

**Activation pattern and modulation of pain related
structures in animal models of migraine**

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Summary of Ph.D. Thesis

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- II.** Fejes-Szabó A, **Bohár Z,** Nagy-Grócz G, Vámos E, Tar L, Pödör B, Tajti J, Toldi J, Vécsei L, Párdutz A.
Effect of Probenecid on the Pain-Related Behaviour and Morphological Markers in Orofacial Formalin Test of the Rat.
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- III. Bohár Z,** Fejes-Szabó A, Tar L, Varga H, Tajti J, Párdutz Á, Vécsei L.
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Introduction

Migraine is defined as a neurological disease characterised by spontaneous recurrent attacks of headache, lasting for 4-72 hours without treatment with accompanying symptoms of nausea, vomiting, photo- and phonophobia. Migraine belongs to the family of primary headache disorders and despite the enormous research effort the exact pathomechanism of the disorder is still unknown.

This disease affects 14.7% of the population in Europe, the economical burden caused by the disease is significant, and underlines the need for further research seeking answers for open questions in its pathomechanism.

Several theories emerged to explain the pathomechanism of migraine, however no theory proved to be completely applicable to explain the cause and all the detailed aspects of the disease. The first concept was proposed in the 1950's and hypothesized, that intracranial vasospasm is causing the aura symptoms, while extracranial vasodilatation is responsible for the migraine pain. The neurogenic theory stated that the primary origin of the migraine attack is neuronal. The vascular and neuronal theory was combined, resulting in the view that the primary cause of the headache derived from the activation of the primary afferent neuronal fibres, which released inflammatory substances leading to plasma protein extravasation, resulting in neurogenic inflammation. The initiating step, however is not completely known in these models.

During spontaneous migraine attacks the dorsolateral pons and the dorsal midbrain involving the nuclei nucleus raphe magnus (NRM), nucleus raphe dorsalis (DR) locus coeruleus (LC) and the periaqueductal grey matter (PAG) are activated and their activation persist even after the successful pain control with sumatriptan, a specific drug used in migraine therapy. This activation was shown to be migraine specific, and provided the basis of the theory that the above mentioned nuclei initiate the migraine attack, they are the so called "migraine generators". All of these nuclei are important elements of the pain modulating system, however their initiating role in the migraine attack remains elusive.

To understand the mechanism of headache generation during the attack, the knowledge about the functioning of the trigeminal system, which conveys sensory information from the head, is essential. The trigeminal nerve is divided into three branches, nociceptive information is transmitted throughout the sensory ganglion of the trigeminal system, the trigeminal or Gasserian ganglion. The central projections of the primary nociceptive neurons terminate in the superficial layers of the upper cervical segments and in

the caudal part of the spinal trigeminal nucleus (TNC), forming the trigemino-cervical complex.

In the research of migraine pathomechanism animal models are used, one of which is the electrical stimulation of the trigeminal ganglion (ESTG). ESTG activates both first and second order trigeminal neurons, it causes plasma protein extravasation and neuropeptide release in the periphery, resulting in neurogenic inflammation, while in the TNC it increases the number of c-Fos immunoreactive cells, demonstrating neuronal activation. The amount of c-Fos immunoreactive cells in the TNC is highly dependent on the applied stimulation intensity and frequency indicating that the utilization of several stimulation settings may be advisable.

Subcutaneous injection of formalin in to the orofacial area is used to examine inflammatory pain reactions in the trigeminal system in animals both at the behavioural and at the molecular level. The behavioural response of the animals can be measured by the time spent scratching of the injected area. The behavioural response to formalin is biphasic, the first phase is more intense and short lasting, while the second phase is prolonged but less intense.

The purine molecule ATP was proposed to have an important role in the regulation of nociceptive transmission, their receptors are divided into two main classes, the P2X and P2Y receptors. Among the ligand-gated P2X receptors the P2X7 receptor (P2X7-R) has been intensively studied in different pain states. The P2X7-R is a non-selective cation channel, and was proposed to be involved in nociceptive processing. Brilliant Blue G (BBG) is a selective, non-competitive P2X7-R antagonist, with good blood-brain barrier permeability and was earlier proposed to be effective in neuropathic pain and also in the nitroglycerin (NTG) induced migraine model. These results lend support to the theory that P2X7-Rs may play a crucial part in the development of headache disorders.

Probenecid (PROB, *p*-(di-*n*-propylsulfamyl)benzoic acid) is a nonselective organic anion transporter and multidrug resistance-associated protein inhibitor, and previously was shown to be effective also in the NTG migraine model, suggesting that PROB may have anti-inflammatory and anti-nociceptive properties.

Aims

Our aims in the present study were

- I. To evaluate the activation pattern of the migraine generator nuclei after electrical stimulation of the trigeminal ganglion at different survival times in the rat.
- II. To examine the effects of a purinergic P2X7 receptor antagonist, BBG on the activation in the TNC caused by two different electrical stimulation settings.
- III. To test the effects of BBG in the orofacial formalin test of the rat both at the behavioural and on the molecular level.
- IV. To detect the effects of probenecid on behaviour and on nociceptive activation in the orofacial formalin test of the rat.

Materials and methods

Experiments were approved by the Committee of Animal Research at the University of Szeged (I-74-14-16/2008; I-74-12/2012) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XI./15.1/02384/001/2007; XXIV/352/2012). Adult male Sprague-Dawley rats were used.

Activation pattern after trigeminal stimulation

Twenty-six rats were divided into two groups. In the first group (n=13) a concentric bipolar electrode was lowered to the right trigeminal ganglion of the animals and was maintained in it for 30 min during deep chloral hydrate anaesthesia. The animals in the second group (n=13) were stimulated for 30 min with square pulses at 10 Hz, 0.5 mA, with a pulse duration of 5 ms.

From both groups 7 animals were perfused transcardially two hours after the placement of the electrode, while 6 animals were perfused four hours after the electrode placement. The brains and cervical spinal cord were removed and postfixed overnight.

After cryoprotection, 30 μm thick serial sections were cut from the TNC and from the brainstem and were processed for c-Fos immunohistochemistry. Cells were counted in the TNC, NRM, PAG, DR and in the LC, and the number of cells per area (μm^2) was calculated.

Effects of BBG after trigeminal activation

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 was injected with physiological saline. Two hours after the BBG or saline injection, the animals were deeply anesthetized with chloral hydrate (400 mg/kg). Half of the animals from the saline-treated group (5SSstim) 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. 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 maintained under deep anaesthesia for 2 hours, then were transcardially perfused and processed for c-Fos and CGRP immunohistochemistry.

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 for 4 hours from the beginning of the stimulation. These parameters are equivalent to the ones applied in the activation pattern experiments.

Overview of the robust stimulation groups:

Saline + 30-min sham: 30SSham (n=6)

Saline + 30-min stimulation: 30SSstim (n=5)

BBG + 30-min sham: 30BSham (n=4)

BBG + 30-min stimulation: 30BStim (n=6)

Orofacial formalin test

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 30x30x30 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 45-min period was divided into 15x3-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. Four hours after the formalin or saline injections the animals were transcardially perfused and processed for c-Fos and CGRP immunohistochemistry.

In all groups c-Fos immunoreactive cells were counted at the different levels of the TNC, while the area covered by CGRP immunoreactive fibres was measured by ImageProPlus 6.2 software. Data from different levels of the TNC were handled separately, and analysed by two-way repeated measures ANOVA. The group were 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.

Effects of probenecid after trigeminal activation

Behavioural test and immunohistochemistry

Sixty rats weighing 200-250 g were used, they were divided into two groups (n=30 per group). The animals in the Placebo group received only intraperitoneal (i.p.) vehicle solution (physiological saline, 1.5 mL) as pre-treatment. In the PROB group, the rats were pre-treated with an i.p. injection of PROB (Sigma-Aldrich; 1 mmol/kg body weight, diluted to 1.5 mL, pH 7.4). One hour after the PROB or vehicle pre-treatment, half of the animals in both groups received a s.c. injection of 50 µL 1.5% formalin solution into the right whisker pad (Placebo-Form and PROB-Form), while the other half of the rats were injected with s.c. 50 µL physiological saline without formalin (Placebo-Phys and PROB-Phys).

The behavioural experiments were conducted as described previously at the BBG experiments, the habituation period starting fifty minutes after the pre-treatments.

Four hours after the formalin or saline injections animals (n=10) were transcardially perfused and processed for c-Fos and neuronal nitric oxide synthase (nNOS) immunohistochemistry. Cell counting and statistical analysis was performed as previously described.

Western blotting

Four hours after the formalin or physiological saline injection, the rats (n=5 per subgroup) were deeply anaesthetized with chloral hydrate, perfused transcardially with 100 mL ice cold PBS and the ipsi- and contralateral side of the TNC between 0 and -4 mm from the obex was removed and processed for IL-1 β and β -actin Western blotting.

Results

I. Activation pattern after trigeminal stimulation

Activation of the TNC after trigeminal ganglion stimulation was previously demonstrated by several studies. Our results also show the clear activation of the ipsilateral side of the TNC, while on the contralateral side and in the sham animals no significant increase in c-Fos cell number could be detected. The different survival times did not show significant alterations in c-Fos positive cells, however at 4 h a decreasing tendency in the cell numbers could be observed.

Among the brainstem migraine generator nuclei we found significant increase in the number of c-Fos positive cells only in the NRM, the other nuclei (LC, DR, PAG) did not show alterations in c-Fos expression after stimulation. Furthermore, no side difference could be noticed in these nuclei, the cell numbers on the stimulated and on the control sides were similar. The different survival times did not influence the cell numbers in the different nuclei, with one exception, in the LC the c-Fos positivity decreased after 4 hours of survival compared to the 2 hours survival group. The linear regression analysis did not reveal a significant connection between the increased cell number in the TNC and NRM ($R^2 = 0.252$; $F=3.708$; $p=0.80$).

II. Effects of BBG after trigeminal activation

Mild stimulation procedure

The comparisons of the cell numbers from the control (left) sides for each of the four treatment groups did not reveal any significant changes. Electrical stimulation of the ganglion caused a significant increase in the number of c-Fos-immunoreactive cells along the whole extent of the examined region of the TNC. 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.

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.

Robust stimulation procedure

There was no significant difference between the control sides of the four treatment groups. Robust stimulation caused a marked increase in the number of c-Fos immunoreactive cells in the saline-treated animals. BBG had a significant attenuating effect on this increase.

CGRP expression was not altered by stimulation or BBG administration in any of the animal groups in the robust stimulation setup.

Orofacial formalin test

The analysis revealed that the nociceptive scores of the saline-injected groups (SSal and BSal) did not differ from each other at any time point. The injection of formalin caused a significant increase in the nociceptive scores in blocks 1 and 5-7. 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. The BBG-treated animals spent less time rubbing their formalin-injected side in both blocks 1 and 5-7, but this difference was not significant compared with the SForm group. 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.

The two phases of the formalin test were distinguishable in our experiments, so we examined the data not just block-by-block, but also in the two phases separately. In the first phase of the formalin test an increase in the nociceptive scores can be seen in both the SForm and BForm groups. In the second phase a similar pattern could be observed, showing more clearly the lack of effect of the BBG treatment on formalin induced nociceptive behaviour.

There was no significant difference when either the control (left) sides or the control and saline-injected sides were compared at any level regarding the c-Fos cell numbers. The injection of formalin increased the number of c-Fos immunoreactive cells significantly at the levels -16.59 mm 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. There was no significant difference between the SForm and BForm groups at any level.

No group difference was found in any of the measured parameters regarding the CGRP immunoreactivity.

III. Effects of probenecid after trigeminal activation

The behavioural pattern observed in our experiments was in accordance with previous findings, as animals showed increased grooming activity after the formalin injection. We found significant differences in the first and fifth to eleventh blocks between the Placebo-Phys and Placebo-Form groups. The pre-treatment with PROB decreased the formalin-induced nociceptive behaviour significantly in each block. However, PROB pre-treatment did not abolish the effect of formalin completely in the first block, where the time spent on rubbing was still significantly higher than that in the Placebo-Phys group. There was no significant difference between the Placebo-Phys and PROB-Phys animals in any block, nor was between the PROB-Form and PROB-Phys subgroups in any other block apart from the first.

C-Fos immunohistochemistry revealed that in the Placebo-Form group, unilateral formalin injection produced an increase in the number of c-Fos-IR neurones in the dorsal, superficial area of the transverse sections of the ipsilateral TNC as compared with the non-treated contralateral side. This increase was significant at different levels along the rostrocaudal axis (between -0.3 and -3.3 mm from the beginning of the TNC), in accordance with the somatotopic representation of the injected area. In the PROB-Form group, the number of c-Fos-IR neurones at the different levels along the rostrocaudal axis also increased in the ipsilateral TNC. This effect was similar, but less pronounced than that in the Placebo-Form group, i.e. PROB significantly decreased the formalin-related activation of the second order trigeminal neurones at several levels of the TNC (between -0.3 and -2.4 mm). On the contralateral sides of the TNCs, no significant differences were noted either between the groups or between the different levels along the rostrocaudal axis.

The nNOS immunohistochemistry resulted in neurones showing cytoplasmic and dendritic staining. The unilateral s.c. formalin injection gave rise to an increase in the number of nNOS-IR neurones in the dorsal, superficial area of the ipsilateral TNC compared to the non-treated contralateral side in the Placebo-Form group. Along the rostrocaudal axis, significant increase can be observed at different levels (between -2.1 and -2.7 and between -3.3 and -3.9 mm). In the PROB-Form group, there was no difference in the number of nNOS-IR neurones between ipsilateral and contralateral TNC, PROB significantly decreased the number of the second order trigeminal neurones expressing nNOS at several levels of the

TNC after formalin injection (between -2.1 and -2.7 and between -3.3 and -3.9 mm). On the contralateral sides of the TNCs, no significant differences were noted either between the subgroups or between the different levels along the rostrocaudal axis.

The Western blot analysis of the TNC segments did not show significant difference between the contra- and ipsilateral sides for any of the groups in IL-1 β , which means that IL-1 β expression did not change four hours after the formalin injection and PROB pre-treatment did not have any modulatory effect on the expression of IL-1 β .

Discussion

Electrical stimulation of the trigeminal ganglion has a direct effect on the primary trigeminal sensory neuron causing alterations in both the peripheral and the central endings. In the periphery, mediators from the nerve endings around the meningeal vessels are released, which results in plasma protein extravasation and eventually neurogenic inflammation. In the central arm, there is a marked activation of the second-order neurones in the TNC. Our results are in accordance with these previous findings as we found marked increase in the number of c-Fos IR cells in the ipsilateral TNC both two and four hours after stimulation. This pronounced increase may arise directly from the electrical stimulation, or is a secondary phenomenon originating from the periphery due to the triggered dural extravasation and inflammation.

We were interested in the activation pattern of the migraine generator nuclei after ESTG, to examine if this model is suitable for mimicking the functional pattern of these nuclei observed in human spontaneous migraine attacks.

We observed a significant increase in neuronal activity in the NRM in the two hour survival group, which persisted even four hours after the stimulation. Direct TNC projections to the NRM are sparse, and the superficial laminae, which are mainly activated in response to electrical stimulation, display even fewer connections to the NRM; accordingly activation of the TNC may not evoke a direct NRM activation. This hypothesis is further supported by the fact that we did not find any correlation between the increased cell activities in the two nuclei neither after 2 nor after 4 hours, suggesting that the increased function of the NRM may not be a direct consequence of the activation of the TNC. Although, there is no correlation between the activation patterns of these two nuclei, the increase in their activity is present simultaneously at both 2 and 4 hours after stimulation.

The NRM is thought to be the main output of the descending pain modulatory system, providing dense innervations to the spinal cord dorsal horn and to the spinal trigeminal nuclei. We assume that the increased activity of the NRM may be a consequence of activation of this system. Increased function could result from the cortical and thalamic input of the NRM, or it could originate from the PAG.

No change in the number of c-Fos immunoreactive nuclei was noticed in any region of the PAG. There is a direct connection between the TNC and the PAG, thus it is surprising that a strong stimulus like we applied caused no alteration in this area. Previous studies indicated an increase in activity of the PAG after nociceptive stimulation, although the stimulation parameters and settings differed from those we applied.

Activity of the cells in the LC did not change, however the activity of this nucleus was already pronounced in the sham group two hours after the stimulation, suggesting that the surgical procedure itself may hide the changes caused by the electrical stimulation. After four hours of survival the activity level of the LC is decreased both in the sham and stimulated groups, indicating that the activation of the LC is due to the invasive procedure.

There is no evidence that the TNC projects directly to the DR. The DR forms part of the pain modulatory system, and receives dense afferents from the NRM, but cortical input can also modulate the activity of this nucleus. We did not find significant change in the number of c-Fos IR cells in the DR after ESTG. Our results suggest that the activation of the trigeminal system does not cause the activation of the DR, thus in the rat this nucleus may not participate in the short-term regulation of trigeminal nociceptive processing.

If it is assumed that the changes seen in our experiments are secondary, the lack of direct projections from the TNC to most of the above-mentioned nuclei supports this hypothesis. In this concept, the stimulation activates the ascending nociceptive routes to the thalamus and to the cortex, and these structures react to the nociceptive input by activating the descending modulatory system. After trigeminal stimulation, the examined nuclei failed to exhibit the uniform activation detected during a migraine attack suggesting that this activation pattern of the migraine generator nuclei may be exclusively present in migraine.

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, and this was supported by our results.

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. 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 and in the TNC, 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. At the central level, BBG could affect P2X7-Rs on central presynaptic terminals and modulate glutamate release, 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 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. 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 resynthesized and for the changes in CGRP-immunoreactivity seen immediately after stimulation to

normalize. 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, as also seen in our experiments. 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. Furthermore, BBG was earlier shown to be hyperalgesic in the modulation of acute nociception in the hot-plate test. These results suggest that 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 hours after formalin injection, c-Fos immunohistochemistry revealed a clear activation in the TNC. The activity 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 and even BBG 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 hours 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, and our results agree with this. However, in the nitroglycerin model, changes in CGRP immunoreactivity were seen 4 hours 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.

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 functions and the pathomechanism of headaches.

In our experiments PROB produced a pronounced anti-nociceptive behavioural effect in the orofacial formalin test especially in the second phase. PROB exhibited no effect on the baseline activity of the control animals, which received a s.c. saline injection in the whisker pad. In addition to the modulation of the nociceptive behaviour, PROB also mitigated the formalin induced c-Fos and nNOS expression in the TNC – considered markers of activation and sensitization of these neurons.

Previously PROB proved to be effective after systemic nitroglycerin administration, decreasing the levels of sensitization markers and modulating CGRP levels in the TNC. The anti-nociceptive effect of PROB along with its inhibitory effect on the trigeminal activation and sensitization can be the result of various mechanisms:

PROB inhibits MRP4, which releases key inflammatory mediators such as PGE₁ and PGE₂ from cells in a time- and ATP-dependent manner. PGE₂ can sensitize transient receptor potential channel subtype V member 1 (TRPV1), a key factor in the development of sensitization during nociception. Moreover, PGE₂ can induce CGRP release from cultured trigeminal ganglion cells. PGE₂ can also act on the central site of the trigeminal system by enhancing the capsaicin-induced CGRP release in slice preparations of the TNC. The expression of cyclooxygenase-2, the inducible enzyme of prostaglandin synthesis has also been detected in the neurones and glial cells in the trigeminal ganglion and in the neurones of the TNC. In addition, prostaglandin receptors are present in the trigeminal ganglion, co-expressed with TRPV1, with a functional interaction between them in the trigeminal system, and are involved in mediating CGRP release from cultured rat trigeminal neurones. Quantitative autoradiography has revealed that TNC has a moderate density of PGE₂ binding sites as well. Accordingly, a possible mechanism of action of PROB is the inhibition of the release of PGE₂, a key player in trigeminal nociception and an important molecule in migraine genesis through the blockade of MRP4.

Data suggest that PROB is able to activate some of the transient receptor potential channels, including TRPV2 and TRPA1 which play an important role in pain perception and are present in the trigeminal system. However, the agonist-mediated desensitization of these receptors is also known, which can cause an anti-nociceptive effect. PROB can desensitize the TRPA1 and may act similarly on TRPV2 as well, which might account of its anti-nociceptive properties in the present experiments.

PROB also inhibits the pannexin-1 channel, which is expressed at several sites in the central nervous system; and is crucial in caspase-1 activation leading to the production and release of interleukin-1beta (IL-1 β) in neurones, astrocytes and macrophages. The release of

IL-1 β from trigeminal ganglion satellite cells may contribute to the mechanisms underlying trigeminal inflammatory hyperalgesia. Up-regulation of astrocytic IL-1 β in the TNC can lead to central sensitization via its receptors present on trigeminal neurones in the same area. These results clearly indicate that IL-1 β plays a role in trigeminal activation. However the results of our Western blot measurement showed that there were no changes in the expression of IL-1 β at the level of TNC four hours after s.c. formalin administration and suggesting that PROB may not influences IL-1 β in the TNC, but one can not exclude the possibility that PROB affects IL-1 β in the peripheral sites of the trigeminal system.

PROB can increase the concentration of kynurenic acid, an endogenous tryptophan metabolite in the central nervous system. This phenomenon can be caused by inhibition of organic anion transporters by PROB, which are involved in the transport of kynurenic acid from brain through the blood-brain barrier or by increasing the concentration of tryptophan in the central nervous system. Experimental data suggest that kynurenic acid can affect nociception and elevation in its concentration in the brain can inhibit the trigeminal activation, probably due to an antagonistic effect on ionotropic glutamate receptors or due an agonistic action on G-protein-coupled receptor-35.

Conclusions

ESTG caused clear activation only in the NRM, but not in the other migraine generator nuclei. This activation pattern does not correspond to what was seen in migraine patients during the attack. The origin of the brainstem activation is not known, it can be the consequence of pain, but the persistent activation of these structures after headache cessation suggests, that the activation of these nuclei is specific for migraine. Our result support this hypothesis, as the activation of the trigeminal system not resulted in the activation of the migraine generator nuclei. However, the exact mechanisms of brainstem activation in migraine patients needs to be further elucidated. Our result in the rat demonstrate that the descending pain modulatory system can be studied in this model, and the further characterization of molecular mechanism in the rat may provide useful information about headache pathomechanism.

Blocking the P2X7-R by BBG was able to modulate trigeminal activation only after the robust stimulation, suggesting that the role of P2X7-Rs in trigeminal nociception is complex, and may depend on the stimulus applied. Similar controversial effects were

observed in an earlier work with BBG, supporting that a more detailed evaluation of P2X7 receptor functioning in the trigeminal system is needed.

PROB showed clear antinociceptive effect in the formalin test, and also in previous experiments, however it is not clear which of its molecular targets is responsible for this effect.

Our result provide important information about the functioning of the trigeminal system, thus contribute to the understanding of pathological processes underlying headache and migraine.

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