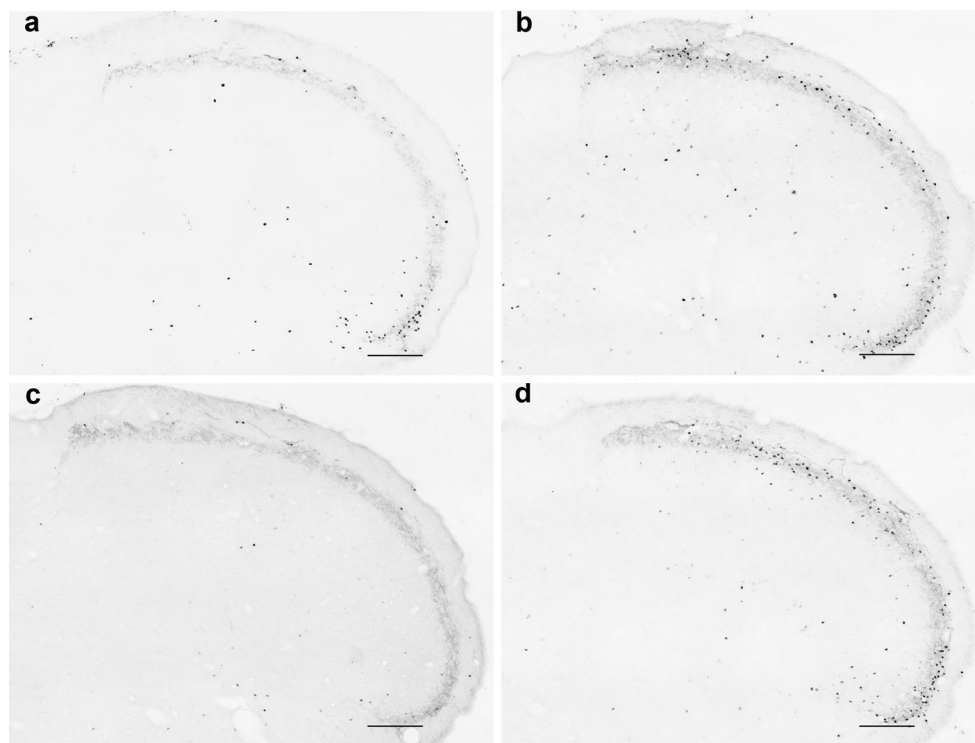
Animals were perfused with 0.1 M phosphate-buffered saline (PBS), followed by 4 % paraformaldehyde in 0.1 M phosphate buffer. The whole brain and the upper cervical spinal cord were removed and postfixed overnight in the same fixative. The correct placement of the electrode was checked during autopsy, the electrode was in the ganglion in all cases. Cryoprotection was performed, using gradient sucrose solutions up to 30 %. Sections from the TNC were prepared from the block, ranging from 1 mm rostral to 4 mm caudal from the obex, and the ventral part of the control (left) side of the blocks was marked by a small incision to enable side discrimination on the sections. 30 µm thick transverse sections were cut and serially collected in 18 wells containing PBS with 0.1 % sodium azide, the overall distance therefore being 540 µm between consecutive sections. Free-floating sections were immersed in 0.3 % H₂O₂ in PBS to block endogenous peroxidase activity. After several washes in PBS containing 1 % Triton X-100 (PBS-T), they were incubated for 1 h in PBS-T containing 10 % normal goat serum. The sections were then incubated overnight at room temperature in the primary antibody for c-Fos (1:2000, Santa Cruz Biotechnology, sc-52) or CGRP (1:20,000, Sigma-Aldrich C8198). The immunohistochemical reaction was visualised by using the Vectastain Elite avidin–biotin kit (PK6101; Vector Laboratories,) with 3,3'-diaminobenzidine as chromogen (Sigma-Aldrich) intensified with nickel ammonium sulphate (Scharlau Chemie). The specificity of the immune reactions was verified by omitting the primary antisera.

Sections were mounted onto glass slides, air-dried and coverslipped with DPX mounting medium (Scharlau Chemie). On the basis of anatomical observations, sections from the same rostro-caudal level were compared during the statistical evaluation.

An observer blinded to the treatment procedures used a Nikon Optiphot-2 light microscope under a 20× objective to count cells immunopositive for c-Fos. The whole area of the laminae I-II in each section of the TNC was evaluated and counted.

Fig. 1 Summary of the results from the mild stimulation paradigm regarding c-Fos immunostaining. Representative photos from the *right sides* of the four treatment groups after c-Fos immunohistochemistry, taken at 16.05 mm caudally from bregma: **a** 5SSham, **b** 5SStim, **c** 5BSham, **d** 5BStim. Scale bar 200 μ m.

Diagram showing the number of c-Fos-immunoreactive cells across the different levels of the TNC after mild electrical stimulation of the trigeminal ganglion (group mean \pm SEM) (**e**). There was no significant difference between the control (*left*) sides (data not shown). For clarity, only the control side of the saline-treated stimulated group is presented. The *hashmarks* indicate significance in the comparison of the *right sides* of the 5SSham and 5SStim groups at different levels of the TNC ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$). BBG treatment showed an attenuating tendency, though it proved to be significant merely at the level of -13.89 mm ($^{**}p < 0.01$, 5SStim–5BStim)



CGRP-stained sections were photographed with a Zeiss AxioCam MRc Rev.3 digital camera attached to a Zeiss AxioImager M2 microscope. Digital images were taken with a $20\times$ objective in TNC laminae I–II, and the area covered by CGRP-immunoreactive fibres was measured through the use of ImageProPlus 6.2 software (Media Cybernetics Inc.) by an observer blinded to the treatment procedures.

Statistical analysis

The cell count results were aligned according to the rostro-caudal location of the section as mentioned above. Data

from different levels of the TNC were handled separately, and analysed by two-way repeated measures ANOVA. The group was used as the between-subject factor and the levels (-13.89 , -14.43 , -14.97 , -15.51 , -16.05 , -16.59 , -17.13 , -17.67 , -18.21 mm from bregma) as the within-subject factor for the analysis.

When Mauchly's test of sphericity proved to be significant, the Greenhouse-Geisser correction was performed. Pairwise comparisons of group means were performed on the basis of estimated marginal means with Sidak adjustment for multiple comparisons.

The sums of the areas covered by CGRP-immunoreactive fibres were compared between groups according to the

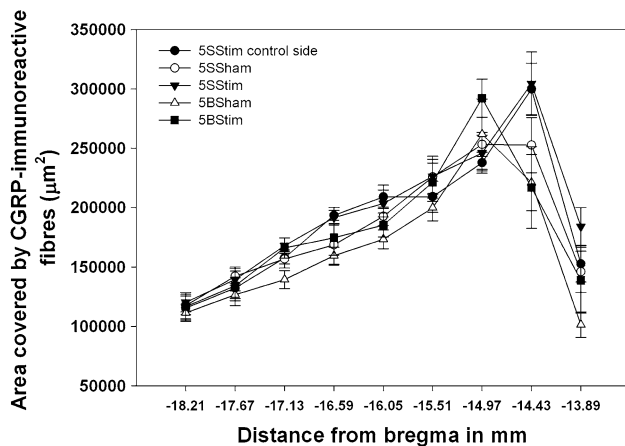


Fig. 2 Diagram showing the area covered by CGRP-immunoreactive fibres at the different levels of the TNC in the different treatment groups after mild electrical stimulation of the trigeminal ganglion (group mean \pm SEM). There was no significant difference between either the control or the stimulated sides or the different groups. For clarity, only the control side of the saline-treated stimulated group is presented

different levels, two-way repeated measures ANOVA being used as detailed above.

Nociceptive scores from the behavioural study were compared block by block through two-way repeated measures ANOVA. Groups were used as between-subject factor and blocks (1–15) as within-subject factor for the analysis. Other statistical parameters were identical to those mentioned above.

Statistical analyses were carried out with IBM SPSS Statistics, version 20 (IBM Corporation) software. All tests were two-sided, and $p < 0.05$ was considered to be statistically significant. Graphs were prepared by using SigmaPlot 12.0 (Systat Software Inc.). Data are reported as mean \pm SEM.

Results

Mild stimulation procedure

As a significant interaction was found between the two investigated factors (levels and groups, $p < 0.01$) for the number of c-Fos-immunoreactive cells in the mild stimulation paradigm, both effects could not be reported independently, whereas the group differences could be examined separately across different levels on the basis of the estimated marginal means for multiple comparisons.

Lowering of the electrode to the trigeminal ganglion for 5 min without stimulation (5SSSham group) did not cause any significant change in the number of c-Fos-immunoreactive cells in the TNC relative to the control side. The comparisons of the cell numbers from the control (left

sides for each of the four treatment groups did not reveal any significant changes (data not shown), and therefore only the data for the stimulated (right) sides of the groups are presented in Fig. 1a–d. Electrical stimulation of the ganglion caused a significant increase in the number of c-Fos-immunoreactive (IR) cells along the whole extent of the examined region of the TNC (Fig. 1a, b, e). BBG exhibited a significant effect ($p < 0.01$) compared with the saline-treated stimulated animals only at the level of -13.89 mm from bregma (Fig. 1b, c, e).

A significant interaction was found between the groups and levels, when the area values from the CGRP measurements were examined. However, the comparisons of the groups and stimulated-control sides at different levels did not reveal any significant alteration (Fig. 2).

Robust stimulation procedure

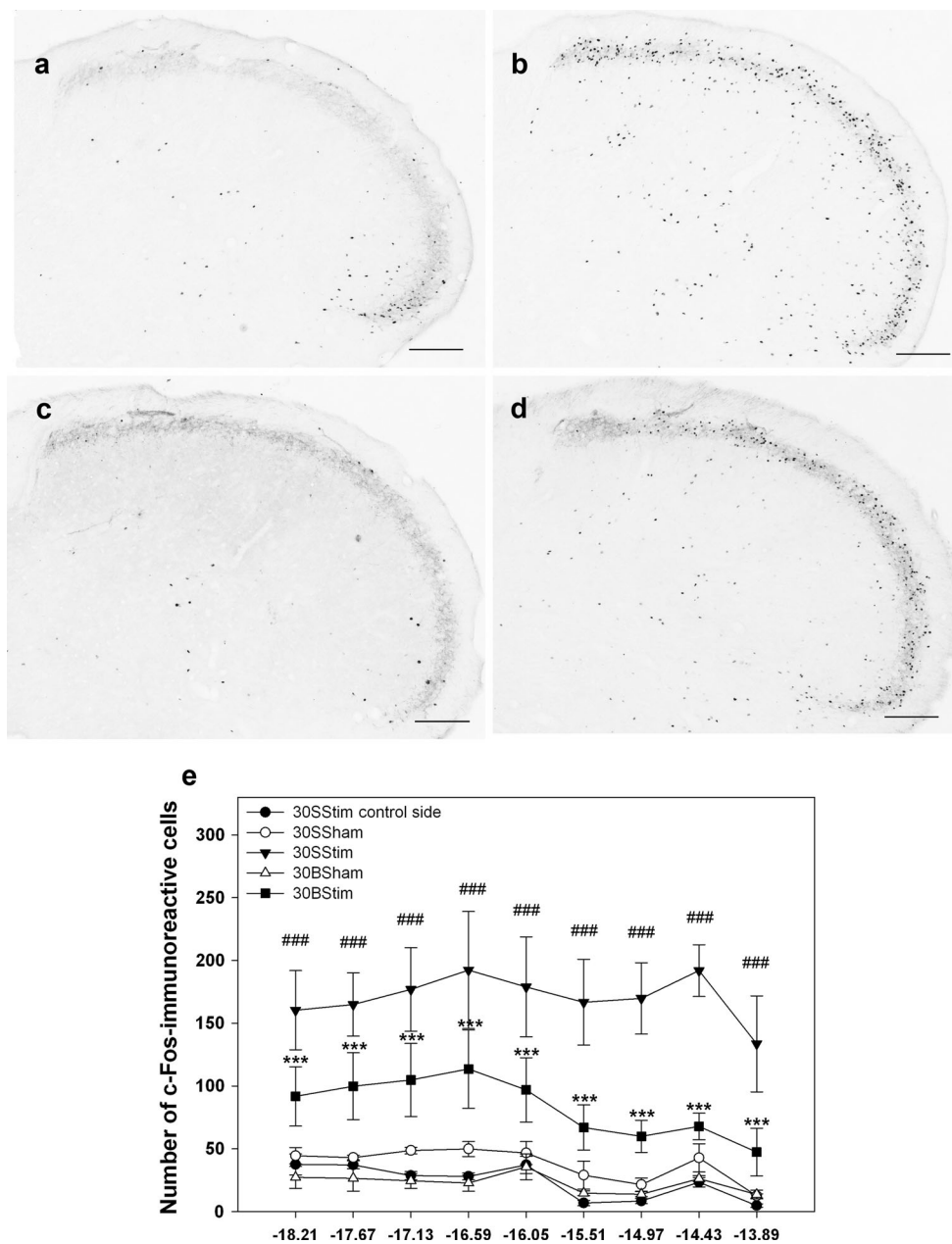
A significant interaction was not found between the investigated factors of levels and groups for the number of c-Fos IR cells in the robust stimulation paradigm. The levels had a significant effect ($p < 0.001$), and there was also a significant difference between the groups ($p < 0.001$). Pairwise comparisons revealed that there was no significant difference between the control sides of the four treatment groups (data not shown). Lowering of the electrode without stimulation did not cause significant changes in the number of c-Fos IR cells in either sham-treated group as compared with the control (Fig. 3e, 30SSham and 30BSham with the 30SSStim control side). Robust stimulation caused a marked increase in the number of c-Fos IR cells in the saline-treated animals (Fig. 3a, b, e, 30SSStim compared with 30SSSham, $^{\#}p < 0.001$). BBG had a significant attenuating effect on this increase (Fig. 3b, d, e, 30SSStim compared with 30BStim).

CGRP expression was not altered by stimulation or BBG administration in any of the animal groups (Fig. 4).

Orofacial formalin test

A significant interaction was found between time and groups ($p < 0.05$) during the analysis of the behavioural data from the orofacial formalin test. The pairwise comparison revealed that the nociceptive scores of the saline-injected groups (SSal and BSal) did not differ from each other at any time point (Fig. 5). The injection of formalin caused a significant increase in the nociceptive scores in blocks 1 and 5–7 (Fig. 5, SForm compared with SSal). After the injection of formalin into the whisker pad, the BBG-treated animals demonstrated significantly increased nociceptive scores in blocks 1, 6 and 8 as compared with the control (Fig. 5, $^{\$}p < 0.05$). The BBG-treated animals spent less time rubbing their formalin-injected side in both

Fig. 3 Summary of the results from the robust stimulation paradigm regarding c-Fos immunostaining. Representative photos from the *right sides* of the four treatment groups after c-Fos immunohistochemistry, taken at 16.05 mm caudally from bregma: **a** 30SSham, **b** 30SStim, **c** 30BSham, **d** 30BStim. Scale bar 200 μ m. Diagram showing the number of c-Fos-immunoreactive cells at different levels along the rostro-caudal axis in the TNC (group mean \pm SEM) in the robust stimulation paradigm (e). There was no significant difference between the control sides (data not shown). For clarity, only the control side of the saline-treated stimulated group is presented. Electrical stimulation of the right trigeminal ganglion caused a significant increase in the number of c-Fos-positive cells as compared with the right side of the sham group at all levels examined ($###p < 0.001$ 30SSham–30SStim). BBG treatment had a significant decreasing effect on the cell counts ($***p < 0.001$ 30SStim–30BStim)



blocks 1 and 5–7, but this difference was not significant compared with the SForm group. In block 8, BBG-treated animals spent significantly more time rubbing their whisker pad as did the animals in the formalin group (Fig. 5). In our experiments, the second phase of the formalin test subsided more quickly as expected in the SForm group, while it was more prolonged in the BForm group, and this difference may account for the significant effect of BBG in block 8.

A significant interaction was observed between the levels and groups ($p < 0.001$) for the number of c-Fos IR cells in the orofacial formalin test. There was no significant difference when either the control (left) sides or the control

and saline-injected sides were compared at any level (Fig. 6e). The injection of formalin increased the number of c-Fos-IR cells significantly at the levels -16.59 to -15.51 mm, mainly in the central part of the TNC as compared with the saline-injected side in the SSal group. In the BForm group, a similar pattern was observed, except that the difference involved one additional level (-14.97 mm, Fig. 6e). There was no significant difference between the SForm and BForm groups at any level (Fig. 6b, d).

No group difference was found in any of the measured parameters as regards the CGRP immunoreactivity (Fig. 7).

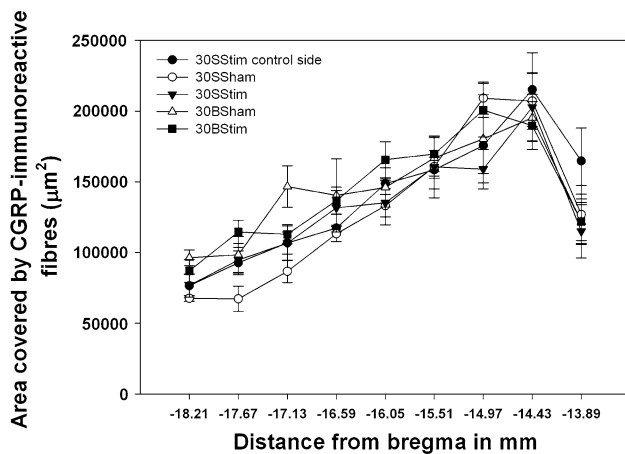


Fig. 4 Diagram showing the area covered by CGRP-immunoreactive fibres at the different levels of the TNC in the different treatment groups after robust electrical stimulation of the trigeminal ganglion (group mean \pm SEM). There was no significant difference between either the control or the stimulated sides or the different groups. For clarity, only the control side of the saline-treated stimulated group is presented

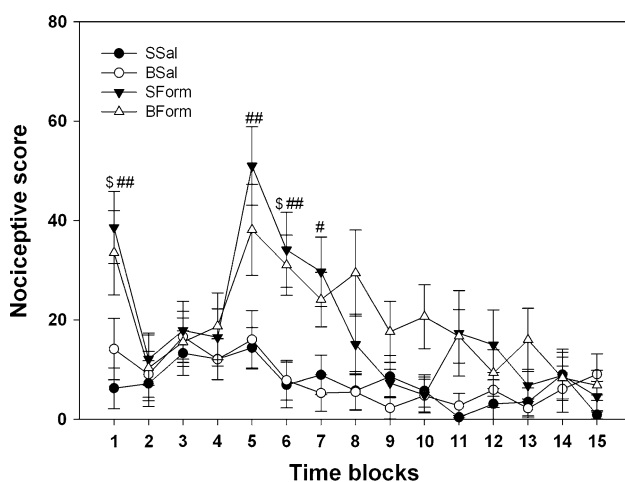


Fig. 5 Diagram showing the mean nociceptive scores in different time blocks from the four treatment groups (group mean \pm SEM, $n = 13$) in the orofacial formalin test. The injection of formalin increased the nociceptive score in blocks 1 and 5–7 as compared with the saline-injected animals ($^{\#}p < 0.05$, $^{##}p < 0.01$). BBG treatment did not affect the nociceptive scores after saline injection, and did not modify the normal behaviour. The nociceptive scores of the BBG and saline-treated formalin-injected groups did not differ significantly in any block

Discussion

Numerous stimulation parameters and stimulation times have been applied in previous experiments, and we therefore decided to make use of two stimulation procedures, a short, mild stimulation and a longer, robust stimulation, in order to examine the possible effects of P2X7-R antagonism on trigeminal activation. As P2X7-R blockade was

previously found to be effective in inflammatory conditions, we additionally examined the effects of BBG in a model of orofacial inflammation, the orofacial formalin test.

Mild, short electrical stimulation of the trigeminal ganglion has been reported to lead to activation of the trigeminal system (Takemura et al. 2000), and this was supported by our results. The activation may be direct, stemming from depolarization of the central terminals of the primary trigeminal afferents, or it may be indirect, resulting from the release of peripheral mediators (histamine, bradykinin, substance P or CGRP).

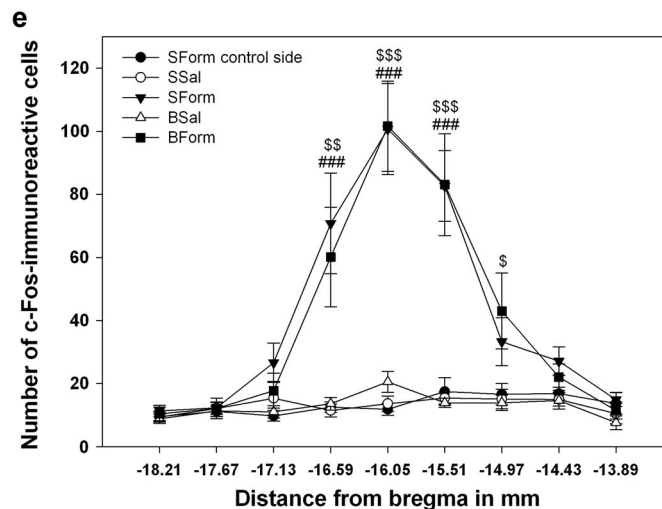
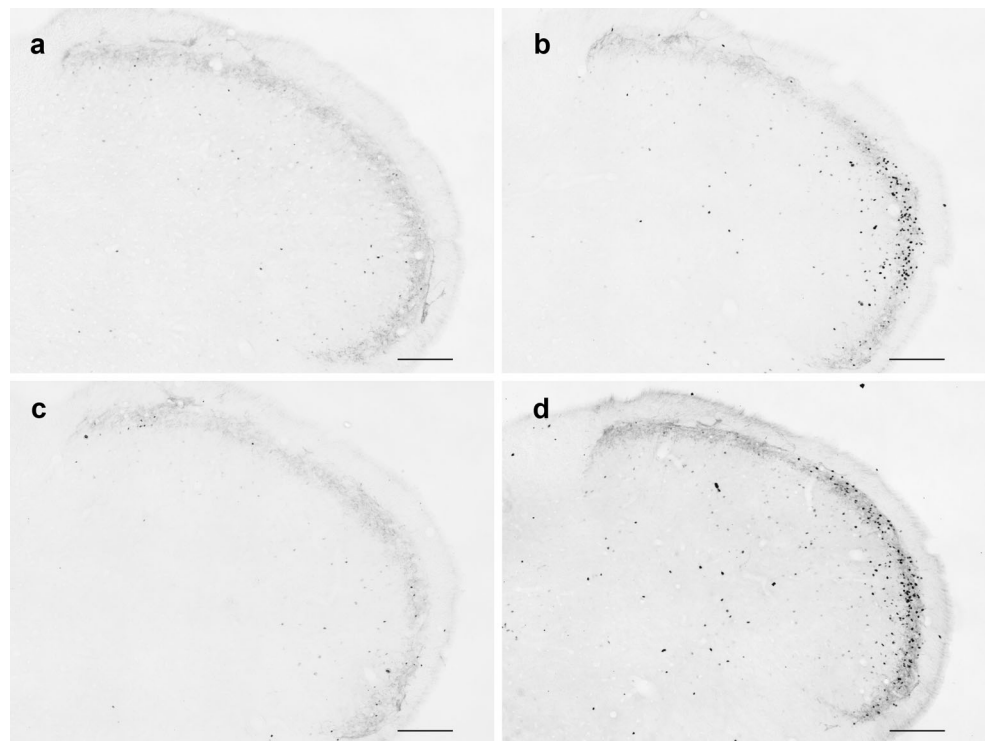
We observed a similar pattern in the robust stimulation paradigm, the number of c-Fos IR cells increasing profoundly, indicating the activation of the trigeminal system.

The main difference seen between the two paradigms was in the number of cells activated after stimulation. Following the robust stimulation procedure more c-Fos IR cells were found in the TNC, suggesting a higher degree of activation than in the mild stimulation procedure. This higher degree of activation may be attributed to the higher frequency applied in the robust paradigm, which can lead to the more rapid firing of the primary trigeminal neurons. An increased firing rate may cause increased levels of transmitter release at both central and peripheral terminals, resulting in a higher degree of activation at the TNC level (Samsam et al. 1999). It is also plausible that the longer stimulation interval leads to more primary trigeminal cells being activated in the TG, and hence in the TNC. We assume that in our experimental setting both the increased frequency and the increased stimulation interval contributed to the higher activation level in the TNC.

Pre-treatment with the P2X7-R antagonist BBG was effective only in the robust stimulation paradigm, resulting in a decrease of the activity, reflected by the c-Fos expression. P2X7-Rs can be found in the trigeminal system, both in the ganglion (Teixeira et al. 2010) and in the TNC (D'Amico et al. 2010), therefore BBG may modulate peripheral and central processes. BBG could modulate the nociceptive processing by interfering with the peripheral neurogenic inflammation, or by modulating non-synaptic communication within the ganglion (Matsuka et al. 2001). At the central level, BBG could affect P2X7-Rs on central presynaptic terminals and modulate glutamate release (D'Amico et al. 2010), and thereby influence nociceptive transmission. Presumably, after the robust stimulation, where the more pronounced peripheral activation and more severe inflammation also involve P2X7-Rs, the blocking effect of BBG manifests, while in the mild paradigm due to the minor changes the effect of BBG does not emerge.

Neither the mild nor the robust stimulation procedure caused alterations in the levels of CGRP. It was earlier found that electrical stimulation of the trigeminal ganglion

Fig. 6 Summary of the results from the orofacial formalin test regarding c-Fos immunostaining. Representative photos from the right (injected) sides of the four treatment groups after c-Fos immunohistochemistry taken at 16.05 mm caudally from bregma: **a** SSal, **b** SForm, **c** BSal, **d** BForm. Scale bar 200 μ m. Diagram showing the mean number of c-Fos immunoreactive cells along the rostro-caudal axis in the TNC in the orofacial formalin test (group mean \pm SEM) (**e**). Injection of saline did not cause any increase in the number of c-Fos-positive cells (SSal and BSal groups). The effect of formalin was visible only on the injected side; the control sides were similar to those in the saline-injected controls. For clarity, only the control side of the SForm animals is presented. Formalin caused an increase in c-Fos cell number on the ipsilateral side as compared with the saline-injected side of the controls (SForm compared with SSal), significant at the levels -16.59 to -15.51 mm ($###p < 0.001$). In the BBG-treated rats, formalin had a similar activity-increasing effect, significant at the levels -16.59 to -14.97 mm ($^s p < 0.05$; $^{ss} p < 0.01$; $^{sss} p < 0.001$). The groups SForm and BForm did not differ significantly from each other

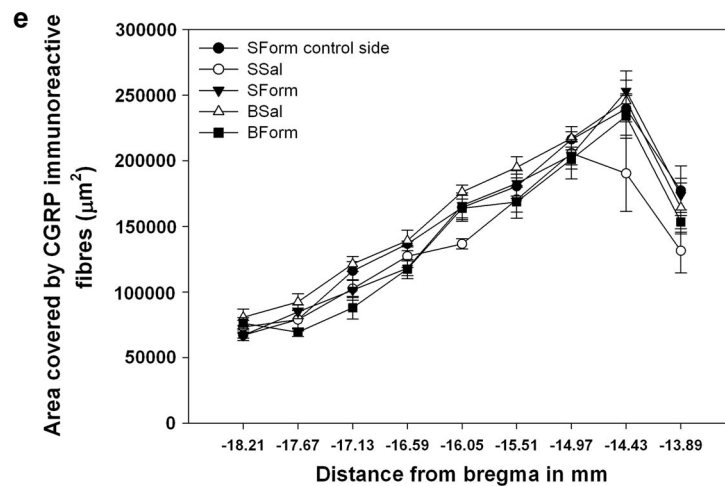
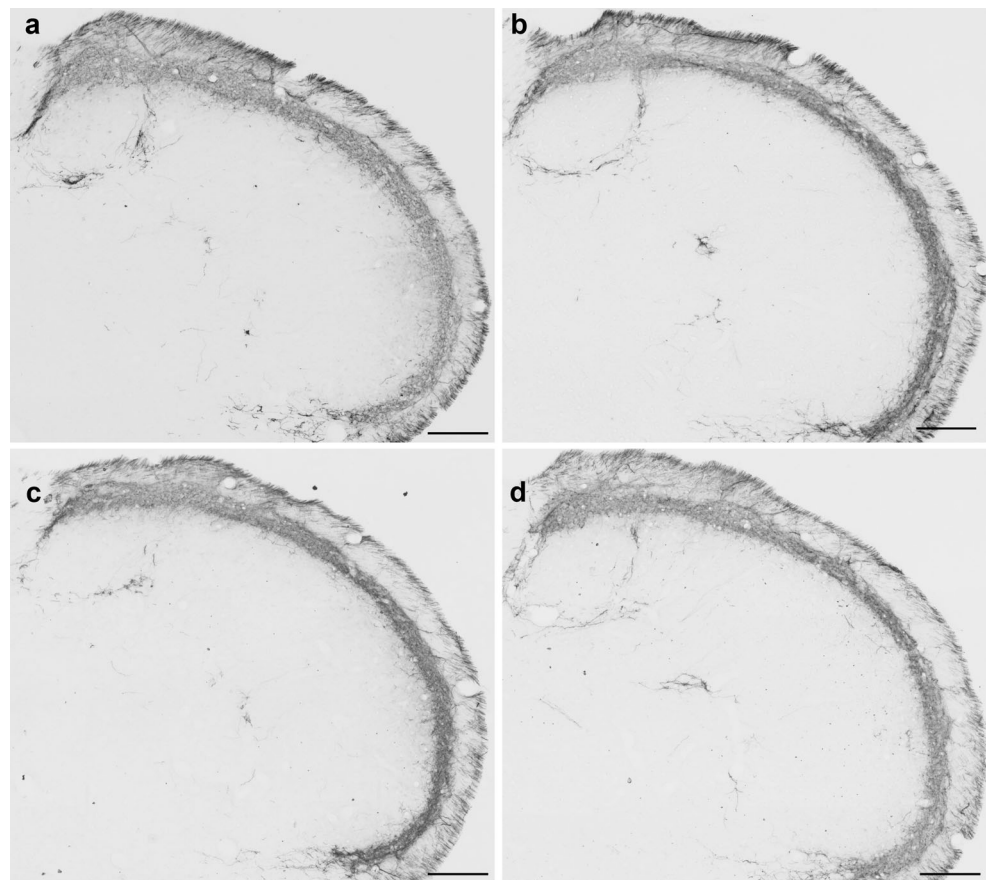


with parameters similar to our robust stimulation, led to the depletion of CGRP from the medial one-third of the central terminals of the trigeminal afferents (Knyihar-Csillik et al. 1998). However, those examinations were conducted immediately after stimulation of the trigeminal ganglion, whereas in our experiments a 2 or a 4 h survival time was included for better observability of the activity changes (c-Fos). These periods might be sufficient for the depleted CGRP to be resynthesised and for the changes in CGRP immunoreactivity seen immediately after stimulation to normalise. BBG treatment did not modify the levels of CGRP in either the sham or the stimulated group.

The injection of formalin into the whisker pad causes a biphasic behavioural effect (Clavelou et al. 1989), as also

seen in our experiments. The first short and intense phase is thought to be caused by the immediate activation of the A δ and C fibres and may be referred as acute pain. The second phase is less intense, but prolonged, and probably caused by sensitization of the trigeminal system due to the inflammatory processes occurring at the periphery. BBG did not exhibit any effect in the first phase of the formalin response. When formalin was applied to the hind paw and was combined with a selective P2X7-R antagonist, A-438079, in previous work, protective effect was exerted only in the second phase of the formalin test (McGaraughty et al. 2007). Furthermore, BBG was earlier shown to be hyperalgesic in the modulation of acute nociception in the hot-plate test (Ando et al. 2010). These results suggest that

Fig. 7 Summary of the results after CGRP immunostaining in the orofacial formalin test. Representative photos of CGRP immunohistochemistry on the *right sides* of the TNC from the four treatment groups taken at 14.97 mm caudally from bregma **a** 5SSham, **b** 5SStim, **c** 5BSham, **d** 5BStim. Scale bar 200 μm . Diagram showing the area covered by CGRP-immunoreactive fibres at the different levels of the TNC in the different treatment groups in the orofacial formalin test (group mean \pm SEM) (**e**). There was no significant difference between either the control or injected sides or the different groups. For clarity, only the control side of the saline-treated formalin-injected group is presented



BBG and blockade of the P2X7-Rs may not be effective against acute nociception.

In the second phase of the formalin test, BBG did not demonstrate any obvious effect. At the beginning of the second phase (in blocks 5–7), the nociceptive scores revealed a decreasing tendency, while in the later blocks the opposite could be observed. Since another P2X7-R antagonist was effective when formalin was applied at the hind paws, our results suggest that the role of the P2X7-Rs in the sensory system is not uniform.

Four h after formalin injection, c-Fos immunohistochemistry revealed that the TNC displays clear activation. The pattern of activation corresponds to the somatotopic projection pattern of the injected area. BBG had no effect on the activation of the trigeminal system after formalin. Our results in the orofacial formalin test are somewhat surprising, considering that other antagonists of the P2X7-Rs (Borsani et al. 2010; Honore et al. 2006; McGaraughty et al. 2007) and even BBG (Ando et al. 2010) have proven effective in numerous inflammatory models. However,

none of these experiments related to the trigeminal system, and our results are the first regarding the effects of blockade of the P2X7-Rs by BBG in this area after inflammation caused by formalin.

The levels of CGRP were not altered 4 h after formalin injection, and following treatment with BBG. Alterations in CGRP usually occur immediately during or after the applied stimulus and cease within a matter of hours (Buzzi et al. 1991; Greco et al. 2008), and our results agree with this. However, in the nitroglycerin model, changes in CGRP immunoreactivity were seen 4 h after nitroglycerin administration, suggesting that the alterations in CGRP levels can be long-term. The effect of P2X7-R antagonism on the expression of CGRP in the formalin test should be further elucidated with regard to the time scale.

BBG in the micromolar range was previously shown to inhibit voltage-dependent sodium channels *in vitro* (Jo and Bean 2011), and it might, therefore, be possible that this feature of BBG contributes to its effects in our experiments. This is rather unlikely, considering that we applied BBG in a single dose, which has been shown not to reach micromolar levels even after continuous administration in mice (Diaz-Hernandez et al. 2012).

In conclusion, our results suggest that P2X7-Rs have a role in the modulation of trigeminal nociceptive processing. Further investigations of the relations of the trigeminal system and P2X7-R signalling may provide important details concerning trigeminal nociceptive processing and the pathomechanism of headaches.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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