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Kseniia Koroleva

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ABSTRACT

Migraine is a widespread disease, accompanied by a severe headache that adversely affect the quality of life. Due to the insufficient effectiveness of existing drugs, the search for new methods of treatment and prevention of migraine pain remains highly relevant. Multiple studies assign the leading role of the trigeminovascular system (TGV) in generation of a pain signal. TGV includes meningeal vessels, trigeminal afferents, and local mast cells that form clusters around nerves and blood vessels. According to the purinergic theory of migraine, extracellular ATP released from endothelium, mast cells, platelets and other meningeal cells plays one of the leading roles in migraine pathology and in pain transmission. However, the molecular mechanisms of ATP action in meninges are still not fully understood. Hydrogen sulfide (H_2S) is a recently discovered gasotransmitter also involved in nociception. However, the role of H_2S in meningeal nociception and its action on ATP-mediated nociception were not investigated.

The aims of this project were to analyze the novel cellular and receptors mechanisms of meningeal nociception induced by ATP and its interactions with the signaling pathways activated by the gaseous transmitter H_2S.

In this project, using the electrophysiological approach, we found that ATP induced the nociceptive firing in rat and mouse trigeminal afferents in meninges. Using mice lacking mast cells we discovered that ATP induced nociceptive activity essentially depends on degranulation of dural mast cells with subsequent release of serotonin which, 5-HT3 receptors, amplifies the excitatory effect of ATP. Indeed, the specific inhibitor of 5-HT3 receptors MDL7222 significantly decreased ATP induced nociceptive firing. Our data suggest that extracellular ATP activates nociceptive firing in meningeal trigeminal afferents directly via P2X3 receptors and indirectly via mast cell-born serotonin operating via 5-HT3 receptors on the nerve terminals.

We also show that the H_2S donor NaHS transiently increased the frequency of spiking activity in trigeminal nerve fibers mainly by activation of pro-nociceptive

TRPV1 receptors. These findings suggested the pro-nociceptive of this gaseous transmitter. However, the pre-application of NaHS suppressed the nociceptive effect of ATP indicating the anti-nociceptive action of H_2S. This modulatory effect was independent from TRPV1 receptor activation. In isolated trigeminal neurons, NaHS decreased inward currents and Ca^{2+} transients, evoked by activation of purinergic P2X3 receptors. Moreover, NaHS prevented ATP-induced degranulation of meningeal mast cells mediated by purinergic P2X7 receptors. Finally, NaHS decreased the concentration of extracellular ATP in meningeal preparation. Thus, H_2S exerted the protective effect against the nociceptive effects of ATP via reduced purinergic activation of trigeminal afferents and stabilization of meningeal mast cells.

In conclusion, we revealed the new mechanisms of pro-nociceptive effects of ATP and its interaction with H_2S in the peripheral trigeminal nociceptive system. The data obtained can be used to develop novel pharmacological approaches for the treatment and prevention of pain generation in migraine.

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TIIVISTELMÄ

Migreeni on laajalle levinnyt sairaus, johon liittyy voimakas päänsärky ja joka vaikuttaa kielteisesti elämänlaatuun. Koska nykyiset lääkkeet eivät ole riittävän tehokkaita, uusien hoitomenetelmien etsiminen ja migreenikipujen ehkäisy on edelleen erittäin tärkeää. Useat tutkimukset osoittavat trigemino-verisuonijärjestelmän (TGV) olevan merkittävä tekijä kivusignaalin luomisessa. TGV sisältää meningeaaliset verisuonet, kolmenväliset afferenssit ja paikalliset syöttösolut, jotka muodostavat klustereita hermojen ja verisuonten ympärille. Migreenin purinergisen teorian mukaan endoteelistä, syöttösoluista, verihiutaleista ja muista meningeaalisista soluista vapautuvalla solunulkoisella ATP: llä on yksi johtava rooli migreeni-patologian ja kivun leviämisessä. ATP: n vaikutusta molekyylimekanismeista aivolisäkkeissä ei kuitenkaan vielä ole täysin ymmärretty. Rikkivety (H_2S) on äskettäin löydetty kaasulähetin, joka osallistuu myös valutukseen. H_2S: n merkitystä meningeaalisessa valutuksessa ja sen vaikutusta ATP-välitteiseen valutukseen ei kuitenkaan tutkittu.

Tämän projektin tavoitteena oli analysoida ATP: n indusoimia meningeaalisen valutuksen uusia solun - ja reseptorimekanismeja ja sen vuorovaikutusta kaasumaisen lähettimen H_2S aktivoimien signalointireittien kanssa.

Tässä projektissa käyttämällä elektrofysiologista lähestymistapaa havaittiin, että ATP indusoi valutuksen ampumista rotan ja hiiren kolmoisherkkyydessä aivokalvoissa. Laulamalla hiiriä, joilla ei ole syöttösoluja, havaitsimme, että ATP: n indusoima särkyvä aktiivisuus riippuu olennaisesti duraalisten syöttösolujen degranulaatiosta myöhemmin serotoniinin vapautumisen kanssa, joka 5-HT3-reseptoreilla vahvistaa ATP: n virittävää vaikutusta. Itse asiassa 5-HT3-reseptorien spesifinen inhibiittori MDL7222 vähensi merkittävästi ATP: n indusoimaa valutusta. Tietojemme mukaan solunulkoinen ATP aktivoi valkosolujen kolmenvälisissä aferensseissä notsetsiptiivista ampumista suoraan P2X3-reseptorien kautta ja

epäsuorasti syöttösoluissa syntyneen serotoniinin välityksellä, joka toimii hermostopääntöjen 5-HT3-reseptorien kautta.

Osoitamme myös H_2S-luovuttajan NaHS lisänneen ohimenevästi kolmoishermostossa esiintyvän aktiivisuuden esiintymistiheyttä lähinnä aktivoimalla posiskeptiivisiä TRPV1-reseptoreja. Nämä havainnot viittasivat tämän kaasumaisen lähettimen progesiniskeptiseen vaikutukseen. NaHS: n esisovellus kuitenkin tukahdutti ATP: n notseptiivisen vaikutuksen, mikä osoitti H_2S: n antiseptiivisen vaikutuksen. Tämä modulaatiovaikutus oli riippumaton TRPV1-reseptorin aktivoinnista. Eristetyissä kolmoishermosoluissa NaHS vähensi sisäänpäin suuntautuvia virtauksia ja Ca^{2+} -siirtoja, jotka aiheuttivat purinergisten P2X3-reseptorien aktivoitumisen. Lisäksi NaHS esti purinergisten P2X7-reseptoreiden välittämien meningeaalisten syöttösolujen ATP-indusoiman degranulaation. Lopuksi NaHS laski solunulkoisen ATP: n pitoisuutta meningeaalivalmisteissa. Siten H_2S suoritti suojaavan vaikutuksen ATP: n nokkiseptiivisiä vaikutuksia vastaan vähentämällä kolmoisherkkyysreferenssien purinergistä aktivointia ja stabiloimalla meningeaaliset syöttösolut.

Yhteenvetona voimme todeta, että ATP: n uusien posiceptisten vaikutusten uudet mekanismit ja niiden vuorovaikutus H_2S: n kanssa perifeerisessä kolmoisrenkaassa on syöpäjärjestelmä. Saatuja tietoja voidaan käyttää kehittämään uusia farmakologisia lähestymistapoja kivun muodostumisen hoitamiseksi ja ehkäisemiseksi migreenissä.

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LIST OF ORIGINAL PUBLICATIONS

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I **Koroleva K.,** Mustafina A., Yakovlev A., Hermann A., Giniatullin R., Sitdikova G. Receptor mechanisms mediating the pro-nociceptive action of hydrogen sulfide in rat trigeminal neurons and meningeal afferents. *Frontiers in Cellular Neuroscience,* 11: 226, 2017.

II **Koroleva K.,** Gafurov O., Guselnikova V., Nurkhametova D., Giniatullina R., Matilla O., Lindsberg P., Malm T., Giniatullin R. Meningeal mast cells contribute to ATP-induced nociceptive firing in trigeminal nerve terminals: direct and indirect purinergic mechanisms triggering migraine pain. *Frontiers in Cellular Neuroscience,* 13: 195, 2019.

III **Koroleva K**., Ermakova E., Mustafina A., Giniatullina R., Giniatullin R., Sitdikova G. Protective effects of hydrogen sulfide against the ATP-induced meningeal nociception. *Frontiers in Cellular Neuroscience,* 14: 266, 2020.

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Other publications:

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Zakharov A., **Koroleva K.,** Giniatullin R. Clustering analysis for sorting ATP-induced nociceptive firing in rat meninges. *BioNanoScience*, 6(4), 508-512, 2016.

Suleimanova A, Talanov M, Gafurov O, Gafarov F**, Koroleva K,** Virenque A, Noè F, Mikhailov N, Nistri A, Giniatullin F. Modeling a nociceptive neuro-immune synapse activated by ATP and 5-HT in meninges: novel clues on transduction of chemical signals into persistent or rhythmic neuronal firing. *Frontiers in Cellular Neuroscience,* 14: 135, 2020

CONTENTS

ABBREVIATIONS

5-HT	5-Hydroxytryptamine
ATP	Adenosine Triphosphate
CBS	Cystathionine-β-synthase
CGRP	Calcitonin Gene Related Peptide
CSD	Cortical Spreading Depression
CSE	Cystathionine γ-lyase
DRG	Dorsal Root Ganglion
H_2S	Hydrogen Sulfide
MMA	Middle Meningeal Artery
NaHS	Sodium Hydrosulfide
P2X	Purinergic ligand-gated ion Receptor
TNFα	Tumor Necrosis Factor
TRPA1	Transient Receptor Potential Ankyrin 1
TRPV1	Transient Receptor Potential Cation Channels, Capsaicin Receptor
α,β-meATP	P2X1/3 Receptor Agonist

1 INTRODUCTION

Migraine is a chronic disorder characterized by a complex of symptoms, including unilateral throbbing and prolonged headache, often accompanied by nausea or vomiting, as well as photophobia and phonophobia [ICHD 3d edition, 2018]. In 2015, The Global Burden of Disease Study recognized migraine as the third most common neurological disease and the seventh-largest cause of disability worldwide [Steiner et al., 2016]. Migraine with its complex pathogenesis including changes in the nervous, immune and cardiovascular systems, along with the limited effectiveness of drug treatment, is one of the most pressing unsolved issues in modern medicine.

Clinical and experimental observations indicate that the menigeal and trigeminal nerves play a leading role in migraine pain, and local vessels primarily contribute to the formation of the nociceptive signal [Olesen et al., 2009; Schueler et al., 2014]. The trigeminal nerve endings and blood vessels make up the trigeminovascular system (TGV), where the initial pain signal is initiated before transmission to the higher centers of the nociceptive system [Noseda & Burstein, 2013]. According to the purinergic theory of migraine proposed by Burnstock [Burnstock, 1981], extracellular ATP and its degradation products mediate vasodilation of meningeal blood vessels, triggering a migraine attack. We have already shown that ATP (but not ADP) stimulates the primary nerve endings located in the dura mater, which increases the frequency of action potentials in the trigeminal nerve, exerting a pro-nociceptive effect [Koroleva et al., 2019; Yegutkin et al., 2016]. However, the receptor mechanisms of ATP action in trigeminal afferents and its potential effect on local mast cells, which have been proposed as migraine triggers, are not well understood. Study of the direct and indirect mechanisms of ATP action in the trigeminovascular system should allow new molecular targets for therapeutic interventions and the prevention of migraine to be identified.

Emerging data suggest the participation of endogenous gaseous hydrogen sulfide (H_2S) in nociception [Kawabata et al., 2007; Okubo et al., 2012]. Immunohistochemical studies have shown expression of the H_2S synthesis enzyme cystathionine-β-synthase (CBS) in sensory ganglia neurons (DRG and trigeminal ganglia) [Xu et al., 2009; Feng et al., 2013]. Moreover, the level of CBS expression increases with the development of inflammation [Bhatia, 2015]. Notably, H_2S is able to modulate neuronal excitability [Kimura, 2002] suggesting it has a modulatiory role in nociception. However, the available data on the role of H_2S in the nociceptive system are contradictory [Bhatia, et al., 2005; Cunha & Verri, 2007]. On the one hand, it has been shown that H_2S is able to activate a number of ion channels such as the TRPV1 and TRPA1 receptors and T-type Ca^{2+} channels involved in the initiation of peripheral pain stimuli [Trevisani et al., 2016]. On the other hand, H_2S activates ATP - dependent potassium channels, or large-conductance Ca^{2+}-activated potassium channels, which results in membrane hyperpolarization and reduced neuronal excitability [Mustafina et al., 2015]. This gaseous transmitter also stabilizes

mast cells [Rodrigues et al., 2017; Matsui et al., 2019;]. In addition, recent studies indicate the antioxidant and anti-inflammatory potential of H_2S [Mannelli et al., 2017; Xu et al., 2019]. Co-expression of CBS and ATP-activated P2X receptors has been detected in sensory ganglia [Xu et al., 2009]. This fact indicates a possible interaction between signaling cascades activated by H_2S and ATP via P2X receptors. However, data on the effect of H_2S on purinergic mechanisms of nociception and, in particular, with migraine are absent.

Our study has strengths and limitations. Our data contribute to the development of fundamental knowledge on the purinergic mechanisms of nociception in the trigeminal innervation of meninges, generating pain signaling during migraine. Direct and indirect mechanisms of ATP action on peripheral trigeminal afferents were revealed. In particular, the role of ionotropic P2X receptors, mast cell degranulation, resulting in release of the pro-nociceptive agents such as serotonin were established. Another novelty is that the novel anti-nociceptive action of the gaseous transmitter hydrogen sulfide against ATP signaling has been shown. This expands our knowledge on the role of hydrogen sulfide in control of pain stimuli in the trigeminal system. Taken together, obtained results contribute to understanding the pathogenesis of migraine, in particular, they establish the pro-nociceptive role of P2X2/3, P2X7 and 5-HT3 receptors, acting in concert to produce migraine pain. This novel information can be used to develop new approaches for developing anti-nociceptive drugs for treatment and prevention of migraine pain.

Some limitations of the project should be mentioned. Experiments were performed at room temperature using ex vivo preparation of meninges, and we used only male rats to record the activity of the trigeminal nerve. Mast cells release different mediators, among which we took in consideration only serotonin. It would be interesting to use slow-releasing hydrogen sulfide donors to follow its effect in a longer time scale. We also did not study the vascular mechanisms, which may also have impact on the effects of ATP and hydrogen sulfide. Future experiments should consider these points by validating in vivo the mechanisms revealed in our study.

2 REVIEW OF THE LITERATURE

2.1 GENERAL CHARACTERISTICS AND PREVALENCE (EPIDEMIOLOGY) OF MIGRAINE

According to the International Classification of Headache Disorders (ICHD-3), migraine is a common form of primary headache, with 6 clinical forms, of which migraine without aura (about 70% of cases) and migraine with aura (about 30%) are the most prevalent. According to a recent global study by the World Health Organization, migraine is one of the most common neurological diseases leading to disability [Feigin et al., 2017].

Migraine without aura is characterized by a recurring headache, which manifests itself in the form of episodes lasting from 4 to 72 hours. It is characterized by a unilateral throbbing headache, moderate or severe in intensity, exacerbated by routine physical activity, accompanied by nausea and/or photophobia and phonophobia. Migraine without aura is characterized by a high frequency of episodes and is often more debilatating than migraine with aura. The frequency of migraine attacks in different people varies widely: some have several episodes per month, others are prone to attacks much less often (up to a year between episodes) [Bille, 1997]. The impact of migraine attacks on a person's social life ranges from moderate deterioration to an inability to carry out any social and physical activities.

A distinctive feature of migraine with aura is a complex of visual disturbances, including loss of visual fields, double vision, flashes of light, and flickering lines in front of the eyes. Tingling, numbness of half of the face or body are less common symptoms. As a rule, all these symptoms occur before the onset of a headache and last no more than 1 hour. Migraine attacks can be caused by a number of factors, among which, stress, hormonal changes [MacGregor, 2009], oral contraceptives, lack of food intake and lack of sleep are most often mentioned [Kelman, 2007].

2.2 CENTRAL AND PERIPHERAL MECHANISMS OF MIGRAINE

Comprehensive study of the mechanisms of migraine pathogenesis helps to better understand the changes in the body that precede or occur during episodes. There are several theories of the development of migraine associated with either a change in vascular tone or a change in the sensitivity (sensitization) of central or peripheral structures mediating nociception, there is also a theory combining vascular and neuronal changes [Goadsby, 2007; 2009; Jacobs & Dussor, 2016].

Wolff [Wolff, 1948] proposed a vascular theory of migraine, according to which migraine pain is associated with dilatation of the cranial vessels [reviewed in Goadsby, 2009]. Experimental, physiological, pharmacological and clinical data were used as evidence of this vascular theory. Wolff showed that changes in the intensity

of pulsations in the occipital and superficial temporal branches of the external carotid arteries correlate with the intensity of the headache during migraine. Notably, factors that reduce the intensity of vascular pulsation also reduced the intensity of the headache [Tunis et al., 1952; 1953].

Using modern bioinformatics, it was found that the largest group of genes associated with migraine also causes a predisposition to vascular diseases [Gormley et al., 2016], which is genetic evidence for the role of the vascular system in migraine. However, further studies opposing the role of the vascular system in the pathogenesis of migraine have shown that the release of humoral factors play a leading role and trigger the activation of sensory, sympathetic and parasympathetic neurons that tightly innervate the surrounding meningeal tissues [Jacobs & Dussor, 2016].

Olesen (1981) proposed another mechanism for the pathogenesis of migraine with aura, suggesting a secondary nature of vascular changes. His research suggests that migraine aura symptoms (such as hemianopsia, paresthesia, visual impairment, and speech impairment) develop as a result of a slowly propagating wave of neuronal depolarization in the cerebral cortex, this is called cortical spreading depression (CSD) [Olesen et al., 1981]. It was shown that CSD initially leads to narrowing and then to excessive vasodilation of the vessels in the pia mater, accompanied by protein extravasation, platelet aggregation, and mast cell degranulation. Developing this idea, further studies suggested that in migraine, pro-inflammatory mediators are released, such as histamine, bradykinin, serotonin, Calcitonin Gene-Related Peptide (CGRP), substance P, neurokinin A and prostaglandins acting on vessels of the meninges [Moskowitz et al., 1984; 1993; Bolay et al., 2002; Karatas et al., 2013]. An increase of pro-inflammatory mediators leads to sensitization which is an increase in the excitability of neurons due to the interaction of compounds with membrane receptors, followed by activation of second messenger cascades and phosphorylation of the receptors. The development of this cascade of reactions may explain some of the symptoms of migraine [Strassman et al., 1996].

The pathogenesis of migraine often includes structural and functional changes in the brain (Figure 1). Using positron emission tomography, it was possible to identify the brain structures with pathological changes in activity during a migraine. Structures with abnormal increased activity include the periaqueductal gray, red nucleus, substantia nigra, dorsal suture, locus coeruleus nuclei, hypothalamus, and posterior thalamus. On the contrary, a decrease in activity was observed in the somatosensory cortex, sphenoid nucleus, caudate nucleus, putamen, and globus pallidus [Weiller et al., 1995; Pintov et al., 1997].

In 1984, Moskowitz proposed a trigeminovascular theory of migraine, which combined the neuronal and vascular mechanisms of the pathogenesis [Moskowitz, 1984]. He suggested that a disturbance of the interactions between the cranial vessels, the trigeminal nerve fibers and the central nervous system leads to the development of sterile neurogenic inflammation in the meninges [Moskowitz, 1984]. The trigeminal nerve plays the main role in this pathological process, as the initiator of

the neurogenic inflammation and the conductor of nociceptive information to the central nervous system [Edvinsson et al., 2012]. A characteristic feature of the trigeminovascular system is the high content of vasoactive neuropeptides, including CGRP, which are stored in the sensory nerve endings innervating intracranial blood vessels [Link et al., 2008]. In addition, a close connection was found between the trigeminal and sympathetic and parasympathetic nerve fibers, which innervate dural vessels, and can also contribute to the mechanisms of meningeal nociception [Akerman et al., 2017]. The main finding was that during an episode of migraine with aura, an increase in the concentration of CGRP in the external jugular vein occurs [Goadsby et al., 1990; 1993]. One of the main properties of CGRP is vasodilation of the extracranial vasculature [Goadsby & Edvinsson, 1993].

The purinergic theory of migraine development was proposed by G. Burnstock in 1981. The theory was based on the widespread distribution of purinergic receptors in the vascular and nervous systems. Later, purinergic (ATP) receptors were found in the sensory ganglia (DRG and trigeminal ganglia) [reviewed in Burnstock, 2009]. According to the purinergic theory, ATP and its metabolites are involved in the pathogenesis of migraine, mediating vasodilation of cranial vessels [Burnstock, 1981,1982; 2013].

Interest in the pathogenesis of migraine is related to the search for effective methods of treatment and prevention. Current migraine therapy includes pharmacological, invasive, and non-invasive instrumental treatments [Schuster & Rappoport, 2016]. Among the most common medications for migraine are triptans, selective agonists of serotonin 5-HT1B/1D receptors, which have powerful vasoconstrictive effects on cranial vessels, leading to relief of migraine pain [Stepień et al., 2003; Wolff et al., 1987]. Mechanisms of triptans action are based on the inhibition of CGRP release to cranial blood vessels and, in the CNS: in the trigeminal nucleus caudalis and ventrolateral periaqueductal gray. This action of triptans serves as an evidence of the vascular tone contribution into the pathogenesis of migraine. However, the use of triptans has several limitations associated with their vasoconstrictive effect and the risk of side effects on the cardiovascular system [Obaidi et al., 2013].

Thus, the main therapeutic target in the treatment of primary headaches, including migraine, is CGRP and its receptors. There are two large groups of CGRP and its receptors antagonists: monoclonal antibodies (eptinezumab, fremanezumab and galcanezumab) [Raffaelli, et.al. 2019] and CGRP receptor antagonists named gepants (ubrogepant, MK-8031). Successful clinical trials with these compounds represent a significant step in the treatment of migraine along with the use of traditional triptans [Schuster & Rapoport, 2016].

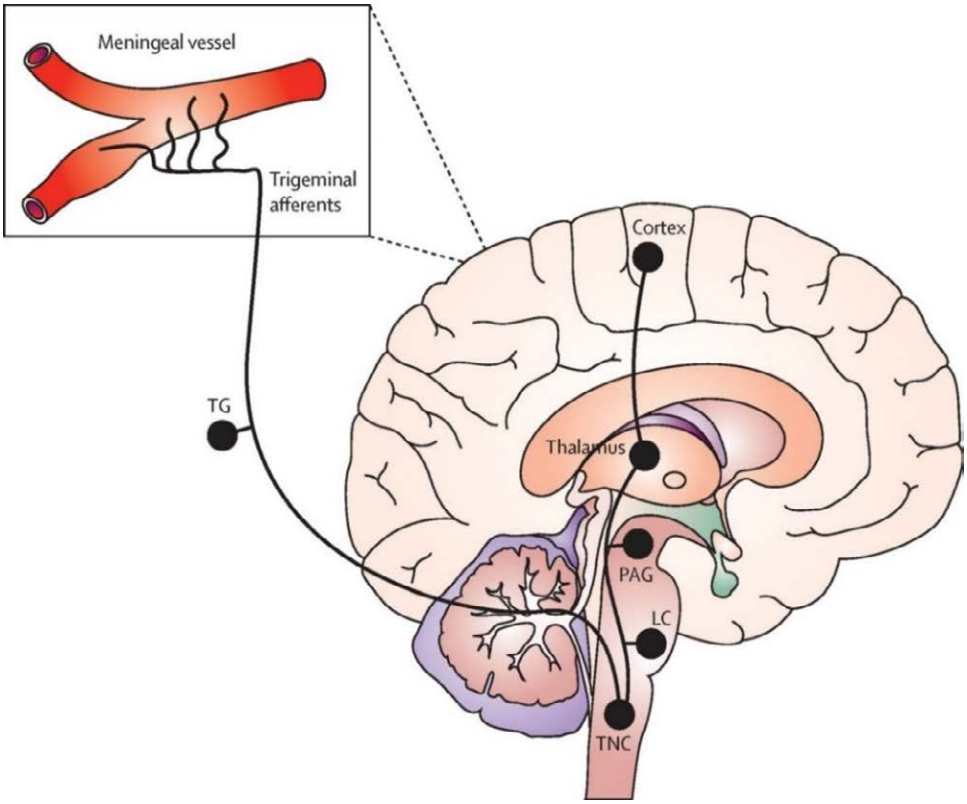

Figure 1. Schematic representation of the main brain structures involved in the pathogenesis of migraine. The trigeminovascular system consists of nociceptive sensory trigeminal afferents surrounding the vessels of the skull. After activation of the trigeminal afferents innervating the vessels, the signal passes through the trigeminal ganglion to the neurons of the trigeminocervical complex. Then nociceptive signals are transmitted to the thalamus. Modulation of the input signal occurs with the participation of the periaqueductal grey matter of the midbrain and locus coeruleus. TG – trigeminal ganglion. PAG – periaqueductal grey matter. LC – locus coeruleus. TNC – trigeminocervical complex. *Figure from* Ferrari *et al.,* 2015.

In cases of ineffective drug treatment, neurostimulation is currently being applied for pain relief using external and implantable devices. Depending on the device used, the cerebral cortex, and cranial nerves (including the branches of the trigeminal nerve and vagus nerves) can be stimulated. The advantages of this method include precise positioning and effectiveness in cases of drug-tolerant chronic migraine. The disadvantages include the high cost of stimulators, and the invasiveness of the installation procedure.

2.3 THE TRIGEMINAL SYSTEM FROM THE PERIPHERY TO THE CENTER. THE ROLE OF PERIPHERAL NERVE ENDINGS IN THE MECHANISMS OF MIGRAINE

The peripheral branch of the trigeminal nerve, called nervus spinosus, innervates the dura and pia mater of the brain meninges, including the large arteries, and venous sinuses. The use of anterograde dyes made it possible to reveal the topography of this branch of the trigeminal nerve from the trigeminal ganglion to the most distal branches. It was revealed that the nervus spinosus comes to the meninges from the middle cranial fossa, it crosses the middle meningeal artery (MMA) and then divides into 4-5 main final branches. One of the branches goes to the petrosquamous fissure and is divides into two smaller branches. The remaining branches pass in the parietal direction along the MMA [Schueler, 2014]. Thus, there is a dense innervation of the parietal-temporal membranes of meninges from which migraine pain can originate (Figure 2).

Classical histological and immunohistochemical methods confirm the close relationship between the MMA and meningeal nerve fibers [Dowgjallo, 1929; Penfield & McNaughton, 1940; Strassman et al., 2004; Messlinger et al., 1993]. Electron microscopy of the peripheral process of the trigeminal nerve, both in rats and in humans, showed the presence of numerous myelinated nerve fibers, including Aβ-, Aδ- and non-myelinated C-fibers [Messlinger et al., 1993; Strassman et al., 2004; Schueler et al., 2014]. There is an assumption that mechanosensitive Aβ fibers can be activated by sharp movement of the head [Strassman et al., 2004, Liu et al., 2008], but their role in the generation of headaches remains not fully understood [Schueler M. et al., 2014]. Aδ and C-fibers containing vasoactive neuropeptides (substance P, CGRP) in their axons conduct nociceptive information to the central parts of the trigeminal nociceptive system [McCulloch et al., 1986].

Activation of trigeminal neurons activates the trigeminocervical complex (TCC), which mediates the central mechanisms of pain [Goadsby et al., 2007].

The TCC sends the nociceptive information along ascending trigeminothalamic projections to the thalamic nuclei (posterior, lateral, ventral and posterior medial thalamus nuclei) and through them to the cerebral cortex [Noseda & Burstein, 2013].

The function of the peripheral trigeminovascular system is regulated by descending control from the endogenous antinociceptive system, which includes the locus coeruleus (noradrenergic system), nucleus raphes dorsalis (serotonergic system), and periaqueductal gray [Burgos-Vega et al., 2015]. Disfunction of the antinociceptive system can lead to prolonged maintenance of increased neuronal excitability and, as a result, contribute to enhanced transmission of nociceptive information to the central nervous system [Noseda & Burstein, 2013].

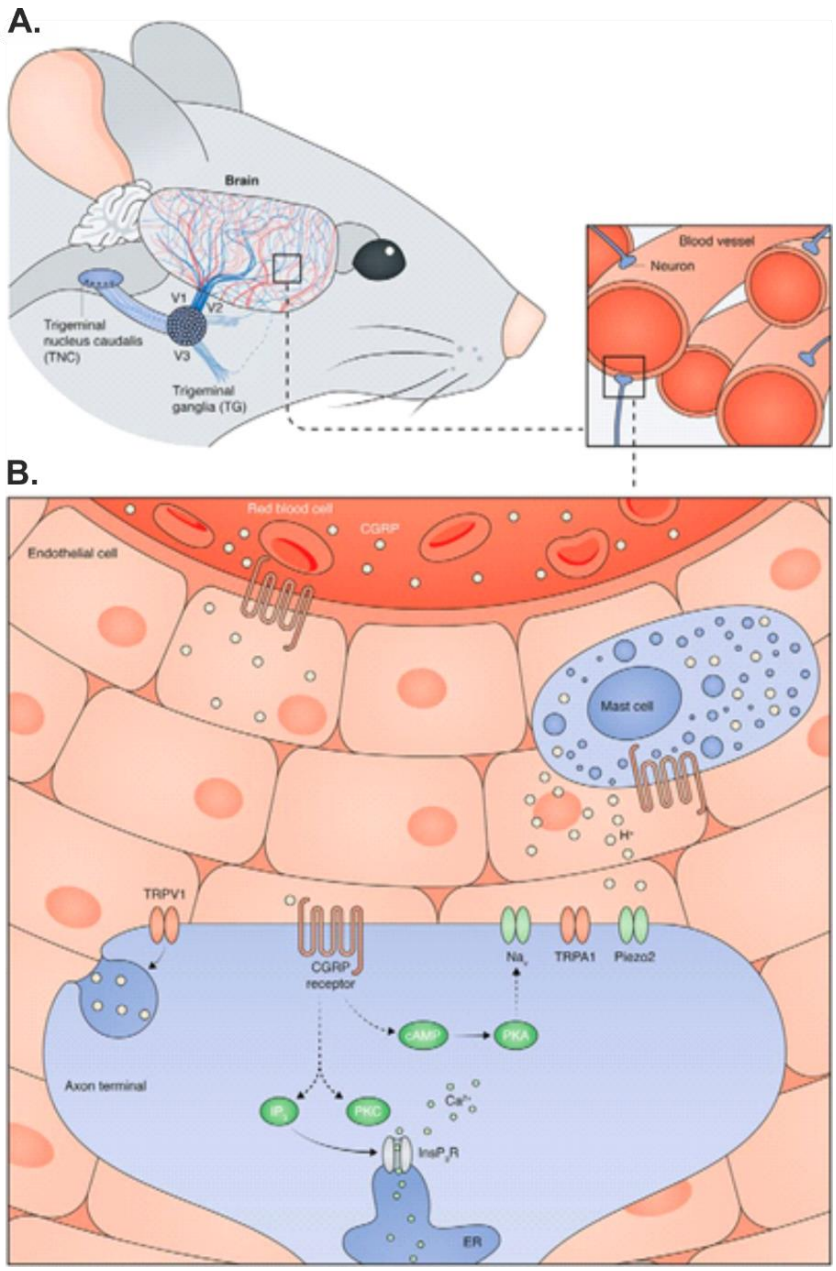

Figure 2. Schematic representation of the rat trigeminovascular system. A. schematic representation of the vessels and afferents of the V1 branch of the meningeal trigeminal nerve of the rat brain; B. A diagram of local vessel innervation by the nerve endings of the trigeminal nerve.
Figure adapted from Moehring & Sadler, 2019.

2.4 MIGRAINE PAIN MEDIATORS

Currently, there are a huge number of active substances that can cause pain or are involved in the modulation of pain processes. These can be divided into two large groups: direct algogens and pain promoters. The first group includes substances (ATP, capsaicin, protons, etc.) directly involved in the generation of a nociceptive signal as the result of activation of ionotropic pain receptors. The second group includes endogenous compounds such as neurotrophic factors, cytokines, and prostaglandins that enhance nociceptive transmission through metabotropic receptors and various intracellular signaling cascades leading to neuronal sensitization [Giniatullin et al., 2013].

2.4.1 Calcitonin Gene-Related Peptide (CGRP)

Calcitoinin gene-related peptide (CGRP), a member of the calcitonin peptide family, is a widespread neuropeptide that plays a key role in migraine. CGRP is among the compounds that cause the strongest vasodilation of peripheral and cerebral blood vessels [Durham, 2006]. Two forms of this neuropeptide are known:

- α-CGRP - is the predominant form in the peripheral nervous system, especially in the trigeminal nerve ganglia [Eftekhari et al., 2010];
- β-CGRP - is present in the nervous system of internal organs [Recober & Russo, 2009].

The CGRP receptor is a heteromeric receptor consisting of calcitonin receptor-like receptor (CALCRL), a G protein, and RAMP1 a receptor activity modifying protein. These receptors are found throughout the body and can mediate various physiological functions [Feuerstein et al., 1995]. The functions of CGRP receptors (modulation of gene expression and regulation of the activity of membrane receptors and ion channels by phosphorylation) are carried out mainly through activation of the cAMP signaling pathway [Charles, 2012].

The involvement of the CGRP receptor in the pathogenesis of migraine is confirmed by the effectiveness of selective antagonists of this receptor in the treatment of migraines, which not only relieve headaches, but also alleviate other migraine-related symptoms [Burns, 2008]. CGRP can increase the sensitivity of nociceptive neurons both in the central nervous system and on the periphery, thereby enhancing the processing of pain signals in migraine [Russo, 2015]. For example, in neurons of the dorsal horn, CGRP enhances glutamatergic neurotransmission [Marvizón et al., 2007; Seybold, 2009]. CSD can serve as a signal for activation of the CGRP gene [Tozzi et al., 2012] with a subsequent increase in CGRP concentration and the creation of a feedback loop: CSD promotes the synthesis and release of CGRP, which, in turn, increases the likelihood of subsequent CSD and the development of migraine [Russo, 2015].

At the periphery, CGRP has a strong vasodilating effect, which is especially pronounced in the intracranial arteries [Brain & Grant, 2004]. In most vessels, CGRP acts directly on smooth muscle cells, activating protein kinase A and ATP-dependent

potassium channels or stimulating the release of NO by endothelial cells [Brain & Grant, 2004]. One of the most important functions of CGRP at the periphery is to initiate mast cell degranulation, which results in the release of pro-inflammatory compounds [Theoharides et al., 2005; Ottosson & Edvinsson, 1997]. CGRP regulates its own synthesis in trigeminal ganglion neurons by paracrine and autocrine mechanisms. Isolation of CGRP from neurons causes the release of tumor necrosis factor-α (TNFα) from satellite cells [Thalakoti et al., 2007], which in turn enhances the synthesis of CGRP in neurons [Bowen et al., 2006].

Despite the significant contribution of CGRP to the pathogenesis of migraine, CGRP receptor antagonists are effective in treating only two-thirds of patients [Russo, 2015], which also indicates that there are other mechanisms at play.

2.4.2 Serotonin (5-hydroxytryptamine, 5-HT) in migraine

Serotonin is a biogenic amine that belongs to the tryptamine class of transmitters [Gasparini et al., 2017]. Serotonin receptors are found in neuronal tissues, the cardiovascular system, the gastrointestinal tract, and the kidneys [Hoyer et al., 2002]. Serotonin has a pronounced vasoconstrictor effect and regulates a wide range of physiological functions through G-protein coupled receptors (except for one type of ionotropic 5-HT3 receptor), which activate secondary mediator systems modulating neurotransmission [Gothert & Schlicker, 1987].

An increase in plasma serotonin levels during migraine and its decline between episodes has been noted [Ferrari et al., 1989]. Pain caused by the activation of 5-HT receptors is partially mediated by the activation of perivascular nociceptive fibers innervating the arteries of the meninges [Noseda & Burstein, 2013] through the activation of 5-HT3 receptors [Kilinc et al., 2017]. One of the main sources of extracellular serotonin is dural mast cells [Theoharides et al., 1982].

The 5-HT3 receptor, the only ionotropic receptor in the 5-HT receptor family, causes depolarization in central and peripheral sensory neurons [Cervantes-Duran et al., 2013; Hicks et al., 2002]. There is conflicting evidence on the functional role of 5-HT3 receptors: experimental data indicate both pro- and antinociceptive effects of 5-HT3 receptor activation [Green et al., 2000; Oatway et al., 2004]. About 20% of descending serotonergic endings form inhibitory axo-axonal contacts with primary afferents [Zhang et al., 2012], which implies their participation in the presynaptic control of peripheral inputs. On the other hand, 5-HT3 receptor antagonists are commonly used for anti-nociceptive therapy [Sagalajev et al., 2015; Greenshaw & Silverstone, 1997].

2.5 ATP AND PURINERGIC RECEPTORS IN NOCICEPTION

2.5.1 Characteristics of purinergic receptors

Purinergic receptors are receptors that bind adenosine and (or) purine nucleotides (ATP, ADP, AMP, UTP, UDP). They are present on the plasma membranes of most

human cells, but their role is especially important in the nervous system, where they are part of the purinergic neurotransmitter system [Abbracchio et al., 2009].

The concept of purinergic neurotransmission was developed in the 80s, after it was shown that adenosine 5'-triphosphate (ATP) is a neurotransmitter in non-adrenergic, non-cholinergic neurons [Burnstock, 1972]. Burnstock provided convincing evidence that ATP meets all the requirements that are imposed on classical neurotransmitters, and proposed calling nerves that secrete ATP "purinergic nerves" and the respective ATP receptors "purinoreceptors" [Burnstock, 1972; reviewed in Ziganshin, 2003]. ATP is a co-mediator in both sympathetic and parasympathetic nerves [Burnstock, 1976], as well as in synapses of the central nervous system [Burnstock, 2007; 2009]. ATP acts as an excitatory neurotransmitter and neuromodulator, contributing to the proliferation, differentiation and apoptosis of cells during development and regeneration, and also contributes to the regulation of the vascular and nociceptive systems [Abbracchio & Burnstock, 1998; Burnstock & Verkhratsky, 2010; Zimmermann, 2006]. Thus, it was proven that ATP is not only the main source of intracellular energy, but also a neurotransmitter that controls the functions of cells through purinergic receptors [Burnstock, 1972; Burnstock & Kennedy, 1985].

Abbracchio and Burnstock (1994) proposed the classification of purinergic receptors into ionotropic P2X and metabotropic P2Y receptors. 7 subtypes of P2X and 8 subtypes of P2Y receptors have been described, including receptors sensitive to pyrimidines and purines [Fredholm et al., 1994]. Ionotropic receptors are widely represented in various tissues [Burnstock, 2013] and are involved in various physiological processes, including nociception [North, 2002; Surprenant & North, 2009].

Purinergic signaling acts to integrate the functional activity between neurons, glial and vascular cells in the central nervous system in various conditions from normal to pathological [Abbracchio & Burnstock, 1998; Fields & Burnstock, 2006; Matute & Cavaliere, 2011; Parpura & Zorec, 2010; Verderio & Matteoli, 2011]. Studies of purine nucleotides offered a novel explanation of intercellular signaling between neurons and glial cells, because both secrete purine nucleotides into the extracellular space and both are equipped with various types of purinergic receptors [Burnstock, 2011].

2.5.2 The prevalence and function of P2X receptors in the central nervous system

In the central nervous system, fast purinergic synaptic transmission was first detected in the medial frenum, and medial habenula [Edwards et al., 1992; Khakh, 2001]. Later it was described in several other areas of the central nervous system, including the spinal cord [Bardoni et al., 1997], locus coeruleus [Nieber et al., 1997], hippocampus [Mori et al., 2001; Pankratov et al., 1999] and somatosensory neurons of the cortex [Pankratov et al., 2002].

P2X2, P2X4, and P2X6 receptors are widely distributed in the brain and often form heteromultimers [Burnstock, 2011]. P2X1 receptors are found in the cerebellum, P2X3, in the brain stem, and P2X7 are often localized in presynaptic membranes [Burnstock, 2011]. There is evidence of expression and localization of P2X5 receptors in the mouse brain [Guo et al., 2008]. All the P2X subtypes, and several metabotropic purinergic receptors, P2Y1, P2Y2, P2Y4, P2Y6 and P2Y12, have been found in the hippocampus.

In addition to their expression in neurons, purinergic receptors are also expressed in glial cells — astrocytes, oligodendrocytes, and microglia [Burnstock & Knight, 2004; Verkhratsky et al., 2009]. Astrocytes in the cerebral cortex and cerebellum express P2Y13, P2Y1 and P2X2 receptors [Carrasquero et al., 2005]. In astrocytes in close contact with the endothelium of blood vessels, the concentration of P2Y2 and P2Y4 receptors is especially high [Paemeleire & Leybaert, 2000; Simard et al., 2003]. At least six of the seven cloned P2X receptors in mammals are expressed in sensory ganglia [Khakh, 2001; Nörenberg & Illes, 2000]. Sensory neurons, spinal (DRG) neurons and the trigeminal ganglion neurons express receptor subtypes from P2X1 to P2X6 [Vulchanova L. et al., 1998]. It has been shown that in small-diameter sensory neurons, P2X3 receptors predominate over other P2X receptors [Xiang et al., 2008].

Data from the literature indicate that ATP is released from cells by several mechanisms. One of these is quantal release of ATP from vesicles [Pankratov et al., 2007]. Other mechanisms include release through connexins or pannexins, through gap junctions, and through the pore of P2X7 receptors [Dubyak, 2006; Scemes et al., 2007]. Studies confirm the vesicular release of ATP from astrocytes [Coco et al., 2003; Stout et al., 2002; Gourine et al., 2010], possibly with the participation of lysosomes [Zhang et al., 2007], and release of ATP through gap junctions made of connexins [Stout et al., 2002].

Pannexin 1 (Panx1) channels are permeable to ATP and can be activated at rest potential by activation of P2Y receptors, and Panx1 can also be activated by elevated cytoplasmic calcium [Locovei et al., 2006].

Upon binding to ATP, the P2X7 receptor forms a pore through which the outflow of intracellular ATP, glutamate and GABA from glial cells in the central nervous system takes place [Duan & Neary, 2006]. In addition, activation of P2X7 receptors promotes the release of several cytokines such as TNFα [Hide et al., 2000], IL-1β; [Ferrari et al., 2006], and cysteinyl-leukotriene [Ballerini et al., 2005], which promote inflammation. These cytokines release from immune cells through secretory lysosomes [Andrei et al., 2004] or membrane vesicles [Bianco et al., 2005] and their release requires the activation of intracellular secondary messengers targeting the C- terminus of the P2X7 receptor [Ferrari et al., 2006]. Data from the literature indicate that P2X7-mediated release of cytokines from macrophages involves the activation of Panx1. Selective blockade of the Panx1 protein reduces P2X7-induced dye uptake in HEK cells and macrophages [Pelegrin & Surprenant, 2006].

The breakdown of extracellular ATP to adenosine is carried out by the coordinated work of a number of enzymes: ATP diphosphohydrolases,

exo- 5′- nucleotidases/CD73, ectonucleotide pyrophosphatases/phosphodiesterases, alkaline phosphatases and other exoenzymes expressed in different types of cells [Burnstock & Ralevic, 2014]. ATP degradation has important physiological significance, since some ATP metabolites such as ADP and adenosine act as physiological stimuli for various purinergic receptors [Zimmermann, 2006]. Thus, adenosine aroused from the hydrolysis of ATP has a pharmacological effect that is opposite to ATP. In addition to hydrolysis of ATP as a source of adenosine, some central neurons and astrocytes directly secrete adenosine [Wall & Dale, 2013].

2.5.3 Activation and desensitization of P2X receptors

Extracellular ATP activates the P2X receptors which open within milliseconds of ATP binding to the ion channels, which are preferably permeable to sodium, potassium and calcium, and changing the membrane potential towards depolarization [Nörenberg & Illes, 2000; North, 2002; Ziganshin, 2003]. In smooth muscle cells, activation of P2X1 receptors mediates the initiation of contractile activity [Hattori & Gouaux, 2012]. In primary afferent nerve endings, P2X receptors are involved in the generation of action potentials, whereas in immune cells activation of P2X receptors triggers the release of cytokines [North, 2016].

P2X7 receptors have properties that are different from other subtypes of P2X receptors, firstly, they have a low affinity for ATP compared to other P2X receptors, and require a higher concentration (> 100 μM) of ATP for activation, secondly, ATP-mediated activation of P2X7 receptors is significantly enhanced by decreased extracellular concentration of divalent cations (Ca^{2+} or Mg^{2+}). This property indicates that unbound extracellular ATP^{4} may be an active ligand. Since most of the extracellular ATP in normal physiological media forms a complex with Mg^{2+} or Ca^{2+}, the proportion of free ATP^{4} is generally low. Thus, for the activation of the P2X7 receptor, a high level of ATP is a prerequisite. It is also possible that divalent ions bind to the receptor and exhibit allosteric inhibition [Duan & Neary, 2006].

The mechanisms of P2X receptors activation may be modulated through interaction with other types of receptors, leading to either an increase or decrease in pain sensitivity. Both TRPV1 [Szallasi et al., 2007], and P2X [Wirkner, et.al., 2007] receptors are involved in visceral pain, neuropathic pain, migraine and cancer pain. Activation of P2X3 receptors in trigeminal neurons leads to activation of Ca^{2+}-dependent kinases such as CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) and phosphorylation of TRPV1 receptors, with subsequent neuronal sensitization which underlies mechanical hyperalgesia [Saloman et al., 2013]. On the other hand, the response of P2X3 receptors can be decreased in the continuous presence of TRPV1 agonists, which is associated with direct physical interaction between receptors using the C-terminal of P2X3 receptors [Stanchev et al., 2009]. Thus, as a result of the interaction between TRPV1 and P2X3 receptors, a decrease in excessive pain caused by strong simultaneous activation of both receptors can be observed [Stanchev et al., 2009].

There is evidence in the literature that activation of P2X3 receptors is involved in the development of mechanical hyperalgesia in the masticatory muscle, where P2X3 and TRPV1 receptors are co-expressed in the afferents of this muscle. P2X3 receptor activation enhances TRPV1-induced responses in trigeminal ganglion neurons due to phosphorylation of TRPV1 receptors, suggesting that P2X3-induced hyperalgesia is TRPV1-dependent [Saloman, 2013]. Similar data were published in a model of inflammatory pain caused by formalin injection into the hind paw in rats. In this study, it was demonstrated that activation of P2X3 receptors induces inflammatory pain, and this involves the activation of TRPA1, 5-HT3 and 5-HT1A receptors in peripheral tissues [Krimon et al., 2013].

Purinergic receptors are characterized by the phenomenon of desensitization, which implies a loss of receptor sensitivity that develops with the prolonged presence of an agonist [Giniatullin & Nistri, 2013].

P2X receptors can be classified into fast (P2X1, P2X3) and slow (P2X2, P2X5, P2X6, P2X7) desensitizing receptors [Burnstock & Ralevic, 2014]. There are also two P2X2 isoforms with different desensitization rates which is related to their C-terminal regions [Illes & Ribeiro, 2004].

Desensitization can be observed in both metabotropic and ionotropic receptors. In contrast to metabotropic receptors, which desensitize mainly through adaptation of the membrane receptor, temporarily turning off elements that are sensitive to extracellular stimuli [Elneil et al., 2001], desensitization of ionotropic receptors involves structural and functional changes in the membrane ion channel [Giniatullin & Nistri, 2013]. The duration of desensitization and the subsequent recovery processes also varies greatly within the P2X receptor group [North, 2002]. Slow recovery after fast desensitization is characteristic of P2X1 and P2X3 receptors [North, 2002; Coddou et al., 2011], whereas other P2X subtypes are less susceptible to desensitization and recover faster [Giniatullin & Nistri, 2013].

2.5.4 The role of P2X receptors in the perception and transmission of nociceptive stimuli in the development of migraine

The abovementioned "purinergic" hypothesis of migraine proposed by G. Burnstock presumed that both extracellular ATP and its degradation products mediate vasodilation after the initial occurrence of vascular spasm during a migraine episode. Indeed, studies have shown that ATP is involved in both the initial and later phases of vasodilation [Burnstock, 2013]. According to this view, ATP can exert dual control with respect to vascular tone as follows: release of ATP as a co-mediator with norepinephrine from perivascular sympathetic nerve endings, followed by activation of P2X1 receptors on smooth muscle cells, leading to vasoconstriction. Further, the action of ATP on vascular endothelial cells (which can result from changes in blood flow or hypoxia) activates the P2X and P2Y receptors, triggering the synthesis of endothelial vascular relaxation factors (nitric oxide, NO). This cascade of reactions leads to vasodilation. The sources of extracellular ATP are the endothelial cells and platelet aggregates [Burnstock, 1981; 1989; 2013].

In addition, ATP is involved in the stimulation of primary afferent nerve endings located in the outer layer of vessels in the brain. ATP sensitive P2X3 receptors are expressed in sensory neurons and were found on the membranes of primary afferent nerve endings innervating the vessels of the brain and originating in the trigeminal, nodal (nodous) and spinal ganglia [Burnstock, 1996; 2001; Chen et al., 1995]. Peripheral sensitization of DRG neurons via P2X3 receptors has also been described [Waeber & Moskowitz, 2003]. However, the role of P2X3 receptors in the activation of primary trigeminal afferents, which is relevant to migraine pain, is poorly understood.

The algogenic (pro-nociceptive) effect of CGRP, which is expressed in trigeminal neurons and released during migraine attacks, may be associated with sensitization of nociceptive P2X3 receptors in sensory neurons. It has been suggested that neuronal P2X3 receptors may be a potential target of CGRP in the early phase of a migraine attack [Fabbretti et al., 2006]. The analgesic effect of the non-steroidal anti-inflammatory drug naproxen, which is widely used to treat migraine attacks, may be explained by blockade of P2X3 receptors in trigeminal neurons, along with its anti-inflammatory effect [Hautaniemi et al., 2012].

It is known that the R192Q mutation in familial hemiplegic migraine in mice (FHM1 R192Q KI) leads to hyperfunction of the $Ca_V2.1$ channel ($Ca_V2.1$, P/Q type) and increased glutamatergic neurotransmission in the cerebral cortex, which may explain the increased susceptibility to CSD in these animals [Tottene et al., 2009; Maagdenberg et al., 2004; Eikermann-Haerter et al., 2009; Marchenkova et al., 2016]. Notably, the same mutation affects the trigeminovascular system, which plays a significant role in the transduction of pain during migraine [Gasparini & Griffiths, 2013]. Thus, the abnormal functioning of the $Ca_V2.1$ channel leads to increased secretion of CGRP [Fioretti et al., 2011; Ceruti et al., 2011] and hyper-excitability of trigeminal neurons [Hullugundi et al., 2014]. In other words, the R192Q mutation affects the sensitization of P2X3 receptors in sensory neurons of the trigeminal ganglion, which once again emphasizes the role of P2X3 receptors in peripheral nociception, including in the pathophysiology of migraine pain [Wirkner et al., 2007; Nair et al., 2010].

Table 1. Summary for P2X Receptor Involvement in Acute and Chronic Pain

Receptor	Main Distribution	Main Functions
P2X3	Sensory neurons, NTS, some sympathetic neurons	Mediates sensory transmission; facilitates glutamate release in CNS
P2X4	CNS, testes, colon	Modulates chronic inflammatory and neuropathic pain
P2X7	Apoptotic cells, immune cells, pancreas, skin	Mediates apoptosis, cell proliferation and proinflammatory cytokine release

In chronic pain in humans, especially of an inflammatory nature, P2X3 receptors can also participate in the generation of nociceptive signals [Burnstock, 2001] by modulating the desensitization of P2X3 receptors. The limiting excitation role of

desensitization can be bypassed by several cellular and molecular mechanisms, which include hydrolysis of extracellular ATP by local enzymes, reduction of high-affinity desensitization, acceleration of recovery after desensitization, and the formation of heteromeres with non-desensitizing P2X2 receptors [North, 2004].

Thus, the study of the properties of P2X3 receptors and search for selective antagonists are of great physiological importance due to the accumulating evidence that these receptors are involved in chronic pain [Burnstock, 2001; North, 2004] and likely in the formation of pain in migraine.

2.6 HYDROGEN SULFIDE AND ITS ROLE IN NOCICEPTION

2.6.1 Synthesis and physiological role

Hydrogen sulfide (H_2S), along with NO and CO, is a common gaseous transmitter that regulates many physiological and pathophysiological processes. The toxic effect of H_2S on body functions was described as early as 1713 [Ramazzini, 2001]. The physiological role of H_2S was first assumed considered in 1989 after the discovery of high concentrations of this transmitter in the brain of rats and humans [Savage et al., 1990]. In aqueous solutions, H_2S dissociates to form H^+, HS^- and S^{2-}. Under standard physiological conditions (37°C, pH 7.4), about 10-20% of the dissolved gas is present in the form of H_2S, while HS^- and S^{2-} are present only in trace concentrations [Olas, 2014; 2015; Olson et al., 2014].

In mammals, H_2S is generated from sulfur-containing amino acids, primarily from L-cysteine and L-homocysteine by the enzymes cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopropionate sulfotransferase [Mikami et al., 2011; Jung & Jeong, 2014]. CBS is the main enzyme for the synthesis of H_2S in the nervous tissue, while in the cardiovascular system, liver and kidneys, the main synthesis enzyme is CSE [Kimura, 2017]. An alternative substrate for the synthesis of H_2S is D-cysteine, which is converted to 3-mercaptopiruvate using the enzyme D-amino oxidase [Shibuya & Kimura, 2013]. This route of H_2S synthesis has been described in the cerebellum and kidneys [Kimura, 2002]. In addition, H_2S can be synthesized by intestinal microflora in significant concentrations [Motta et al., 2015]. Like other gaseous transmitters, H_2S does not accumulate in synaptic vesicles and easily passes through the plasma membrane, exerting local auto- and paracrine action [Olson, 2013]. An increase in H_2S concentration occurs in response to various physiological stimuli, followed by a rapid decrease of its level as a result of degradation and binding to surrounding buffering molecules [Russo et al., 2000; Kimura, 2002].

The physiological role of H_2S includes regulation of apoptosis and the cell cycle, and participation in redox reactions [Tang et al., 2010; Moore & Whiteman, 2015]. H_2S has cardioprotective and neuroprotective properties [Beard & Bearden, 2011]. This transmitter is also involved in the induction of long-term potentiation in the hippocampus [Abe & Kimura, 1996; Yakovlev et al., 2017]. It can modulate inflammatory processes [Patacchini et al., 2005] and gastrointestinal functions [Tang

et al., 2010; Moore & Whiteman, 2015]. H_2S is involved in regulation of corticotropin-releasing hormone secretion in the hypothalamus [Matsunami et al., 2009]. H_2S can regulate calcium signaling in all types of brain cells, namely, neurons, microglia, and astrocytes [Nagai et al., 2004; Tang, 2010]. H_2S is involved in energy processes, and, depending on concentration, it can either be an electron donor, or bind and inhibit cytochrome *C* oxidase [Dorman et al., 2002].

The molecular mechanisms of H_2S action are mediated by chemical modification of protein molecules, which includes direct attachment of a sulfur atom to the thiol group (-SH) of a protein and its transformation into a hydropersulfide residue (-SSH), and reduction of disulfide bonds (S=S) [Fiorucci et al., 2007; Wallace et al., 2012; Matsunami et al., 2009] with subsequent changes in protein conformation and activity.

H_2S, due to its anti-inflammatory and antioxidant effects, plays a neuroprotective role in neurological diseases such as Alzheimer's disease, Parkinson's disease, traumatic brain injury, subarachnoid hemorrhage and ischemic stroke [Zhao et al., 2017]. It has been demonstrated that intracranial hemorrhage significantly suppresses the production of endogenous H_2S in the rat brain due to a decrease in the level of CBS. The administration of NaHS, the classic exogenous H_2S donor, not only restored H_2S content in the brain and plasma, but also weakened cerebral edema, neutrophil infiltration, microglia accumulation, neurological deficit and apoptosis of neurons [Galli et al., 2005].

2.6.2 The role of H_2S in perception and transmission in nociceptive stimuli

Numerous studies indicate that, in various tissues, H_2S can produce either pro- or antinociceptive effects, resulting from its interaction with distinct cellular targets. These opposite effects apparently depend on the level of expression of the enzymes producing this gas, and on the concentration of H_2S [Bhatia et al., 2005].

In addition to regulation of excitability due to a direct effect on potassium channels, low-threshold T-type Ca^{2+}-channels, and TRP receptors, H_2S also affects the development of inflammation and related processes such as migration of neutrophils and cytokine production [Matsui et al., 2019]. Up-regulation of T-type Ca^{2+} channels by H_2S likely underlies the enhanced transmission of pain stimuli [Garattini et al., 2019]. Several studies have shown that in inflammation, there is increased expression of H_2S synthesizing enzymes and an increase in H_2S concentration, which promotes the pain response. Thus, in a temporo-mandibular joint model of inflammation, enhanced expression of CBS and CSE was shown, as well as an increase in the rate of endogenous H_2S production, while the H_2S synthesis inhibitor, propargylglycine (PAG), prevented these changes [Kida et al., 2015; Chen et al., 2019].

It was found that the H_2S synthesizing enzymes CBS and CSE are expressed in rat peritoneal mast cells and involved in the regulation of intracellular calcium concentration and mast cell homeostasis [Irmak, 2019]. It has been shown that the influx of calcium into mast cells is associated with the activation of pain related TRP channels. However, even though H_2S donors induce an increase in intracellular

calcium, no obvious effect on mast cell degranulation has been found [Theoharides & Kalogeromitros, 2006; Gri et al., 2012; Sismanopoulos et al., 2012].

A modulating effect of H_2S on the level of expression of P2X3 receptors was detected in DRG neurons [Wang et al., 2015; Xu et al., 2009]. Hydrogen sulfide suppresses ATP-induced inflammation and the formation of β-amyloid 1-42 (Aβ1-42) in microglia cells [Karatas et al., 2013].

On the other hand, there are data indicating that H_2S exhibits not only anti-inflammatory, but also antinociceptive activity. Thus, H_2S reduced swelling of the paw [Zanardo et al., 2006], joints [Ekundi-Valentim et al., 2010] and the brain [Zhao et al., 2017], caused by various stimuli. This anti-inflammatory activity was due to a decrease in cytokine production [Li et al., 2005], leukocyte recruitment [Ekundi-Valentim et al., 2010] and expression of adhesion molecules [Talaei, 2016]. The antinociceptive effects of H_2S were also evidenced by a decrease in sensitization of nerve endings in rat knee edema models [Ekundi-Valentim et al., 2010], nerve damage [Kida et al., 2015], and opioid withdrawal syndrome [Yang et al., 2014].

In a model of neuropathic pain with chronic damage to the rat sciatic nerve, H_2S prevented the development of allodynia and hyperalgesia, reduced the release of cytokines by activation of the Nrf2/HO-1 (Nuclear factor erythroid-2 (NF-E2) -related factor 2 (Nrf2), hemeoxygenase-1 (HO-1)) signaling pathway in microglia in the spinal cord [Melo et al., 2019]. A slow-release H_2S donor methyl-benzene-carbothioamide also exerted a similar anti-inflammatory and antinociceptive effect in a mouse inflammatory pain model due to a decrease in cytokine production and leukocyte recruitment [Mannelli et al., 2017]. In addition, through the activation of Kv7 potassium channels, H_2S donors reduced the development of the neuropathic pain induced by chemotherapeutic agents [Xu et al., 2019]. The H_2S donor also reduced the sensitivity of visceral afferents in the colon through a NOS-dependent mechanism [Xu et al., 2009; Feng et al., 2013].

Expression of CBS, an endogenous H_2S synthesis enzyme, was detected in DRG and trigeminal sensory neurons [Traina, 2019]. In trigeminal neurons, the expression of CBS increased during inflammation, with a simultaneous increase in excitability due to reduced voltage-gated potassium currents [Miao et al., 2014]. CBS colocalizes with TRPV1 in DRG neurons innervating the large intestine, and H_2S synthesis contributes to the development of visceral hypersensitivity [Xu et al., 2009]. TRPV1 receptors are able to sensitize in response to inflammation, which is associated with increased expression of the enzyme CBS in sensory DRG neurons [Zhu et al., 2015]. Some data showed the ability of H_2S to activate TRPA1 receptors. Thus, it has been shown that H_2S-induced vasodilation is due to the release of vasoactive neuropeptides, such as CGRP and substance P [Hajna et al., 2016; Pozsgai et al., 2012]. Also, after application of H_2S, activation of TRPA1 channels led to mechanical hyperalgesia and allodynia in mice [Okubo et al., 2012]. Notably, TRPA1 receptors were not involved in the pro-nociceptive effects of H_2S in viscera [Andersson et al., 2012].

TRP receptors are a family of channels that are homologous in structure but perform various functions in the body and are one of the targets for the action of H_2S in the CNS including the trigeminal nociceptive system [Cui et al., 2016; Liu et al., 2016; Lee et al., 2016]. The specific responsiveness of TRP channels to a number of physical and chemical stimuli allows these channels to participate in various sensory processes, including vision, hearing, taste, tactile and temperature sensitivity, redox processes, and pain [Voets et al., 2005; Wetsel, 2011; Feng, 2014; Ogawa et al., 2016]. TRP channels are involved in the pathophysiological processes of pain, respiratory reflex hypersensitivity, cardiac hypertrophy, and cell death at the focus of ischemic lesions [Moran et al., 2011]. The TRP channel family is the largest group of nociceptive ion channels, the TRPV1 and TRPA1 subtypes are particularly well-studied [Clapham, 2003; Patapoutian et al., 2009]. Activation of nociceptive TRP channels in sensory ganglia (for example, DRG) leads to the entry of sodium and calcium ions through the cell membrane and, as a result, to depolarization of the neuronal membrane. This positive shift in membrane potential can lead to the activation of multiple voltage-dependent ion channels and the generation of action potentials [Gees et al., 2010], which are transmitted to the spinal cord and the higher central nervous system [Jardín et al., 2017].

TRPV1 channels are expressed in sensory neurons (DRG, TG, and the vagus nerve), and in C- and Aδ- nerve fibers, which contain various neuropeptides, such as substance P and CGRP [Caterina & Julius, 2001]. The phenomenon of desensitization of TRPV1 receptors in sensory neurons was described by Jancsó [Jancsó, 1957; 1961]. In 1961, Jancsó and his colleagues showed that the administration of capsaicin to adult rats at a dose of 80 mg/kg for 1 to 3 days led to a complete loss of chemical sensitivity lasting up to 3 months [Jancsó, 1961].

There are different processes of desensitization of TRPV1 channels presented by acute desensitization, with a rapid loss of receptor activity upon interaction with an agonist, but also so-called tachyphylaxis, characterized by a gradual decrease in response to repeated administration of an agonist [Szallasi & Blumberg, 1999]. The phenomenon of desensitization of TRPV1 receptors underlies the paradoxical analgesic effect of capsaicin and has protective role with a sharp increase in calcium levels [Liu & Simon, 1996; Docherty et al., 1996; Liu et al., 2005]. Acute desensitization of TRPV1 is related to the agonist-induced conformational change that closes the ion channel pore. This process depends on the level of intracellular calcium and, therefore, it can be inactivated by means of intracellular calcium chelators [Caterina et al., 1997; Schwarz et al., 2000]. Studies have shown that acute desensitization of TRPV1 receptors occurs as a result of channel interaction with calmodulin (CaM), where CaM acts as a calcium sensor, thereby decreasing channel activity in response to an increase in intracellular calcium concentration [Rosenbaum et al., 2004].

Tachyphylaxis is a decrease in response to repeated use of an agonist and involves the cyclic operation of the TRPV1 channel between resting and active conformations including numerous non-conducting intermediate states [Liu & Simon, 1996]. That is why tachyphylaxis is seen as a transient state in which the TRPV1 channel is

recovering from an intermediate state to a state of rest, and channels can be activated once again after the agonist binding, that is a process in which calcium and some other factors, such as intracellular ATP and phosphatidylinositol-4,5-bisphosphate (PIP2), can play modulating roles [Lishko et al., 2007; Liu & Simon, 1998; Koplas et al., 1997].

Phosphorylation and dephosphorylation are critical processes in the function of TRPV1 receptors. This is illustrated by the role of the phosphatase calcineurin, which inhibits the desensitization of the TRPV1 channel [Docherty et al., 1996] and the action of calmodulin-dependent kinase CaMKII, which regulates the activity of TRPV1 through the phosphorylation of two residues: Ser502 and Thr704 [Jung et al., 2004]. Lipids may also be involved in the regulation of the activity of TRPV1 channels. Thus, PIP2 performs its regulatory function after activation of phospholipases, such as phospholipase C (PLC). Despite the evidence that PIP2 synthesis is necessary to restore TRPV1 currents after desensitization, there is a disagreement over whether PIP2 increases or decreases the probability of channel opening [Moran et al., 2011]. In sensory neurons, such as DRG or trigeminal neurons, the activation of the enzyme PLC by the pro-inflammatory agents such as ATP, nerve growth factor (NGF), bradykinin or chemokines leads to sensitization of TRPV1 channels [Chuang et al., 2001; Tominaga et al., 2001; Moriyama et al., 2003; Zhang et al., 2005]. This phenomenon enhances the sensitivity of these channels to high temperatures, acids, and capsaicin, which accompany pain-related processes such as tissue irritation or inflammation [Moran et al., 2011].

TRPA1 and TRPV1 receptors are likely involved in the development of migraine attacks and can be activated by a number of agonists, among which are acrolein, formalin, NO, and H_2S [Benemei et al., 2015]. The co-expression of TRPV1 and TRPA1 channels with CBS in the nerve fibers of the dura mater of the brain [Zhu et al., 2015], as well as in the bodies of trigeminal neurons [Feng et al., 2013] has been shown.

2.7 MAST CELLS AND MIGRAINES

Mast cells are widely distributed immune cells that are formed from hematopoietic pluripotent stem cells that can migrate and mature near the endothelium of blood vessels and peripheral nerves [Gupta & Harvima, 2018; Moretti et al., 2014] and also involved in nociception in different systems [Aich et al., 2015]. Almost all vascularized tissues have resident mast cells that produce, contain and release biologically active compounds called "mast cell mediators" [Kempuraj et al., 2004; Theoharides & Kalogeromitros, 2006; Hildebrand et al., 2008; Lennerz et al., 2008; Tore & Tuncel, 2009; Okragly et al., 2018]. Classical mast cell mediators include proteases (tryptase, chymase), bioorganic amines (histamine and serotonin), proteoglycans (heparin), cytokines (TNFα), nitric oxide (NO), prostaglandins, leukotrienes and kinins, neuropeptides (corticotropin releasing factor, endorphins, somatostatin, substance P, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP) [Conti et al., 2018], and growth factors

[Metcalfe et al., 1997; Tore and Tuncel, 2009.] After release, these mediators can modify the state of mast cells themselves through the autocrine effect, whereas through paracrine effects they affect nearby nerves, the vascular smooth muscle endothelium and thereby regulate the function of distant organs [Boyce, 2007].

Mast cells are involved in the innate and adaptive immune responses [Ramachandran et al., 2019], and the pathological processes in the CNS associated with neuroinflammation, including migraine.

The process of mast cell degranulation is preceded by activation. The dynamics of these processes are controlled by the level of intracellular calcium and cAMP [Questrada et al., 2003]. Mast cell activation is mediated by membrane receptors in both an IgE-dependent and IgE-independent manner [Ribatti & Crivellato, 2011]. IgE-independent mast cell activation is mediated by various neuropeptides via specific membrane receptors. These peptides include substance P, somatostatin, vasoactive interstitial peptide, and CGRP [Church, 1991]. As a result of binding to membrane receptors, an increase in the concentration of intracellular calcium occurs, which triggers mast cell degranulation.

Anaphylactic degranulation is a global exocytosis of the contents of all granules. This mechanism is an example of immediate hypersensitivity [Xiang et al., 2001]. Gradual degranulation is carried out through the release of microvesicles into the surrounding space [Ribatti & Crivellato, 2011; Questrada et al., 2003].

Mast cells can be activated by pathogens associated with the IgE antigen, drugs, physical factors such as temperature and pressure through activation of TRPV2 channels, toll-like receptors (TLR, 1–7, 9), nucleotide-binding oligomerizing domain-like receptors (NOD), cytokine receptors, and microbial-associated molecular proteins (MAMP) [Zhang et al., 2012; Traina, 2019]. Notably, mast cells express CRF1 and CRF2, which are receptors for corticotropin releasing hormone, which have a central role in the stress-induced hypersensitivity of internal organs, and sensitization of the local nerve endings [Overman et al., 2012].

Many studies have shown that mast cells and nerve endings form a neuro-immune contact leading to close interactions [Newson et al., 1983]. It should be noted that mast cells can affect the excitability of nerve endings, but also neurotransmitters, released from the nerve endings, can affect on the morphology, number and degranulation level of mast cells [Forsythe & Bienenstock, 2012].

For example, CGRP and substance P released from the nerve endings innervating the meninges can degranulate mast cells with subsequent release of pro-inflammatory compounds, which in turn activate mechanosensitive C-fibers, again leading to repetitive release of CGRP and substance P from these nerve fibers [Julius & Basbaum, 2001; Irmak et al., 2019]. Thus, mast cells support the multiple processes associated with peripheral neurogenic inflammation [Julius & Basbaum, 2001].

The available evidence suggests that mast cells can modulate a cascade of inflammatory responses that lead to activation of trigeminal afferents [Levy et al., 2007; Irmak et al., 2019]. Thus, TNFα, a pro-inflammatory cytokine released from mast cells mediates the sensitization of meningeal nociceptors [Moretti et al., 2014;

Irmak et al., 2019]. In addition, it has been shown that extracellular ATP can affect the functional state of mast cells [Kurashima et al., 2012]. The degranulating mast cells effect of ATP shows that this purinergic messenger acts as an endogenous trigger for neuroinflammation in various neurological disorders, including migraine.

P2X7 receptors initiate the neuroinflammation in various tissues. Activation of P2X7 receptors in the mast cells of the respiratory tract leads to release of various pro-inflammatory cytokines such as IL-1β, IL-18 [Ferrari et al., 2006] and IL-6 [Shieh et al., 2014]. The cytokines make a significant contribution to the development of neuropathic and inflammatory pain [Radley & Grounds, 2006].

ATP-driven mast cell degranulation probably also occurs in migraine with aura, since CSD itself is an inducer of degranulation of meningeal mast cells and the opening of pannexin-1 channels [Karatas et al., 2013] which are permeable to ATP [Nielsen et al., 2019]. In connection with the contribution of mast cells to the development of the inflammatory process, the search for compounds that stabilize mast cell membranes is highly relevant. Compounds that stabilize mast cell membranes that are currently available include certain medications, for example the anti-allergic drug oxatomide (Oxatomide, Tinset) which is classified as a histamine receptor antagonist (H1). It is known to inhibit the release of various pro-inflammatory mediators such as histamine, leukotrienes, prostaglandins and TNFα from mast cells and other leukocytes [Radley & Grounds, 2006].

Cromoline (cromolyn sodium) is a commonly used mast cell stabilizer [Edwards & Howell, 2000] that inhibits mast cell degranulation both in vitro and in vivo by decreasing phosphodiesterase activity and preventing an increase in intracellular calcium concentration [Steiner et al., 2003; Shin et al., 2004]. Cromoline is a safe and widely used asthma medicine [Storms & Kaliner, 2005].

Interestingly, several recent studies indicate that H_2S prevents mast cell degranulation in cases of airway inflammation. It also reduced histamine concentration and suppressed the skin itching caused by the administration of C48/80, a substance leading to mast cell degranulation [Rodrigues et al., 2017].

Thus, analysis of the literature has shown that both central and peripheral mechanisms play a distinct role in the pathogenesis of migraine. Headache is primarily associated with activation of peripheral mechanisms of nociception, namely, meningeal trigeminal afferents. However, the molecular mechanisms of nociceptive firing in these nerve fibers are not fully understood. According to the purinergic theory of migraine, ATP causes vasodilation of the meningeal vessels surrounding the brain and is involved in activation of the nerve endings of the trigeminal nerve and probably mast cells that form clusters around blood vessels and nerve fibers. Mast cell degranulation leading to the release of inflammatory compounds may contribute to the nociceptive action of ATP. However, the molecular mechanisms that trigger mast cell degranulation, and how the mediators of mast cells affect electrical activity in trigeminal nerve fibers, are not clear.

Expression of the H_2S synthesizing enzyme CBS in sensory neurons has been shown. In several tissues, H_2S can exhibit pro-nociceptive properties, affecting the

excitability of neurons, but it can show also protective properties by reducing inflammatory reactions and oxidative stress. In sensory neurons, co-expression of CBS and ATP-sensitive P2X3 receptors was shown, which suggests an interaction between them. However, so far, there are no data on the effects of H_2S on ATP receptors in the trigeminal nerve.

3 AIMS OF THE STUDY

The aim of the study was to analyze the receptor mechanisms underlying the action of ATP on the peripheral trigeminal nerve fibers, as well as to identify the action of H_2S on ATP-dependent generation of nociceptive signals.

To achieve the goal, the following tasks were set:

1. To study the effect of ATP on the electrical activity of the rat and mouse trigeminal nerve, and to reveal the role of P2X3 receptors in the pro-nociceptive action of ATP;
2. To assess the effect of ATP and P2X7 receptor agonists on mast cell degranulation in rat meninges;
3. To analyze, using mast cell deficient mice (C57BL/6J-Kit$^{W\text{-}v}$/J), the role of mast cells and the mediator of mast cells, serotonin, on the ATP-induced electrical activity in trigeminal nerve fibers;
4. To determine the role of TRPV1 and P2X3 receptors in the modulatory effects of H_2S on the pro-nociceptive effect of ATP in the rat trigeminal nerve fibers;
5. To analyze the effect of an H_2S donor on inward currents and calcium signals eliciated by activation of P2X3 receptors in isolated trigeminal ganglion neurons;
6. To evaluate the effect of H_2S on the basal level of extracellular ATP and ATP-induced mast cell degranulation in rat meninges.

4 MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMALS

The experiments were carried out on male Wistar rats aged 9-12 days (P 9-12) for culture of trigeminal ganglion neurons, and 4-6 weeks rats (P 28-42), wild-type mice (JAXC57BL/6J line) and mice with mast cell deficiency (C57BL/6J-Kit$^{W\text{-}v}$/J line) at the age of 3 months for isolated skull preparation. Knockout mice (C57BL/6J-Kit$^{W\text{-}v}$/J, or Kit$^{W\text{-}v}$/J) had pleiotropic defects in pigment cells, germ cells, red blood cells, and mast cells. In the work, males were used to minimize the effect of the changeable hormonal background of females. The major part of the work was done by using rats. Mice were included in the study as a valuable additional model allowing to use genetic knockouts such as mast cell-deficient mice.

Animals from the vivariums of the University of Eastern Finland (Kuopio) and Kazan Federal University were used. Rats and mice were housed in cells with controlled temperature and humidity and a 12-hour light cycle. Food and water were provided ad libitum. The experimental protocols complied with the ethical standards for the humane treatment of animals adopted at the Kazan Federal University and approved by the Local Ethics Committee of KFU (protocol No. 8 dated May 5, 2015), and also complied with the Council of the European Union Directive of September 22, 2010 (2010/63/EEC) and were approved by the Committee for the Use of Animals of the University of Eastern Finland (licenses EKS-004-2014 and EKS-002-2017). All measures were taken to minimize the number of animals used in the experiments.

4.2 OBJECTS OF STUDY

4.2.1 Hemiskull preparation

As an object of ex-vivo research, we used an isolated rat hemiskull with intact dura mater [De Col et al., 2012]. This is the most reliable model for study of the peripheral processes in dura mater to explain mechanisms of migraine pain. The main advantages of this preparation are [Zakharov et al., 2015]:

- preservation of the native morphology of meningeal tissues, including local nerves and blood vessels;
- control of the concentration of the applied substances, which is critical for studying the function of receptors with pronounced desensitization, such as ATP receptors [Giniatullin & Nistri, 2013];
- registration of exclusively peripheral nociceptive transmission [Amir, 1996; Thalakoti, 2007] without involving signals from the soma of trigeminal ganglion neurons.

After decapitation, the rat skull was thoroughly cleaned of the external cranial muscles. The skull was divided by a sagittal section into two halves, and the brain

was carefully removed from the cranium, without touching the dura mater. Processes of the trigeminal nerve (nervus spinosus), innervating the middle meningeal artery (MMA), were preserved in the dura mater. The resulting halves of the skull were placed in artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 120 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 glucose, 24 $NaHPO_4$, 30 $NaHCO_3$ with constant oxygenation of 95% O_2 / 5 % CO_2.

4.2.2 Isolation of trigeminal ganglion neurons

To record P2X3 currents, a trigeminal ganglion cells culture of 9-12 day old Wistar rats was used. The animals were decapitated, the trigeminal ganglia extracted, placed in a cold Ham's F12 Nutrient Mixture and chopped. An enzymatic cocktail containing 0.25 mg/ml trypsin, 1mg/ml collagenase and 0.2 mg/ml DNAse was used to dissociate neurons. Dissociation was carried out in a thermal shaker at a temperature of 37°C, at 1000 rpm, for 25 minutes. Dissociated neurons were placed on coverslips coated with poly-L-lysine and were kept in an incubator at a temperature of 37°C, in 5% CO_2 for 24 hours before the start of the experiments.

4.3 ELECTROPHYSIOLOGY

4.3.1 Activity of the trigeminal nerve peripheral branch

Just before the experiment, the isolated rat skull preparation was placed in an experimental bath with a flow perfusion system (with a flow rate of 6-7 ml/min). Under visual control, the peripheral process of the trigeminal nerve was released from the dura mater and was sucked into a glass electrode (electrode tip diameter 150 μm; Figure 3). The application of substances was carried out in the area of divergence of the middle meningeal artery (MMA) [Schueler, 2014]. The isolated preparation was washed with ACSF solution under constant oxygenation with 95% O_2 / 5% CO_2, the pH was maintained at 7.20-7.35.

Electrical signals were recorded using a DAM 80 amplifier (0.1 Hz – 1 kHz bandwidth; gain 10000; World Precision Instruments, Sarasota, USA). The signals were digitized on a PC using the NI PCI6221 board (National Instruments, USA) and WinEDR v.3.2.7 software (Strathclyde University, UK).

The shape and parameters of the signal (two-phase signal, with a duration in the range of 0.3-1.5 ms and an amplitude of 20 to 150 μV) served as a guideline for determining action potentials. In order to reveal the short-term effect of the action of substances, the experimental data were divided into 2 and 5 min intervals. Data recorded immediately before the application of the substance were used as a control, for comparison with the effect of the substance.

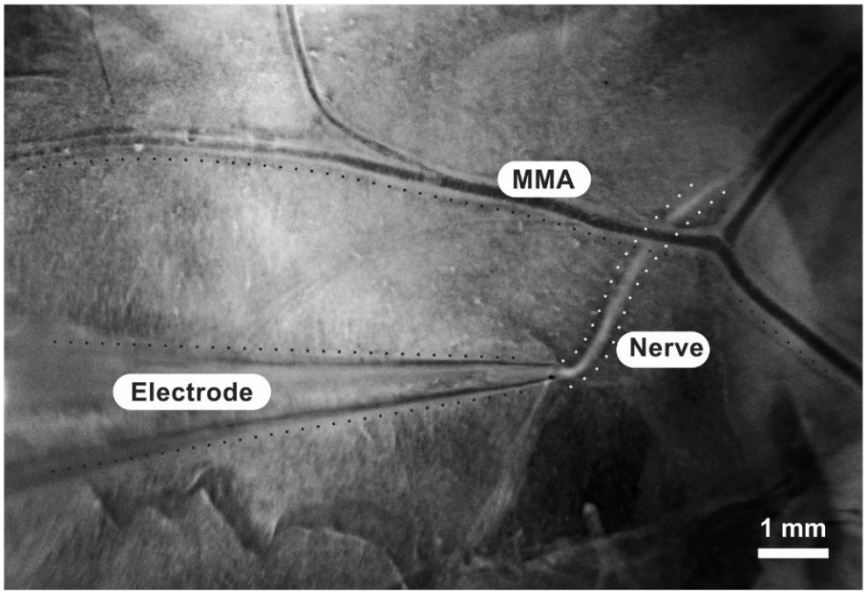

Figure 3. Nociceptive action potentials recordings from the trigeminal nerve. A photograph of the hemiskull preparation with preserved nervus spinosus (nerve), innervating the area of divergence of the middle meningeal artery (MMA). The nerve tip was sucked in a glass electrode.

4.3.2 P2X3 receptor responses in trigeminal ganglion neurons

During the experiment, the cells were constantly perfused with a solution containing (mM): 148 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, 10 D-Glucose, pH 7.2. The intracellular solution contained (mM): 145 KCl, 1 $MgCl_2$, 10 Hepes, 5 EGTA, 0.5 $CaCl_2$, 2 Mg-ATP, 0.5 Na-GTP, 5 KCl, pH 7.2. The solution was supplied using a gravity-controlled perfusion system (ALA Scientific Instruments Westbury, USA). For fast solution exchange (about 300-500 ms) the cells were under the flow of experimental solution from a second pipette.

P2X3-induced currents were recorded using the "whole cell" patch-clamp using Axopatch-200B amplifiers (Axon Instruments/Molecular Devices, USA) and borosilicate glass patch pipettes (Havard Apparatus, USA) with a resistance of 3-10 MΩ.

P2X3 currents were induced by local application of the agonist α,β-meATP at a concentration of 20 μM for 2 s using a rapid perfusion system (Rapid Solution Changer 200, BioLogic Science Instruments, France), with a solution supply of ~ 20 ms. To prevent desensitization of P2X3 receptors, α,β-meATP was applied at intervals of 5 minutes.

Patch-clamp data for recording P2X3-induced currents were analysed using the Clampfit software (Axon Instruments/Molecular Devices, USA). The change in the amplitude of P2X3-induced currents during the experiment was analysed.

4.4 Calcium signals in isolated neurons

Fluo4-AM fluorescent marker (2 μM) was used to visualize calcium signals in cells. Cells were placed in an extracellular solution containing the dye and incubated at 37°C for 30-40 minutes in darkness. Then the cells were placed in a normal extracellular solution for 10 minutes to ensure complete ester esterification, and then fluorescence visualization of stained cells was carried out using an Axio Observer.D1 microscope (Carl Zeiss, Germany). An excitation filter (BP 450-490 nm), a beam splitter (FT 510 nm) and an emission filter (LP 555 nm) were used. Fluorescence images were recorded using an AxioCam MRm high-speed camera (Carl Zeiss, Germany). The test substances were applied using a gravity-controlled perfusion system (ALA Scientific Instruments Westbury, USA). To differentiate neuronal cells, a 100 mM KCl solution was used, which was supplied for 2 s. Image processing software (NIH, USA) was used to process fluorescence images and estimate fluorescence intensity (in relative units, a.u.). Peak amplitude was calculated using the MATLAB software package (The MathWorks, USA).

4.5 Mast cell degranulation

To study mast cell degranulation, meninges staining (Wistar rats P35-40) with Toluidine Blue was used. [Guselnikova et al., 2014]. Hemiskulls were placed in the studied solutions for 20 min, after which they were placed in paraformaldehyde (4%) for 12 hours. Before isolation of the meninges, the skulls were washed in a phosphate-saline buffer solution of the following composition (mM): 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4. Isolated dura mater was fixed on a glass slide. Staining with Toluidin Blue lasted 10 minutes, and then the preparations were fixed with ethanol (95-99%). Pictures from stained meninges were taken at 20x magnification. The rate of degranulation was evaluated visually; the calculation was carried out as a % of the total number of cells (Figure 4).

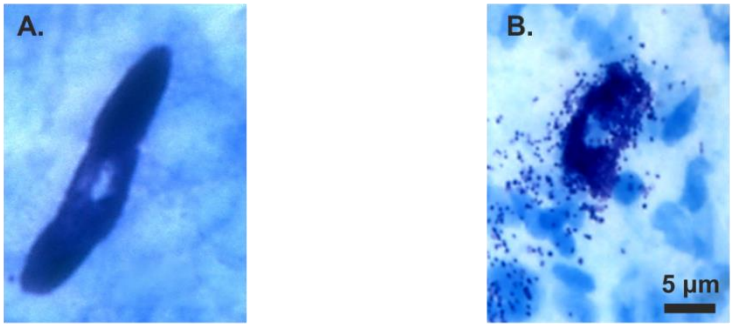

Figure 4. Photographs of meningeal mast cells. A. intact mast cell; B. degranulated mast cell.

4.6 MEASURING THE EXTRACELLULAR LEVEL OF ATP IN THE DURA MATER

The concentration of ATP released from the tissues of the dura mater (P 35-45) was estimated using the ATP luminescence analysis kit (Cat No. 6016941; PerkinElmer, Waltham, USA). Isolated rat hemiskulls were filled with studied compounds. After 20-minute incubation, 100 uL of the media were taken away for analysis. The analysis was performed according to the ATPlite assay kit protocol using 96-well Costar plates (Corning, USA). Luminescence was measured using a POLARstar Optima microplate reader (BMG Labtech GmbH, Germany).

4.7 DRUGS

Table 2. Main reagent specifications

Name	Final concentration, μM	Manufacturer	Description
α,β-meATP	20	Sigma-Aldrich, USA	P2X1/3 receptor agonist
Serotonin (5-Hydroxytryptamine, 5-HT)	2	Tocris, UK	5-HT receptor agonist
A-317491	10	Alomone Labs, IL	$P2X_3$ receptor antagonist
Adenosine triphosphate, ATP	100	Sigma-Aldrich, USA	Nucleotide
BzATP triethylammonium salt	30	Tocris, UK	$P2X_7$ receptor agonist
Capsaicin	1	Sigma-Aldrich, UK	TRPV1 receptor agonist
Capsazepine	10	Sigma-Aldrich, USA	TRPV1 receptor antagonist
HC 030031	50	Tocris, UK	TRPA1 receptor antagonist
MDL7222	10	Tocris, UK	5-HT3 receptor antagonist
Sodium hydrosulfide, (NaHS)	100	Sigma-Aldrich, USA	Hydrogen sulfide donor
Toluidine Blue	10%	Sigma-Aldrich, USA	Due

Sodium hydrosulfide (NaHS) was used as a hydrogen sulfide donor. In solution, NaHS dissociates into HS^- ions and binds to the hydrogen proton H+ to form H_2S. At room temperature (20°C), 22.3% of the total concentration of H_2S is present in the solution [Sitdikova et al., 2014]. Real-time measurements of H_2S concentration using amperometry approach indicated a fast loss of the sulfide after volatilization of H_2S [Deleon et al., 2012; Sitdikova et al., 2014]. In our experiments, NaHS was used at a concentration of 100 μM, which gives about 11 μM H_2S in the perfusion system. NaHS stock solutions were prepared immediately before the experiment and they were kept tightly closed in a dark place until use.

4.8 STATISTICAL ANALYSIS

Statistical data processing was performed using MATLAB and Origin Pro 2015 software (OriginLab, Northampton, USA). To assess reliability, the Student's t-test was used (for dependent (paired) and independent samples). Differences were considered statistically significant at $p<0.05$. All values are indicated as mean±error of the mean (M±SEM).

5 RESULTS

5.1 MECHANISMS OF ATP ACTION ON THE FREQUENCY OF ACTION POTENTIALS IN THE TRIGEMINAL NERVE

Peripheral trigeminal afferents transmit information from the cranial structures to the higher pain centers, which are responsible for the analysis, processing, and integration of pain signals. Stimulation of primary afferents in the meninges leads to an increase in the frequency of action potentials in the nerves and the trigeminal ganglion neurons and sensory trigeminal nuclei in the brainstem [Bartsch & Goadsby, 2002; Bove & Moskowitz, 1997; Burstein et al., 1998]. It was shown that the increased frequency of action potentials in trigeminal afferents correlates with the appearance of a subjective sensation of pain, and this increase in nociceptive firing is under the control of algogens [Edvinsson et al., 2012]. Activation of meningeal afferents is considered as the main mechanism for the occurrence of pain during migraine.

5.1.1 Effects of ATP on the frequency of action potentials in the rat and mouse trigeminal nerve

The peripheral part of the trigeminal nerve in the meninges generates spontaneous activity at rest. In rats, the baseline frequency of action potentials in control was 0.54±0.13 s^{-1}, and in mice – 0.23±0.13 s^{-1}. The key objective of this work was to study the effect of extracellular ATP on the spontaneous activity of the trigeminal nerve fibers. To study the effect of ATP on the electrical activity of the trigeminal nerve, ATP was applied for 10 min to the receptive field of meninges innervated by the trigeminal nerve – at the division of the middle meningeal artery (MMA) which is the largest artery of the dura mater. The large cerebral arteries, venous sinuses and MMA, are all sensitive to nociceptive irritants of various modalities [Dowgjallo, 1929; Penfield & McNaughton, 1940; Strassman et al., 2004; Messlinger et al., 1993]. To identify the neuronal and vascular mechanisms underlying the development of migraine attacks, stimulation of cerebral vessels and the dura mater is typically used [Liu et al., 2008].

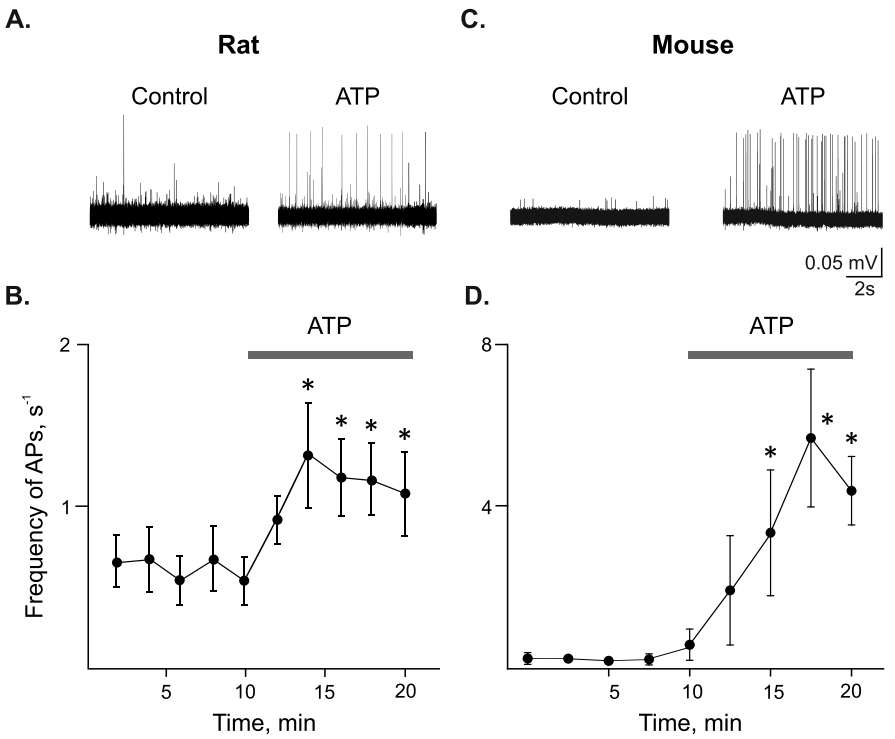

Figure 5. The effect of ATP on the frequency of action potentials of the trigeminal nerve, innervating the dura mater of the rat and mouse brain.
Original recordings of rat trigeminal nerve spiking in control and after the application of ATP (100 μM) in rat (A) and mice (C); Frequency of action potentials during the application of ATP (100 μM) in rat (n=12, B) and mice (n=6, D). mean±standard error; *p<0.05

In our experiments, we found that the application of ATP increased the frequency of action potentials in the trigeminal nerve of rats and mice (Figure 5). The peak of the frequency of action potentials in the presence of ATP (100 μM) in rats was 1.33±0.32 s^{-1} (n=12; p<0.05; Figure 5 A, B); ATP had a more pronounced effect in mice; the maximum frequency of action potentials was 5.66±1.69 s^{-1} (n=6; p<0.05; Figure 5 C, D).

To identify the subtype of ionotropic receptors mediating the effect of ATP, α,β-meATP, an agonist of P2X3 and P2X2/3 receptors was used, α,β-meATP (20 μM) increased the frequency of action potentials in the rat trigeminal nerve to 3.22±1.05 s^{-1} (n=5; p<0.05) from a control value of 0.9±0.12 s^{-1} (n=5; Figure 6). The selective blocker of P2X3 receptors A-317491 (10 μM) did not change the frequency of action potentials by itself, however, subsequent addition of α,β-meATP did not cause the increase in the frequency of action potentials. Thus, in control, the action potential frequency was 1.31±0.25 s^{-1} (n=5); in the presence of A-317491 it was 1.31±0.38 s^{-1}; and during the application of α,β-meATP it was insignificantly changed to 2.05±0.78 s^{-1} (n=5; p<0.05).

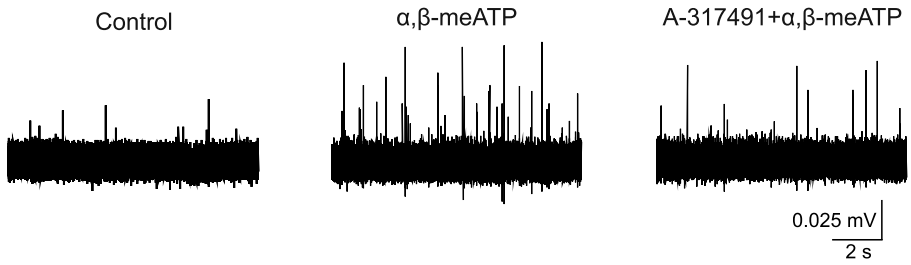

Figure 6. The role of P2X3 receptors in the ATP effect in rat trigeminal nerve.
The electrical activity of the rat trigeminal nerve in control, after application of the P2X3 receptor agonist α,β-meATP (20 μM); and α,β-meATP against the selective antagonist A-317491 (10 μM).

Our data indicate that ATP enhances spiking in the trigeminal nerve in both rats and mice, indicating its nociceptive effect, which promotes the appearance of pain during a migraine attack. It was also shown that the ATP-induced increase in the frequency of action potentials in the trigeminal nerve is mediated by activation of the P2X3 and heteromeric P2X2/3 receptors of trigeminal afferents.

5.1.2 ATP induces degranulation of mast cell in rat meninges

In addition to the direct effect on trigeminal nerve receptors via P2X3 receptors, ATP can cause mast cell degranulation [Radley & Grounds, 2006; Nurkhametova et al., 2018]. Mast cells in the meninges form clusters around blood vessels and nerve fibers. Mast cell degranulation can be caused by several factors, including ATP-induced activation of P2X receptors. Notably, pro-inflammatory and pronociceptive agents can exert their own direct stimulating effect on nerve endings but can also act via other substances released from mast cell vesicles. To study the indirect effect of ATP mediated by mast cell degranulation, the rat dura mater histological specimens were stained with Toluidine Blue (Figure 7). Intact mast cells had clearly defined boundaries, a rounded shape, and were characterized by dense staining. Mast cells were defined as degranulated if their membrane was destroyed, and the content of the cell was partially or completely released into the surrounding space in the form of a "scattering" of colored granules.

In the intact rat meninges, it was found that out of all randomly detected mast cells (100%), only 24.4±1.8% (n=6; $p<0.05$) were degranulated (Figure 7). Incubation of meninges in a solution containing ATP (100 μM) for 20 min led to a doubling of the number of degranulated cells amounting to 53.8±3.7% (n=6; $p<0.05$; Figure 7 B) from the total number of recorded cells.

Since it was shown earlier that P2X7 receptors are expressed in the membrane of mast cells, we hypothesized that their activation could mediate the effects of ATP in the meninges. Incubation of meninges in a solution containing the P2X7 receptor agonist BzATP (30 μM) for 20 min led to an effect similar to the action of ATP, namely, the number of degranulated cells was increased to 75.14±2.8% (n=6; $p<0.05$; Figure 7 B) in relation to the total number of recorded cells.

Thus, we obtained data indicating that ATP caused mast cell degranulation, which presumably promotes the release of the pro-inflammatory mediators contained in mast cells, which may have their own effects on the excitability of trigeminal nerve endings. Based on the action of BzATP we can conclude that the likely target of ATP in meningeal mast cells is the P2X7 receptor.

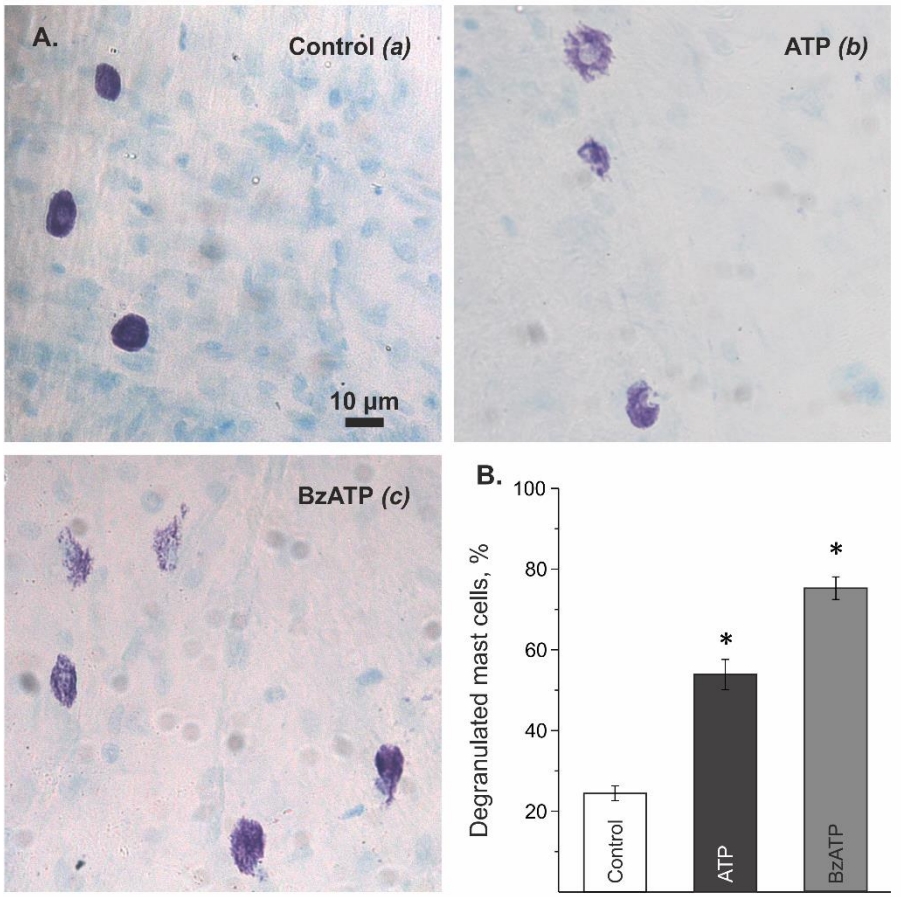

Figure 7. The effect of ATP on mast cell degranulation in the rat dura mater. A. Images of the fixed dura mater of the brain and mast cells stained with Toluidin Blue in control (a), after incubation in ATP (b) and with the P2X7 receptor agonist - BzATP (c); B. The percentage of degranulated rat mast cells in control and after incubation in a solution containing ATP (100 µM) and BzATP (30 µM) relative to the total number of detected cells. *p<0.05

5.1.3 The role of mast cells in the stimulatory effect of ATP on the electrical activity of the trigeminal nerve

To study the role of mast cells in the indirect effects of ATP on the trigeminal nerve spiking, genetically modified mice with a mast cell deficiency (C57BL/6J-Kit$^{W\text{-}v}$/J mice) were used. To confirm the mast cell deficiency, meninges were stained with

Toluidine Blue. In the meninges of wild-type animals (C57BL/6J), mast cells formed clusters, while mast cells were absent in the C57BL/6J-Kit$^{W\text{-}v}$/J line of mice (Figure 8).

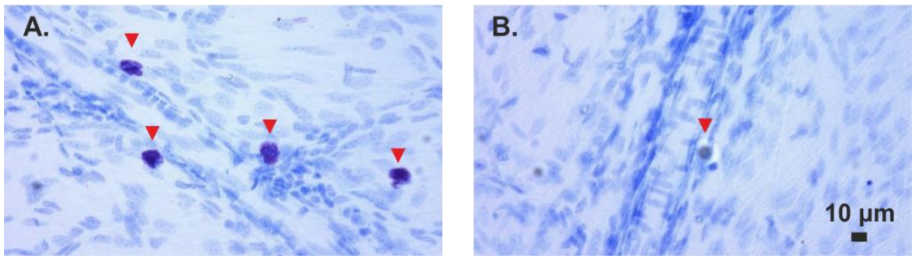

Figure 8. Histological preparations of the dura mater stained with Toluidine Blue. Arrows indicate mast cells in the meninges of wild-type mice (C57BL/6J line) (A) and of genetically modified mice without mast cells (C57BL/6J-Kit$^{W\text{-}v}$/J mice) (B).

Next, we analyzed the spontaneous and ATP-induced electrical activity of the trigeminal nerve in genetically modified mice (Figure 8 B). The baseline frequency of action potentials in the trigeminal nerve of C57BL/6J-Kit$^{W\text{-}v}$/J mice was 0.21±0.08 s^{-1} (n=7), which did not differ significantly from the baseline frequency of the trigeminal nerve in the control C57BL/6J mice (0.23±0.13 s^{-1}, n=6; $p>0.05$).

However, the effect of ATP on the frequency of action potentials was significantly different between control and mast cell deficient mice (Figure 9). In the control group, ATP caused a strong significant increase in the frequency of action potentials after 6 min of application, up to 5.65±1.69 s^{-1} (n=6, $p<0.05$). However, in the C57BL/6J-Kit$^{W\text{-}v}$/J group, the increase of action potential frequency in response to ATP application was significantly less and after 6 min raised up to 1.01±0.36 s^{-1} (n=7; $p<0.05$) (Figure 9). It was suggested that the differences in ATP responses were associated with mast cell degranulation and the subsequent release of pro-nociceptive agents, which does not occur in C57BL/6J-Kit$^{W\text{-}v}$/J mice due to mast cell deficiency.

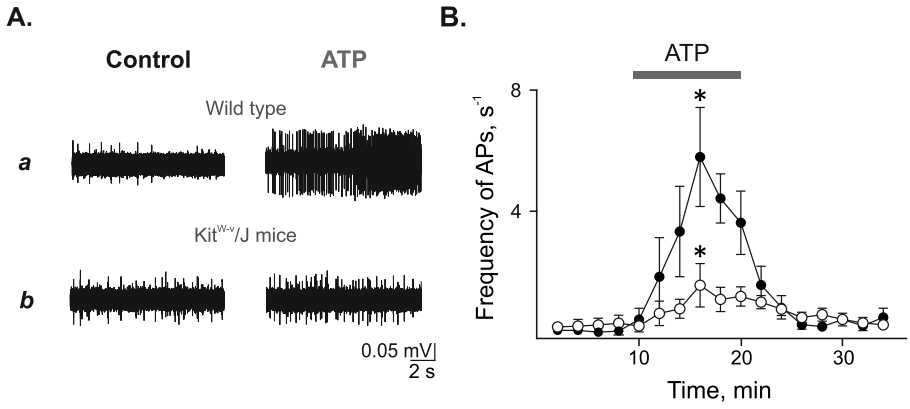

Figure 9. Effect of ATP on the frequency of action potentials in control and C57BL/6J-Kit$^{W\text{-}v}$/J mice. A. An example traces of action potentials from the trigeminal nerve of wild-type mice (a) and C57BL/6J-Kit$^{W\text{-}v}$/J mice (b). B. Frequency of action potentials during ATP

(100 μM) application in wild-type animals (black circles) and in mice without mast cells (C57BL/6J-Kit^{W-v}/J, white circles). *p<0.05

5.1.4 The role of serotonin in the nociceptive effects of ATP in mice trigeminal nerve

One of the pro-inflammatory agents released during mast cell degranulation is serotonin (5-HT), which in rats has a pronounced nociceptive activity in the peripheral trigeminal nerve [Kilinc et al., 2017]. Therefore, we suggested that the release of serotonin from mast cells contributes to the trigeminal nerve activation induced by ATP.

In the first set of experiments, the effect of serotonin on the electrical activity of the trigeminal nerve was analyzed. Serotonin (2 μM) increased the frequency of action potentials to a plateau which was maintained over the 20 min period after the substance was supplied (Figure 10). In control, the frequency of action potentials was 0.1±0.04 s^{-1}. By the 4th min of serotonin application, the frequency of action potentials increased to 0.36±0.15 s^{-1}; whereas by 6 min the frequency reached a value of 0.58±0.23 s^{-1} (n=7; p<0.05). The action potential frequency remained at the same level at 18 min (0.77±0.23 s^{-1}, n=7; p<0.05) compared to control.

To identify the receptor mechanism of serotonin action in mice, the selective 5-HT3 receptor blocker MDL7222 (10 μM) was used [Kilinc et al., 2017]. Incubation with MDL7222 did change the baseline frequency of action potentials in the trigeminal nerve. However, MDL7222 reduced the effect of serotonin on the frequency of action potentials compared to its effect in control (Figure 10 A). Thus, the frequency of action potentials in the control was 0.057±0.038 s^{-1}, whereas after the application of MDL7222 the frequency was 0.014±0.008 s^{-1} (n=7, p>0.05). In the presence of MDL7222, the frequency of action potentials after 8 min of serotonin application was 0.15±0.06 s^{-1} (n=7; p<0.05), and by 18 min it was 0.34±0.14 s^{-1} (n=7, p>0.05).

Thus, MDL7222 significantly reduced the activating effect of serotonin (p<0.05) compared to its control effect, which indicates that 5-HT3 receptors are the main target of serotonin in the peripheral trigeminal afferents in the meninges.

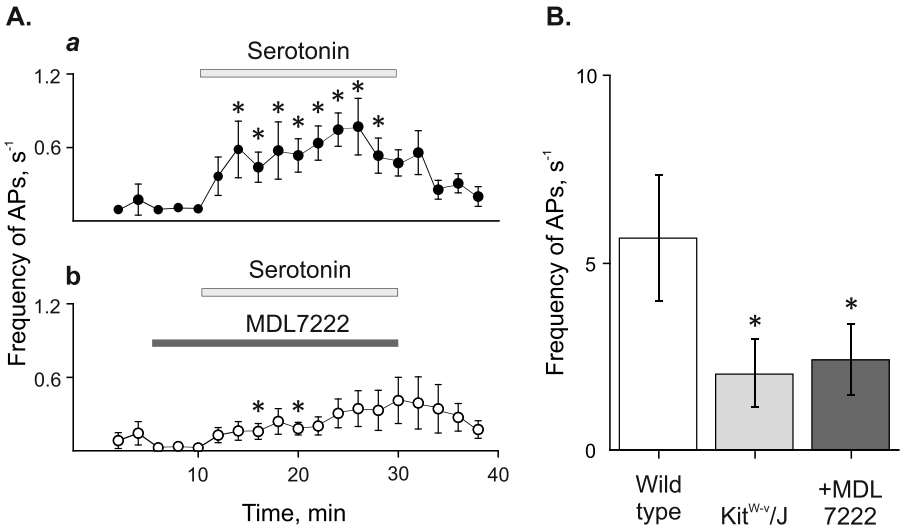

Figure 10. The pro-nociceptive action of 5-HT3 in the mouse trigeminal nerve. A. Effect of serotonin (2 μM) on the frequency of action potentials of mouse trigeminal nerve in control (a) and in the presence of the selective 5-HT3 receptor antagonist MDL7222 (10 μM) (b). B. A histogram showing the frequencies of action potentials in wild-type mice in comparison with Kit^{W-v}/J mice and the ATP effect in wild-type mice in the presence of the 5-HT3 receptor blocker. *p<0.05

Thus, we conclude that ATP promotes mast cell degranulation by activation of P2X7 receptors, followed by the release of serotonin, which, in turn, works as an amplifier of the nociceptive signal. It should be noted that given the abundance of ATP receptors in various cell types, additional mechanisms of ATP action are also possible during the formation of nociceptive signaling, including interaction of ATP with other receptors, vascular effects, and modulation of other local systems.

5.2 THE EFFECT OF HYDROGEN SULFIDE ON THE PRO-NOCICEPTIVE EFFECT OF ATP

In the second part of our study, we analyzed the interactions between the signaling induced by ATP and the gas molecule H_2S, which, in various tissues, has either pro- or antinociceptive effects mediated through multiple cellular targets [Okubo et al., 2012; Matsunami et al., 2009]. Notably, expression of CBS, an enzyme providing the endogenous synthesis of H_2S both in DRG and trigeminal neurons was detected [Xu et al., 2009]. It was also shown that P2X3 receptors colocalize with CBS in DRG neurons innervating the hind limbs, and the expression level of P2X3 receptors in these neurons was related to the activity of the CBS enzyme. These data indicate a possible interaction between P2X3 receptors and the CBS/H_2S axis in DRG neurons and their common participation in the mechanisms of neuropathic pain [Wang et al., 2015].

However, information on the role of H_2S in the pathogenesis of primary headaches, such as migraine, is limited and contradictory [Teicher et al., 2017], and the possible interaction of ATP and H_2S signaling in meningeal membranes has not been studied.

5.2.1 Receptor mechanisms of the hydrogen sulfide action on the electrical activity of the rat trigeminal nerve

Sodium hydrosulfide (NaHS), a common donor of H_2S, applied for 20 min at a concentration of 100 μM increased the electrical activity of meningeal trigeminal nerve fibers (Figure 11 A). After 10 min of NaHS application, the action potential frequency was 1.85±0.2 s^{-1} compared with 0.44±0.06 s^{-1} in control ($p<0.05$; n=5) suggesting a pro-nociceptive effect. After that peak, by 20 min of NaHS action, the frequency of action potentials had decreased to the control level (0.58±0.05 s^{-1}; n=5).

Several studies indicate (see Introduction) that in various tissues, the activating effect of NaHS is mediated by the activation of TRPV1 or TRPA1 receptors. It has been also shown that the H_2S synthesizing enzyme CBS is co-expressed in sensory neurons together with P2X3 receptors [Trevisani et al., 2016]. Therefore, we next investigated the role of TRPV1, TRPA1, and P2X3 receptors in the nociceptive effects of H_2S in the rat trigeminal nerve. To this aim, we used specific blockers of the TRPV1, TRPA1 and P2X3 receptors (Figure 11). The obtained results showed that the selective TRPV1 receptor blocker, capsazepine (25 μM), completely prevented the stimulating effects of NaHS on the peripheral nerve endings of the trigeminal nerve (Figure 11). In the control, the frequency of action potentials was 0.40±0.11 s^{-1} and it did not significantly change during incubation in capsazepine (0.32±0.13 s^{-1}; n=6; $p>0.05$). Subsequent application of NaHS (100 μM) in the presence of capsazepine did not lead to a significant increase in the frequency of action potentials. Thus, the frequency of action potentials was 0.56±0.30 s^{-1} during the first 5 min of application of NaHS; 0.44±0.27 s^{-1} at 10 min and 0.27±0.15 s^{-1} at 15 min, respectively (n=6; $p>0.05$; Figure 11).

Inhibition of TRPA1 receptors with HC-030031 (50 μM) alone did not change the frequency of action potentials (0.65±0.09 s^{-1} in control versus 0.59±0.1 s^{-1} under the action of HC-030031; n=8, $p>0.05$). However, HC-030031 partially reduced the stimulatory effect of NaHS. Thus, the increase in the frequency of action potentials after application of NaHS in the presence of HC-030031 was 1.28±0.31 s^{-1} (n=8; $p<0.05$; Figure 11), which was significantly lower than the frequency of action potentials during NaHS application under control conditions (Figure 11).

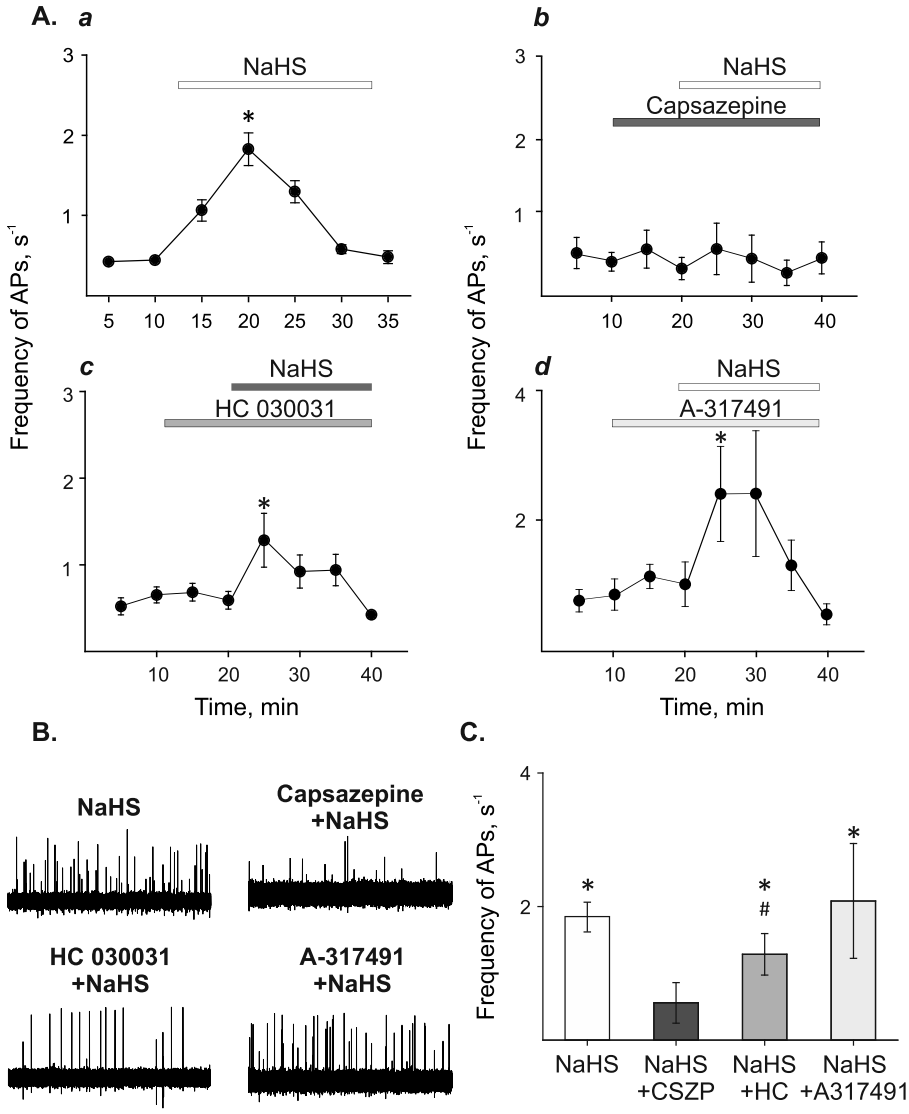

Figure 11. The role of TRPV1, TRPA1, and P2X3 receptors in the effects of NaHS on the electrical activity of the trigeminal nerve. A. The frequency of action potentials during application of NaHS (100 μm) in control (a); after inhibition of TRPV1 receptors by capsazepine (25 μM) (b); in the presence of the selective TRPA1 receptor blocker HC-030031 (50 μM) (c); in the presence of the selective P2X3 receptor blocker A317491 (10 μM) (d); B. Examples traces of action potential recording in the trigeminal nerve of rats with NaHS application in the control and in the presence of TRPV1, TRPA1 and P2X3 receptor blockers; B. Histograms showing the frequencies of NaHS-induced action potentials in the control and in the presence of TRPV1, TRPA1 and P2X3 receptor blockers; *p<0.05 compared with the control; #p<0.05 compared with the NaHS effect.

To study the role of P2X3 receptors in the pro-nociceptive effects of a donor of H_2S, the specific antagonist of P2X3 and P2X2/3 receptors A317491 was used (Figure 11). Preliminary application of the antagonist (10 μM) showed that it does not have

its own pronounced effect on the frequency of action potentials in the trigeminal nerve. Thus, the frequency of action potentials in control was 0.70±0.21 s^{-1}, whereas after application of the antagonist, the frequency was 0.84±0.30 s^{-1}. Subsequent application of the H_2S donor significantly increased the frequency of action potentials in the rat trigeminal nerve to 2.08±0.86 s^{-1} (n=4; $p<0.05$), which did not differ from the effects of NaHS in control. The data obtained indicated that the main mechanism for the increased frequency of action potentials by H_2S is the activation of TRPV1 receptors and, to a lesser extent, TRPA1 receptors. However, no role of P2X3 receptors in the pro-nociceptive action of NaHS has been identified.

5.2.2 Effect of NaHS on the pro-nociceptive effect of ATP in the rat trigeminal nerve. The role of TRPV1 receptor activation

Next, we analyzed the effects of H_2S on the pro-nociceptive effect of ATP in the rat trigeminal nerve. In control, the application of ATP (100 μM) led to a surge in the activity of action potentials in the rat trigeminal nerve (an increase in the frequency of action potentials to 2.51±0.43 s^{-1} after 10 min of application of ATP compared to control (0.75±0.12 s^{-1}; n=5; $p<0.05$)). However, after preliminary application of the H_2S donor NaHS (100 μM) for 20 min, the subsequent application of ATP did not increase the frequency of action potentials. Thus, after 10 min of ATP application it was 0.36±0.13 s^{-1}. Thus, preliminary exposure of the trigeminal afferents to H_2S prevented the pro-nociceptive action of ATP. Co-expression of P2X3 and TRPV1 receptors and the interaction of these two types of nociceptive receptors upon their activation have been shown [Saloman et al., 2013]. Pre-activation of TRPV1 receptors decreased the subsequent activation of P2X3 receptors due to the inhibitory interactions of the C-termini of P2X3 and TRPV1 proteins [Saloman et al., 2013; Ruan et al., 2006; Stanchev et al., 2009].

Since, according to our data, activation of TRPV1 receptors underlies the stimulating effect of H_2S, we suggested that activation of TRPV1 inhibits the function of P2X3 receptors and prevents the pro-nociceptive effect of ATP. Therefore, the effect of ATP was analyzed under conditions of preliminary activation of TRPV1 with capsaicin. In control, the frequency of action potentials was 0.32±0.2 s^{-1}; application of capsaicin (1 μM) sharply increased activity to 3.3±0.98 s^{-1}, followed by a decrease in the frequency of action potentials to values close to control (0.31±0.11 s^{-1}), likely due to the development of desensitization of TRPV1 receptors. The subsequent application of ATP (100 μM) led to an increase in the frequency of action potentials to 1.32±0.53 s^{-1} by 10 min and 20 min to 2.1±0.53 s^{-1} (n=4; $p<0.05$) (Figure 12).

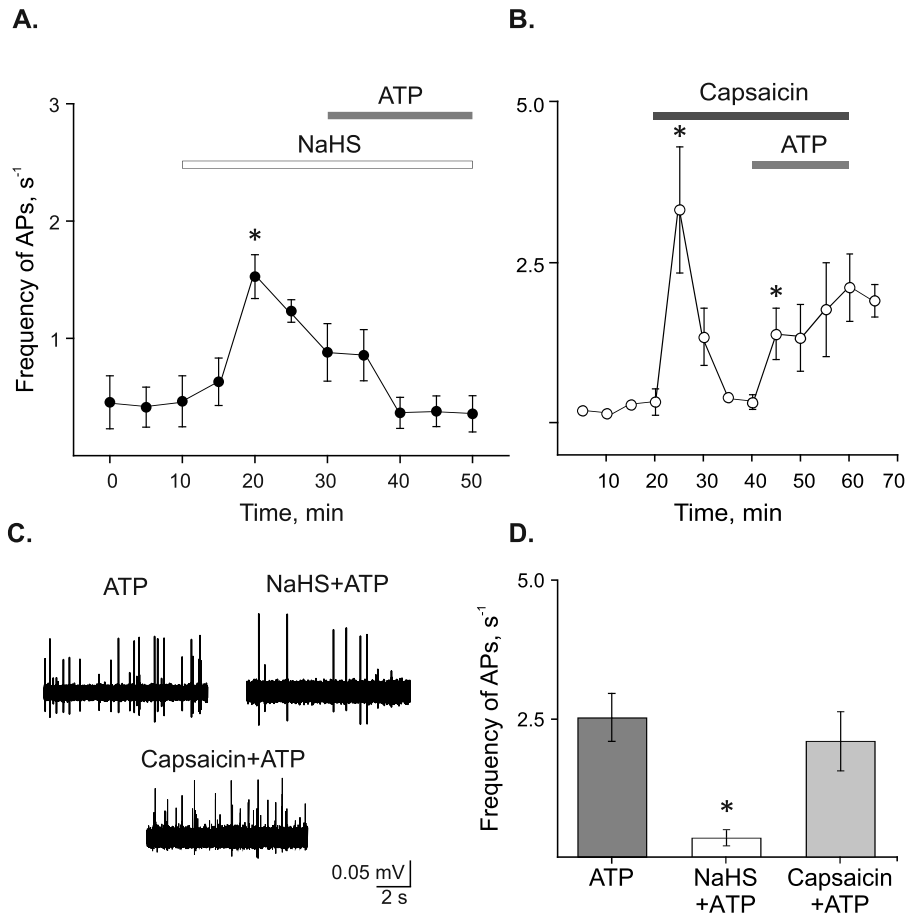

Figure 12. Effect of H_2S on the pro-nociceptive effect of ATP in trigeminal afferents. A. The frequency of action potentials during application of ATP (100 μM) in the presence of NaHS (100 μM); B. The frequency of action potentials during the application of ATP (100 μM) after of preliminary activation of TRPV1 receptors with capsaicin (1 μM); C. Examples trace of action potentials in the trigeminal nerve of a rat with ATP application in the control, after preliminary incubation in NaHS and capsaicin; G. The frequency of action potentials in the trigeminal nerve after application of ATP; ATP in the presence of NaHS; ATP in the presence of capsaicin. *p<0.05

The results indicated that the activation of TRPV1 receptors did not affect the pro-nociceptive effect of ATP. This fact excluded the role of TRPV1 receptors in suppressing the effect of ATP under the action of H_2S.

5.2.3 Effect of NaHS on inward currents mediated by activation of P2X3 receptors in rat trigeminal ganglion neurons

Further study of the mechanisms of the inhibitory effect of the hydrogen sulfide donor on the pro-nociceptive properties of ATP was carried out using cultures of isolated trigeminal ganglion neurons. To this aim, we used patch-clamp recording, and membrane currents in neurons were elicited by application of the P2X3 receptor

agonist α,β-meATP (20 μM) (Figure 13). To prevent desensitization of the P2X3 receptors, α,β-meATP was applied for 2 s via the fast application system with an interval of 5 min.

In control, the application of α,β-meATP (20 μM) caused three types of membrane current: fast, slow and biphasic containing both a fast and slow component. The average amplitude of the fast current and the fast component of the biphasic current was 460.8±78 pA (n=15). The amplitude of the slow current and the slow component of the biphasic current was 200.54±42.26 pA (n=14).

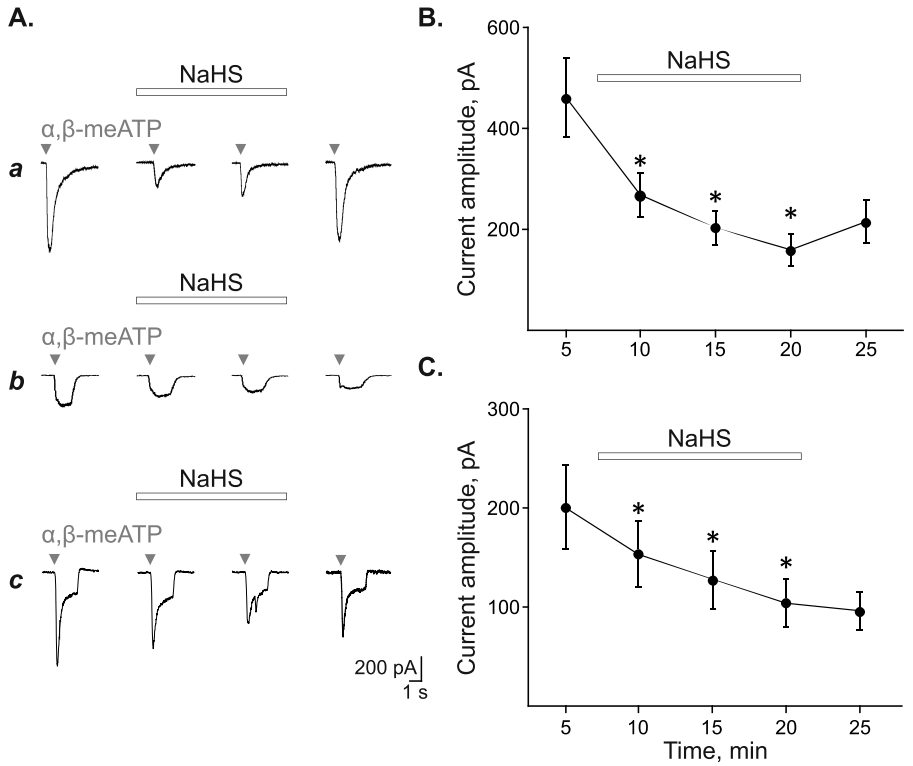

Figure 13. Effect of NaHS (100 μM) on P2X3 mediated currents in trigeminal ganglion neurons. A. Examples of fast P2X3 receptor currents in control and in the presence of NaHS (a); Examples of slow P2X3-induced currents in trigeminal ganglion neurons in control and in the presence of NaHS (b); Examples of biphasic P2X3 receptor currents in control and in the presence of NaHS (c) B. Changes in the amplitude of the fast component; C. Changes in the amplitude of the slow component of P2X3 currents. *p<0.05

Under conditions of cell perfusion with a solution containing NaHS at a concentration of 100 μM, the amplitude of P2X3 receptor responses decreased within 15 min of application. A more significant decrease in the amplitude of the fast component of the current mediated by P2X3 receptors was observed after 5 min of NaHS application (268.2±42.7 pA; n=5, p<0.05). By 10 min, the amplitude of the fast component had decreased to 203.5±32.8 pA; and by 3 min to 158.8±31.1 pA (n=15, p<0.05). The amplitude of the slow component showed a significant decrease to

153.7±33.6 pA (n=14, $p<0.05$) after 5 minutes of application. The wash led to a partial recovery of responses, which did not reach the initial values.

Thus, it was shown that application of the hydrogen sulfide donor NaHS reduced the responses of ATP- sensitive P2X3 receptors.

5.2.4 The effect of NaHS on calcium signals induced by activation of ATP receptors in rat trigeminal ganglion neurons

It is known that activation of ATP receptors in trigeminal ganglion neurons causes a short-term increase in the level of intracellular calcium (the so-called calcium signals) due to the activation of P2X3 receptors. In the next set of experiments, we analyzed the effects of NaHS on calcium signals induced by ATP and α,β-meATP, a P2X3 receptor agonist (Figure 14).

Analysis of the whole population of neurons studied in these experiments showed that an increase in calcium in response to ATP application was observed in 70% of cells (61 of 87 cells) and 41.4% of cells in response to α,β-meATP application (33 of 136 cells). In control experiments, we analyzed changes in the amplitude of calcium signals during repetitive ATP applications with an interval of 5 min to reduce the degree of receptor desensitization. Under these conditions, the average amplitude of the first response to ATP was 3.53±0.45 a.u. the amplitude of the second response was 3.42±0.42 a.u. whereas the amplitude of the third response was 2.93±0.3 a.u. (n=61), thus no significant differences between the responses were observed. The P2X3 receptor agonist α,β-meATP (20 μM) elicited calcium responses with an average amplitude of 3.53±0.45 a.u. at the first application; 3.42±0.42 a.u. - in response to the second application and 2.92±0.3 a.u. (n=33) - in response to the third application. Again, no significant differences between the responses were observed.

Next, calcium signals in response to the application of agonists were studied in the presence of the H_2S donor NaHS. ATP (100 μM) was first applied to the cells for 2 s, causing the first control calcium response. Then, for 5 minutes, the cells were perfused with a solution containing NaHS (100 μM) and ATP receptor agonists were reapplied. To differentiate neuronal and glial cells, a KCl solution (100 mM) was used.

In control, the amplitude of the first response to the application of ATP, was 3.08±0.3 a.u. (n=66). In the presence of NaHS, the ATP response decreased to 2.01±0.27 a.u. (n=66; $p<0.05$), and wash did not significantly change the average amplitude of the response (1.93±0.26 a.u.; n=66; $p>0.05$). In only approximately 30% of the cells the amplitude of the calcium signal increased, in the other cases, the amplitude did not change, or a slight decrease was observed.

Application of α,β-meATP in control induced initial calcium responses with an amplitude of 2.73±0.26 a.u. (n=27), which was reduced by the H_2S donor to 1.69±0.23 a.u. (n=27, $p<0.05$). The subsequent wash did not significantly change the calcium response, the amplitude of which was 1.52±0.2 a.u. (n=27; $p>0.05$). Only in approximately 50% of cases did washing lead to a partial increase in the amplitude of the calcium response.

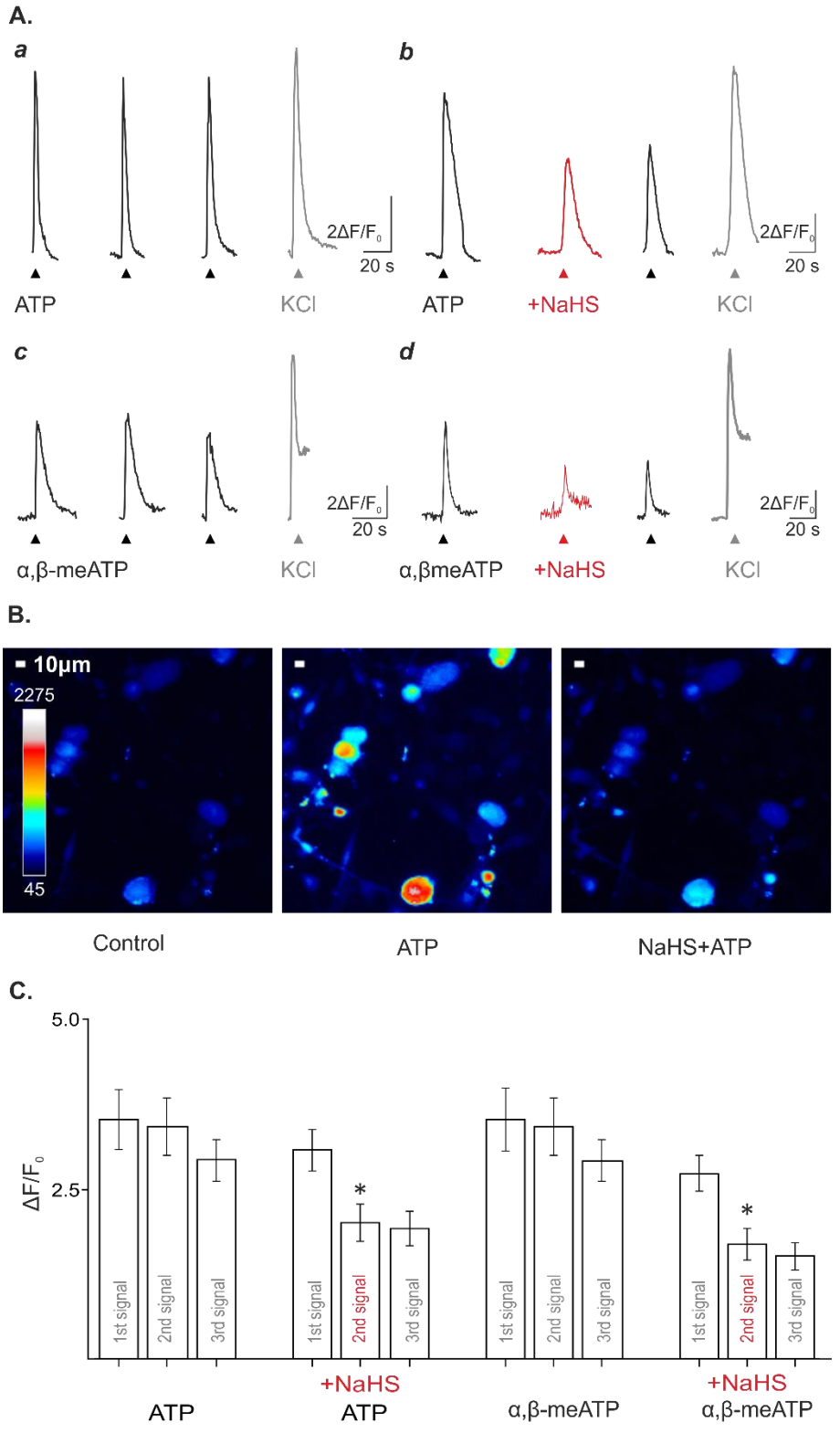
A.
a
b
2ΔF/F0
20 s
ATP
KCl
ATP
+NaHS
KCl
c
d
α,β-meATP
KCl
α,βmeATP
+NaHS
KCl
B.
10μm
2275
45
Control
ATP
NaHS+ATP
C.
5.0
2.5
ΔF/F0
1st signal
2nd signal
3rd signal
*
+NaHS
ATP
α,β-meATP

Figure 14. The effect of NaHS on ATP-induced calcium signals in a culture of isolated rat trigeminal ganglion neurons. A. (a) Examples of calcium signals in response to three repeated ATP applications (100 μM); (b) examples of calcium signals during the application of ATP (100 μM) in control, and in the presence of NaHS (100 μM) ;(c) three consecutive applications of α,β-meATP (20 μM); (d) examples of calcium signals during the application of α,β-meATP (20 μM) in control, and in the presence of NaHS (100 μM). In all cases, a signal is shown in response to the application of KCl for the differentiation of neuronal and glial cells. B. Pseudo-colour images reflecting calcium signals in response to ATP application in the control and after preliminary perfusion in a solution containing NaHS. C. Amplitude of calcium signals in response to three repeated applications of ATP (100 μM) or α,β-meATP (20 μM) in the control and in the presence of NaHS *p<0.05

Thus, H_2S reduced the amplitude of calcium signals caused by the application of ATP or the selective P2X3 receptor agonist in isolated trigeminal ganglion neurons.

5.2.5 The effect of NaHS on the level of extracellular ATP in rat meninges

In the meningeal membranes, the level of ATP in the extracellular space is at low nanomolar concentrations [Yegutkin et al., 2016]. ATP release can occur from nerve endings, astrocytes, platelets, endothelial cells, mast cells, and through exocytosis or pannexin-1 channels [Burnstock et al., 2011; 2014]. We analyzed the level of ATP released from rat meningeal membranes under control conditions and during incubation in NaHS for 20 min. The basic measurement of ATP level in meninges of the control group provided a value of 1.12±0.13 nM, after 20 min this level had not changed (1.04±0.24 nM; n=8). In the experimental group, the initial ATP level was 0.99±0.17 nM, and after incubation in a solution containing 100 μM NaHS, the ATP level significantly decreased to 0.23±0.05 nM (n=8; $p<0.05$; Figure 15). Based on this data, it can be assumed that H_2S reduced ATP release probably by affecting the transport of ATP through the membrane.

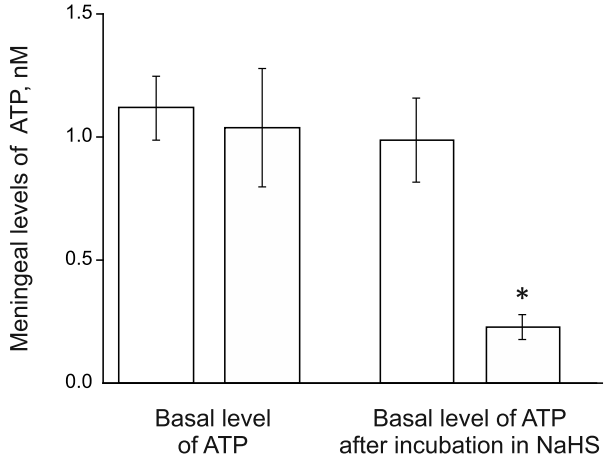

Figure 15. Effect of the hydrogen sulphide donor on the level of extracellular ATP in a rat hemiskull preparation by using the ATP luminescence analysis kit. The ATP level was determined in control (2 measurements with an interval of 20 min) and after incubation in 100 μM NaHS for 20 min. *p<0.05

5.2.6 The effect of the hydrogen sulfide donor on the degranulation of meningeal mast cells

The modulating effect of H_2S on the pro-nociceptive effect of ATP in the trigeminal nerve can be carried out via meningeal mast cells which partially mediate the pro-nociceptive effect of ATP. As we showed earlier, the action of ATP includes not only direct activation of trigeminal afferents, but also degranulation of mast cells, followed by the release of active pro-inflammatory compounds, such as serotonin.

We found that ATP caused a significant increase in the number of degranulated meningeal mast cells (53.8±3.7%; n=6; $p<0.05$) compared with the control group (24.4±1.8%; n=6; $p<0.05$).

To study the effects of H_2S on mast cells, meningeal membranes were incubated in a solution containing 100 μM NaHS for 30 min. Incubation of meninges with the H_2S donor did not change mast cell morphology, and the number of degranulated cells did not exceed the control values (31.8±4.6%; n=6). However, pre-incubation in NaHS for 10 min followed by application of ATP (100 μM) for 20 min did not increase mast cell degranulation (34.7±4.7%; n=6). Thus, the H_2S donor prevented mast cell degranulation by ATP (Figure 16).

We previously showed that mast cell degranulation under the action of ATP is mediated by the activation of P2X7 receptors, which, in addition to responsiveness to this purine, can also transport ATP through the membrane via ion channels of P2X7 receptors and by modulating the activity of pannexin-1 channels [Wareham & Seward, 2016; Kurashima et al., 2012; Iglesias et al., 2006]. Therefore, in subsequent experiments, we analyzed the ability of the P2X7receptor agonist BzATP to activate mast cells and studied the action of the NaHS donor on this effect. We found that 30 μM BzATP increased the number of degranulated cells to 75.14±2.8% (n=6, $p<0.05$; Figure 16). Pre-incubation in NaHS (100 μM) for 10 min prevented P2X7 mediated degranulation of the meningeal mast cells as the number of degranulated mast cells was only 33.4±5.5%; (n=6; Figure 16). Thus, the H_2S donor prevents ATP-induced degranulation of meningeal mast cells, which is manifested by the decreased activity of the P2X7 receptor agonists.

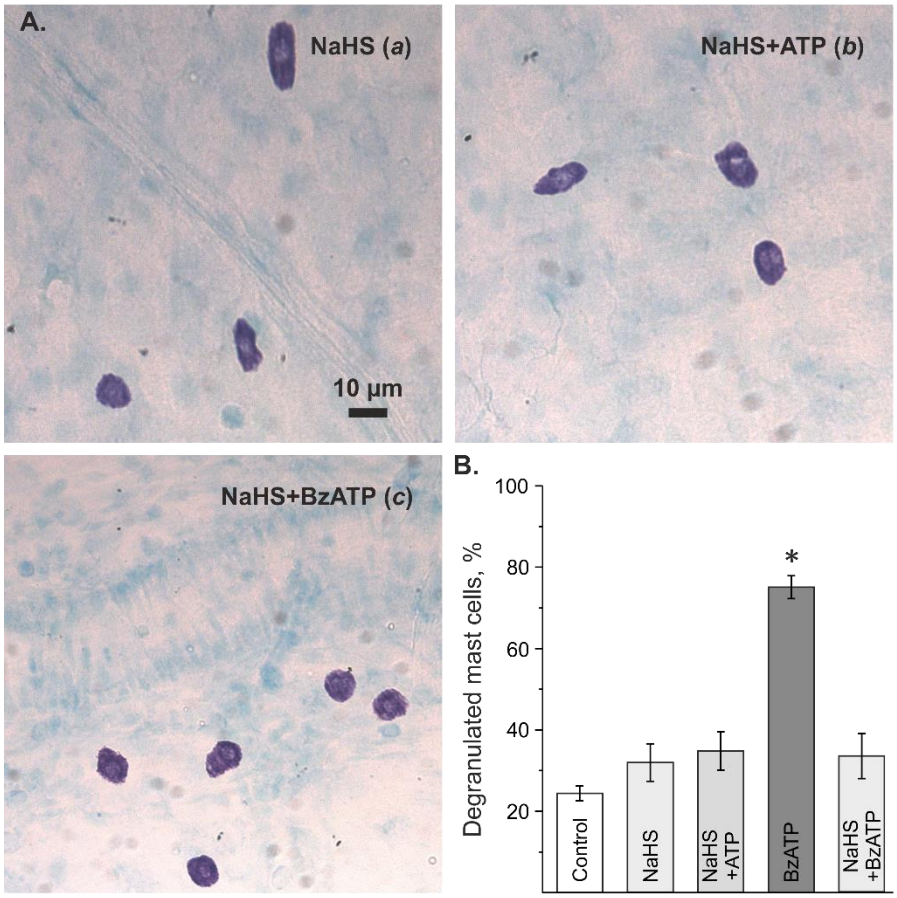

Figure 16. Effect of the NaHS donor on ATP-induced mast cell degranulation. A. Images of Toluidine Blue stained rat meninges after incubation in NaHS (100 µM); NaHS+ATP (100 µM); NaHS+BzATP (30 µM); B. Histograms showing the level of mast cell degranulation under various conditions (n=4 for each group). *p<0.05

6 DISCUSSION

6.1 THE ROLE OF ATP IN THE PATHOGENESIS OF MIGRAINE

Migraine is a widespread neurological disorder with several pronounced symptoms, including severe headache and it is one of the most common causes of temporary disability [Steiner et al., 2016]. Treatment of migraine is often difficult due to the limited efficacy of the available drugs and their side effects [Schuster and Rapoport, 2016]. Thus, the search for new treatments and prevention approaches remains highly relevant. The peripheral part of the trigeminal nerve in the meninges plays a key role in the pathogenesis of migraine pain [Olesen et al., 2009; Schueler et al., 2014] and it represents a potential target for pharmacological interventions in headaches.

The purinergic theory of migraine proposed by Burnstock in 1981 is based on the wide distribution of purine receptors in the vascular and nervous systems [reviewed in Burnstock & Wood, 1996], including the sensory DRG and trigeminal ganglia [Burnstock, 2009]. According to the purinergic theory of migraine, ATP and its metabolites are directly involved in the pathogenesis of migraine, in particular, mediating vasodilation of intracranial vessels [Burnstock, 1981, 1982; 2013].

In the rat meninges and cultures of trigeminal neurons, a very low level of extracellular ATP is observed, which prevents the constant stimulating effect of endogenous ATP on peripheral afferents of sensory neurons [Yegutkin et al., 2016]. In contrast, there are relatively high concentrations of extracellular AMP and adenosine in the meninges and trigeminal ganglia indicating high activity of ATP-degrading ectonucleotidases [Yegutkin et al., 2016]. Such control of nucleotide homeostasis is important for keeping the activity of trigeminal nerves low in the meninges, which are the main locus for the generation of nociceptive signals during migraine [Olesen et al., 2009].

6.1.1 The role of P2X3 receptors of peripheral trigeminal afferents in the pro-nociceptive effect of ATP

A comparative analysis of purines, including ATP and its derivatives, for trigeminal nerve activity showed that only the application of ATP (100 μM) led to an increase in nociceptive activity in the rat trigeminal nerve. In contrast to ATP, its metabolites, ADP, AMP and adenosine, did not show a pronounced nociceptive effect [Yegutkin et al., 2016]. In the present work, we also showed an increase in the frequency of action potentials in the peripheral processes of the trigeminal nerve in both rats and mice after ATP application which indicates the similarity of pro-nociceptive mechanisms in different animal species.

Differences in the effectiveness of ATP and its metabolites may be associated with the expression of various types of purinergic receptors that are present in the peripheral terminals of the trigeminal nerve and in the somata of trigeminal ganglion

neurons. In somata of trigeminal ganglion neurons, ADP can generate action potentials; this is comparable to the action of ATP [Ceruti et al., 2008; Magni et al., 2013; 2015]. Apparently, in the trigeminal ganglion, ADP causes activation of P2X and P2Y receptors on the membranes of glial cells, which contributes to the maintenance of long-term pain in migraine [Ceruti et al., 2008; 2011; Kawaguchi et al., 2015]. We found that adenosine, the final breakdown product of ATP, accumulates in the meninges and cells of the trigeminal ganglion [Yegutkin et al., 2016]. In trigeminal ganglion neurons, adenosine activates A1 receptors and inhibits activity of the trigeminal nucleus caudalis and blocks the release of CGRP [Burnstock, 1989]. Thus, it plays a potentially inhibitory role, as has been shown in other parts of the central nervous system [Dunwiddie & Masino, 2001].

To analyse the receptor mechanisms of ATP action in the peripheral trigeminal afferents, we used P2X3 receptor antagonists and agonists. The agonist of P2X3 receptors α,β-meATP increased the frequency of action potentials in rat meningeal trigeminal nerves, and this effect was blocked by the P2X3 receptor antagonist A-317491. The prolonged generation of action potentials without pronounced desensitization may be associated also with the simultaneous activation of P2X2/3 receptors, which are also blocked by A-317491.

Thus, we showed that ATP increases the frequency of action potentials in the trigeminal nerve in rats and this effect is mediated by direct activation of P2X3 and P2X2/3 receptors].

6.1.2 The role of mast cell degranulation in the pro-nociceptive action of ATP

One of the potential sources of endogenous inflammatory mediators during a migraine attack are the local mast cells [Levy, 2009; Giniatullin et al., 2019]. These immune cells contain many vesicles filled with inflammatory mediators, such as histamine, serotonin, biogenic amines, cytokines, enzymes, lipid metabolites, ATP, neuropeptides, growth factors, and nitric oxide [Johnson & Krenger, 1992]. These are known as the key allergic reaction factors, and modulators of the inflammatory processes in arthritis, bronchial asthma, irritable bowel syndrome and interstitial cystitis [Theoharides & Kalogeromitros, 2006].

Meningeal mast cells are represented by dense clusters around the vessels and nerves in the dura mater [Theoharides et al., 1995, 2005]. As a result of the interaction between mast cells and nerve endings, the functional unit, the mast cell - nerve immune synapse is formed [Bienenstock et al., 1991; Singh et al., 1999; Bauer & Razin, 2000; Suzuki et al., 2001, Giniatullin et al., 2019]. The extensive list of mast cell active substances and their proximity to primary afferents suggests their coordinated participation in the formation of nociceptive signals in nervous fibres.

Clinical studies performed in 1963 confirmed the direct involvement of mast cells in the pathogenesis of migraine. Thus, it has been shown that injection of a substance that degranulates mast cells (Compound 48/80 or C48/80), led to exaggeration of migraine symptoms [Sicuteri, 1963]. Conversely, cromolyn (a mast cell stabilizing

substance) provided a beneficial effect as a prophylactic migraine drug [Monro et al., 1984].

Recent studies using cultured mast cells indicated that ATP could act as a substance that causes mast cell degranulation [Wareham & Seward, 2016]. Indeed, in our work, staining of meningeal mast cells with Toluidine Blue in fixed dura mater preparations confirmed the ability of ATP to degranulate mast cells in these tissues, and this is related to the generation of migraine pain. Mast cell degranulation, in turn, leads to an increase in the concentration of active pro-inflammatory agents, which include serotonin, which can have its own strong effect on meningeal nerve endings.

Importantly, in this project, using a transgenic line of mice with mast cell deficiency, we clearly showed the role of mast cells in ATP-induced generation of action potentials in the trigeminal nerve. In particular, we found that the increase in nociceptive activity induced by ATP in transgenic mice was significantly lower than in the control group.

It is known that serotonin secreted from mast cells exerts a persistent increase in the incidence of action potentials in the rat trigeminal nerve [Kilinc et al., 2017]. Here we obtained similar data on murine hemiskull preparations and hypothesized that serotonin may enhance the pro-nociceptive effect of ATP. Indeed, even at a low concentration, serotonin (2 μM) caused a significant increase in the frequency of action potentials during the entire time serotonin was present in the perfusion solution. The effect of serotonin was reduced after inhibition of the 5-HT3 receptors by the specific blocker MDL7222. These results indicated that in the trigeminal nerve, the pro-nociceptive effect of serotonin is mediated by the activation of 5-HT3 receptors.

The role of endogenous serotonin in the pro-nociceptive action of ATP was confirmed using the 5-HT3 receptor blocker MDL7222. After inhibition of 5-HT3 receptors, the activating effect of ATP on the frequency of action potentials was significantly less than in control conditions and was comparable with the ATP response in genetically modified mast cell deficient mice.

Thus, we have demonstrated that ATP not only directly activates P2X3 receptors but also degranulates mast cells to release serotonin, which in turn enhances nociceptive signalling.

A similar increase in the nociceptive effect of ATP has been shown in a formalin-induced model of inflammatory pain in rats. Subcutaneous injection of formalin into the hind paw in rats led to the development of hyperalgesia, and similar results were obtained after injection of the P2X3 agonist receptor α,β-meATP, whereas the selective antagonist of the P2X3 and P2X2/3 receptors A-317491 prevented the painful effects of formalin. Moreover, local subcutaneous administration of selective TRPA1, 5-HT3 or 5-HT1A receptor antagonists prevented P2X3 agonist-induced nociceptive responses. Thus, it was suggested that activation of P2X3 receptors causes the inflammatory reaction leading to the activation of other types of nociceptors in peripheral tissues [Krimon et al., 2013].

6.2 THE MECHANISMS OF INTERACTION OF ATP AND HYDROGEN SULFIDE SIGNALLING DURING ACTIVATION OF TRIGEMINAL AFFERENTS

In our study, we also analysed the interactions between signalling induced by ATP and by hydrogen sulfide, which has both pro- and anti-nociceptive effects in different tissues, mediated through various cellular targets [Okubo et al., 2012 Matsunami et al., 2009]. In sensory neurons of the DRG and trigeminal ganglia, expression of the enzyme CBS, which generates endogenous H_2S, was detected [Xu et al., 2009; Feng et al., 2013]. In trigeminal ganglia neurons, the expression of CBS was increased under conditions of inflammation with a simultaneous increase in neuronal excitability due to the suppression of potassium currents [Miao et al., 2014]. CBS is colocalized with TRPV1 receptors in DRG neurons innervating the colon and it was proposed that the H_2S synthesis contributes to the development of visceral hypersensitivity [Xu et al., 2009]. TRPV1 receptors acquired the typical property to sensitized by various inflammatory stimuli, which is associated also with increased expression of the enzyme H_2S generating enzyme CBS in DRG neurons [Zhu et al., 2015]. The pro-nociceptive TRPA1 receptor in sensory neurons can also be target for the direct action of H_2S. Thus, it was shown that NaHS-induced vasodilation is due to the release of the vasoactive neuropeptides, CGRP and substance P [Hajna et al., 2016; Pozsgai et al., 2012]. Also, the activation of TRPA1 channels by H_2S led to mechanical hyperalgesia and allodynia in mice [Okubo et al., 2012], while TRPA1 was not involved in the pro-nociceptive effects of H_2S in the visceral tissues [Andersson et al., 2012].

On the other hand, both activating and inhibitory effects of H_2S were shown in neurons of the spinal nucleus of the rat trigeminal nerve [Teicher, 2017]. In addition, the protective effect of H_2S donors has been shown in inflammatory pain models, which is mediated through mast cell stabilization [Rodrigues et al., 2017, Roviezzo et al., 2015]. It has been shown that H_2S increased the level of intracellular calcium without mast cell degranulation [Moustafa & Habara, 2016]. However, there is no data indicating interactions between signalling induced by H_2S and ATP via P2X3 or other ATP receptors.

6.2.1 The role of TRPV1/TRPA1 and P2X3 receptors in the effects of hydrogen sulfide

We show that application of the H_2S donor to the peripheral trigeminal afferents caused a short-term increase in the frequency of action potentials, followed by a return to control values. We used antagonists of TRPV1, TRPA1 and P2X3 receptors to analyse the receptor mechanisms of the nociceptive action of NaHS. The TRPV1 receptor blocker capsazepine completely prevented the burst of activity caused by the application of NaHS. In the presence of the selective TRPA1 receptor antagonist HC-030031, the NaHS induced increase in the frequency of action potentials remains

in a lesser extent, whereas the selective P2X3/P2X2/3 antagonist A-317491 did not significantly change the pro-nociceptive effect of NaHS. These data indicated that the increase in the frequency of action potentials was mainly mediated by activation of TRPV1 and, to a lesser extent, TRPA1 receptors. A similar effect was observed in neurons of the spinal nucleus of the trigeminal nerve when the donor of H_2S was applied to the dura mater, first the spontaneous neuronal activity increased, followed by long-term reduction of activity [Teicher et al., 2017]. Thus, TRPV1 channels are the main target of H_2S in the peripheral trigeminal afferents, and the activation of TRPV1 receptors is accompanied by desensitization, which prevents lasting excessive nociceptive firing.

6.2.2 The role of TRPV1 receptors in the inhibitory effect of hydrogen sulfide on pro-nociceptive ATP action

There are data suggesting interaction between H_2S signalling and P2X3 receptors in DRG neurons, as both hyperalgesia and increased expression of CBS and P2X3 receptors were observed in conditions of lumbar disc herniation [Wang et al., 2015].

However, there is no direct evidence of the action of H_2S on P2X3 receptor activity. In our study, we showed that the application of the NaHS donor on meningeal afferents prevented the increase of action potentials in response to ATP application. Since we have shown that H_2S induced the activation of TRPV1 receptors, we further hypothesized that a sharp increase in the level of intracellular calcium interferes with the pro-nociceptive effect of ATP. It has been shown that 33-58% of all trigeminal ganglia neurons co-express P2X3 and TRPV1, as shown by immunohistochemical studies [Saloman, 2013]. Moreover, both activating and inhibitory interactions between P2X3 and TRPV1 receptors have been shown in DRG neurons. Activation of P2X3 receptors led to phosphorylation and sensitization of TRPV1. An increase in calcium level upon activation of P2X3 receptors contributed to desensitization of TRPV1 receptors. Such calcium-dependent inhibitory interactions between P2X3 and TRPV1 have been described in heterologous expression systems, and in sensory neurons [Piper & Docherty, 2016].

Studies on interactions between P2X3 and TRPV1 receptors in afferents of the masticatory muscles showed that hyperalgesia caused by intramuscular injection of α,β-meATP was prevented by preliminary administration of the of TRPV1 receptor antagonist AMG9810. However, in a subpopulation of P2X3/TRPV1-positive neurons, capsaicin-induced calcium signals were significantly amplified after preliminary activation of P2X3 receptors [Saloman et al., 2013].

Inflammatory mediators are able to enhance the sensitivity of the TRPV1 receptor [Turner et al., 2007] making them more responsive to endogenous pain stimuli. Mast cell degranulation results in the release of protease and tryptase, which can sensitize TRPV1 receptor [Amadesi et al., 2006].

To verify the assumption that the inhibitory effect of H_2S on the stimulatory effects of ATP is associated with NaHS-induced activation of TRPV1 receptors, we activated TRPV1 receptors with the agonist capsaicin followed by application of

ATP. It turned out that the preliminary activation of TRPV1 receptors did not affect the intensity of the pro-nociceptive action of ATP. Thus, we can conclude that the inhibitory effect of H_2S on ATP signalling is not associated with TRPV1-mediated inactivation of P2X3 receptors.

Next, the effects of the H_2S donor on the activity of P2X3 receptors on isolated trigeminal ganglion neurons were analysed using electrophysiological methods and calcium imaging.

We showed that the agonist of P2X3 receptors α,β-meATP induced three types of inward current: fast, slow and mixed currents, consisting of two components with the fast and slow speed of desensitization. It is known that, in sensory neurons, the P2X2 and P2X3 subunits can form the homomeric P2X2, homomeric P2X3, or heteromeric P2X2/3 receptors. Electrophysiological studies indicate that the P2X2 and P2X3 subunits determine the majority of ATP-mediated reactions in mouse sensory and sympathetic ganglia neurons. Co-expression and simultaneous activation of P2X3 and P2X2/3 receptors in trigeminal ganglion neurons is supported by the fact that the responses to the application of α,β-meATP induced inward currents with slower desensitization rate than currents caused by activation of only P2X3 receptors [Cockayne et al., 2005]. We found that the application of NaHS led to a decrease in both the fast and slow components of the currents activated by α, β-meATP.

It is known that as a result of the activation of P2X3 receptors, the non-selective cation ion channel is opened with the permeability for sodium, potassium and calcium ions [North, 2002]. A short application of ATP and the selective P2X3 receptor agonist lead to a short-term increase in the level of intracellular calcium, the so-called "calcium transient". To diminish the effect of desensitization on the amplitude of calcium transients, the three short agonist applications were applied in the control, which did not show a pronounced desensitization. However, after exposure of neurons to the solution containing the H_2S donor, we observed a significant decrease in the amplitude of the transients induced by activation of P2X3 receptors.

The data obtained indicated that the H_2S donor directly affects the function of ionotropic P2X3 receptors in trigeminal ganglion neurons and prevents the pro-nociceptive effect of ATP in peripheral trigeminal afferents.

6.2.3 The role of mast cell degranulation in the inhibitory effects of hydrogen sulfide on the pro-nociceptive action of ATP

As stated above, endogenous ATP and ADP are at very low nanomolar concentrations, which presumably allows excessive activation of sensory nerve endings to be avoided [Yegutkin et al., 2016]. The level of extracellular ATP can be increased due to tissue damage and secretion from the endothelial cells of meningeal vessels as a result of blood flow changes and local hypoxia during a migraine attack. Release of ATP from glial and immune mast cells in response to a mechanical factor can take place through gap junctions or pannexins, as well as via P2X7 receptor channels [Dubyak, 2006]. The low level of endogenous ATP is provided by the

coordinated action of nucleoside triphosphate diphosphohydrolases (NTPDases) that degrade ATP to its metabolites [Burnstock & Ralevic, 2014]. In our experiments with the rat hemiskull preparation, nanomolar ATP concentrations (about 1 nM) were measured in the extracellular solution, which is consistent with previous studies. However, the incubation of hemiskulls in a solution containing NaHS reduced the level of ATP almost 5-fold. This effect may be associated both with a decrease in the release of ATP from various sources, and with the potential effect of H_2S on ATP degradation enzymes.

Meningeal mast cells play an important role in the pathogenesis of migraine due to the pro-inflammatory mediators contained in these immune cells [Wang et al., 2013]. Recent work indicated that H_2S can prevent mast cell degranulation. Thus, in the respiratory system of mice, H_2S prevented mast cell activation in a model of asthma and this effect was absent in mast cell deficient mice [Roviezzo et al., 2015]. In addition, H_2S prevented the development of heart failure caused by administration of isoproterenol in rats by inhibiting the release of renin from mast cells [Liu et al., 2014]. Finally, in models of pruritus and acute skin inflammation, it was found that the donor of H_2S significantly reduced the level of histamine and attenuated C48/80-induced itching, which is probably due to the stabilizing effect of H_2S on mast cells. This protective effect of H_2S donors was also demonstrated using peritoneal mast cells stimulated by C48/80 in vitro [Rodrigues et al., 2017].

In our experiments, incubation of meninges in a solution containing NaHS, a donor of H_2S, resulted in a 2-fold decrease in the number of degranulated mast cells induced by ATP exposure. Thus, this protective effect of H_2S can be an additional factor that reduces the pro-nociceptive activity of ATP in meningeal afferents.

It was shown that activation of P2X7 receptors underlies mast cell degranulation under the action of ATP. P2X7 receptors are expressed in many immune cells and they are usually sensitive to the high concentrations of ATP that can be released during any type of cell stress to regulate innate immune and inflammatory responses [Tewari & Seth, 2015]. Therefore, we can hypothesize that the stabilizing effect of H_2S is associated with decreased activity of P2X7 receptors in mast cells. Indeed, whereas the P2X7 receptor agonist, BzATP significantly increased the number of degranulated mast cells, a preliminary incubation of meningeal membranes with the H_2S donor NaHS, reduced this number. The interaction between H_2S and P2X7 receptors was suggested as a rat model of stroke, in intracerebral hemorrhage associated with local inflammation. It was found that the activation of the NLRP3 (NOD-like receptor (NLR) family pyrin domain-containing 3) inflammasome led to the development of neuroinflammation in this type of stroke, and that P2X7 receptors directly contributed to the activation of NLRP3. In this study, the exogenous donor of H_2S or activation of endogenous H_2S synthesis decreased the inflammatory response due to reduced expression of microglial P2X7 receptors and the H_2S donor decreased the activation of these receptors in vitro [Zhao et al., 2017].

Thus, we can assume that P2X7 receptors in mast cells receptors can serve as another target for the action of H_2S in the dura mater. Inhibition of P2X7 receptors

can prevent the further increase in ATP concentration and the release of other active pro-inflammatory components from mast cells. Thus, H_2S demonstrated protective properties in meningeal mast cells, which are involved in the development of the inflammatory processes in migraine.

7 CONCLUSIONS

In our study, we examined in detail the pro-nociceptive role of ATP in the mechanisms of migraine and revealed new mechanisms of ATP action in the peripheral parts the trigeminal nociceptive system: These data are summarized in Figure 17. Thus, the multiple effects of ATP are mediated by the direct activation of neuronal P2X3 receptors, but also the action of ATP includes mast cell degranulation via P2X7 receptors and the release of the pro-inflammatory mediators such as serotonin. The presented cascade of reactions can be enhanced in conditions of the high level of ATP in the meninges, associated with inflammation. The action of ATP released serotonin on its 5-HT3 receptors in peripheral afferents, should further contribute to persistent pain firing in the trigeminal nerve.

We also analysed the interaction in signalling between ATP and H_2S, a gaseous mediator, which can be synthesized in trigeminal neurons and has its own nociceptive effects. We found that H_2S causes the activation of electrical activity in the trigeminal nerve predominantly due to activation of TRPV1 receptors. However, H_2S prevented the ATP-induced activation of the rat trigeminal nerve showing the anti-nociceptive effect. One mechanism of this effect is likely a decrease in membrane currents through P2X3 receptors along with the suppression of ATP-induced calcium signals in trigeminal ganglion neurons. In addition, H_2S reduced the ATP levels in meninges and prevented ATP-induced mast cell degranulation. These data indicate the important anti-nociceptive role of H_2S, especially in conditions of inflammation, which occurs in the pathogenesis of migraine. The molecular mechanisms of the direct and indirect pro-nociceptive action of ATP that we identified, as well as the anti-nociceptive mechanisms of H_2S, may be used as therapeutic targets for the development of drugs designed for the treatment or prevention of migraine.

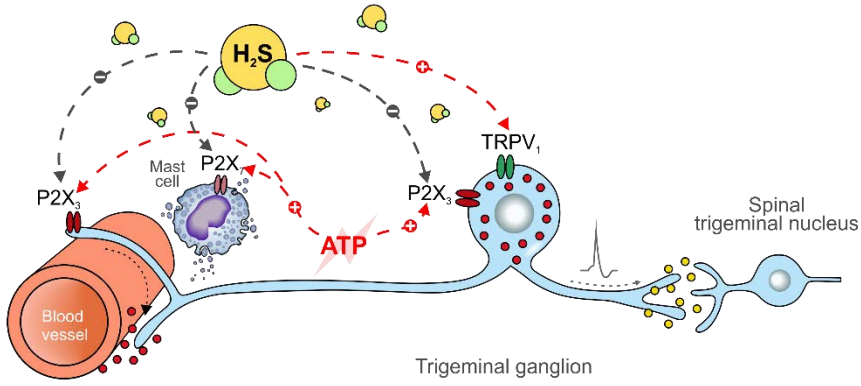

Figure 17. The representation of the mechanism of action of ATP and H_2S in the trigeminal nerve of a rat. Red lines – activating, grey lines – inhibitory effect.

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ORIGINAL PUBLICATIONS (I – III)

I

Receptor mechanisms mediating the pro-nociceptive action of hydrogen sulfide in rat trigeminal neurons and meningeal afferents

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Receptor Mechanisms Mediating the Pro-Nociceptive Action of Hydrogen Sulfide in Rat Trigeminal Neurons and Meningeal Afferents

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Hydrogen sulfide (H_2S), a well-established member of the gasotransmitter family, is involved in a variety of physiological functions, including pro-nociceptive action in the sensory system. Although several reports have shown that H_2S activates sensory neurons, the molecular targets of H_2S action in trigeminal (TG) nociception, implicated in migraine, remains controversial. In this study, using suction electrode recordings, we investigate the effect of the H_2S donor, sodium hydrosulfide (NaHS), on nociceptive firing in rat meningeal TG nerve fibers. The effect of NaHS was also explored with patch-clamp and calcium imaging techniques on isolated TG neurons. NaHS dramatically increased the nociceptive firing in TG nerve fibers. This effect was abolished by the TRPV1 inhibitor capsazepine but was partially prevented by the TRPA1 blocker HC 030031. In a fraction of isolated TG neurons, NaHS transiently increased amplitude of capsaicin-induced currents. Moreover, NaHS by itself induced inward currents in sensory neurons, which were abolished by the TRPV1 inhibitor capsazepine suggesting involvement of TRPV1 receptors. In contrast, the inhibitor of TRPA1 receptors HC 030031 did not prevent the NaHS-induced currents. Imaging of a large population of TG neurons revealed that NaHS induced calcium transients in 41% of tested neurons. Interestingly, this effect of NaHS in some neurons was inhibited by the TRPV1 antagonist capsazepine whereas in others it was sensitive to the TRPA1 blocker HC 030031. Our data suggest that both TRPV1 and TRPA1 receptors play a role in the pro-nociceptive action of NaHS in peripheral TG nerve endings in meninges and in somas of TG neurons. We propose that activation of TRPV1 and TRPA1 receptors by H_2S during neuro-inflammation conditions contributes to the nociceptive firing in primary afferents underlying migraine pain.

Keywords: pain, hydrogen sulfide, trigeminal nerve firing, trigeminal neurons, TRPV1-and TRPA1 receptors, Ca^{2+}-imaging

Abbreviations: CBS, cystathionine beta-synthase; CGRP, calcitonin gene-related peptide; CSE, cystathionine gamma-lyase; H_2S, hydrogen sulfide; NaHS, sodium hydrosulfide; TG, trigeminal.

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INTRODUCTION

Hydrogen sulfide (H_2S), a member of the gasotransmitter family along with nitric oxide (NO) and carbon monoxide, is involved in the regulation of great variety of physiological functions, including nociception and inflammation (Kawabata et al., 2007; Feng et al., 2013). The neuro-modulatory role of H_2S was shown in the central and peripheral nervous system where it promotes the induction of long-term potentiation (LTP) in hippocampus (Abe and Kimura, 1996), inhibits giant depolarizing potentials in neonatal hippocampus (Yakovlev et al., 2017), affects NMDA-mediated currents (Abe and Kimura, 1996; Yakovlev et al., 2017), increases the transmitter release from motor nerve endings (Sitdikova et al., 2011; Gerasimova et al., 2013, 2015), or initiates contractile responses of the rat urinary bladder by stimulation of primary afferent neurons (Patacchini et al., 2004).

Increasing evidence suggests H_2S to play a role in the emergence and conductance of somatic and visceral pain (Okubo et al., 2012). Intracolonical administration of sodium hydrosulfide (NaHS), a H_2S donor, induced nociceptive behavior with abdominal hyperalgesia/allodynia (Matsunami et al., 2009). NaHS produced mechanical hyperalgesia in the rat hind paw in response to intraplantar administration (Kawabata et al., 2007). On the other hand, NaHS activates ATP-dependent K^+ channels in different tissues (Tang et al., 2005; Mustafina et al., 2015) which may underlie the antinociceptive effects of NaHS (Distrutti et al., 2006).

Endogenously H_2S is produced from L-cysteine by the enzymes cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase along with additional contribution of cysteine aminotransferase or D-amino acid oxidase (Abe and Kimura, 1996; Renga, 2011). It was shown that CBS is widely expressed in rat trigeminal (TG) neurons (Feng et al., 2013) and its expression is upregulated in response to inflammatory pain with subsequent increase of the excitability of TG neurons by suppression of K^+ conductance (Miao et al., 2014). It was reported that CBS is colocalized with transient receptor potential, vanilloid 1 (TRPV1) receptors in colon specific dorsal root ganglion (DRG) neurons (Xu et al., 2009; Qu et al., 2013). TRPV1 receptors undergo sensitization in response to inflammation which is mediated by the increased expression of CBS on DRG (Zhu et al., 2015).

Several recent publications indicated the ability of H_2S to activate TRPV1 or TRPA1 receptors *in vitro* and *in vivo* experiments. Thus, the TRPV1 antagonist prevented NaHS-evoked luminal chloride secretion (Storti et al., 2015). NaHS-induced constriction of smooth muscle cells of airways and H_2S-evoked intestinal motility were abolished by the TRPV1 antagonists (Trevisani et al., 2005; Bhatia et al., 2006). Moreover, NaHS increased the afferent neuronal activity in gut and induced inward currents in DRG neurons which were inhibited by TRPV1 antagonists (Lu et al., 2014).

However, there is also evidence indicating activation of TRPA1 receptors by H_2S. Activation of capsaicin-sensitive sensory nerves through TRPA1 receptors by NaHS-induced vasodilatation resulting from the release of the vasoactive neuropeptides calcitonin gene-related peptide (CGRP) and substance P (Pozsgai et al., 2012; Hajna et al., 2016). Indirect evidence shows that activation of TRPA1 channels by H_2S resulted in mechanical hyperalgesia and allodynia in mice (Okubo et al., 2012) whereas TRPA1 did not participate in pro-nociceptive effects of H_2S in visceral tissues (Andersson et al., 2012). There is abundance evidence that H_2S affects TRP channels in sensory neurons, but the molecular target of H_2S action in nociceptive system remains to be determined.

The aim of this study was to explore the role of TRP receptors in the firing of TG nerve fibers induced by NaHS using extracellular recordings of peripheral branches of the TG nerve in isolated rat meninges and patch clamp recordings of TRPV1 currents as well as Ca^{2+}-imaging of rat TG neurons.

MATERIALS AND METHODS

Preparation and Solutions

All animal experiments were performed in accordance with the European Community Council Directive of September 22, 2010 (2010/63/EEC) and approved by the Animal Care and Use Committee of the University of Eastern Finland and the Ethics Committee of Kazan Federal University. Electrical activity of TG nerve was recorded using isolated hemiskull preparations obtained from adult (P35–36) rats as described previously (Shatillo et al., 2013). Firing activity was recorded from the *nervus spinosus* (V3 branch of the TG nerve) which was isolated and cleaned from the *dura mater*. This nerve innervates a region of the medial meningeal artery, supposed to initiate migraine pain and this model is widely used to investigate molecular mechanisms of migraine pain. The isolated preparation was washed for 20 min with Krebs solution containing (in mM): 120 NaCl; 2.5 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 11 glucose; 1 $NaHPO_4$; 24 $NaHCO_3$ constantly gassed with 95% O_2/5% CO_2 and the pH kept at 7.2–7.4.

TG neurons were isolated from P9–P12 rats. Animals were anesthetized and decapitated. TG ganglia were excised and enzymatically dissociated in F12 medium containing 0.25 mg/ml trypsin, 1 mg/ml collagenase, and 0.2 mg/ml DNAase (Sigma) at 37°C. Cells were plated on poly-l-lysine-coated glasses in F12 medium with 10% fetal bovine serum and cultured for 1–2 days at 37°C in an atmosphere containing 5% CO_2. During experiments cells were continuously perfused (at 2 ml/min) with a solution containing (in mM): 148 NaCl; 5 KCl; 1 $MgCl_2$; 2 $CaCl_2$; 10 HEPES; 10 D-Glucose; pH adjusted to 7.2 with NaOH. The intracellular solution for patch clamp experiments contained (in mM): 145 KCl; 2 $MgCl_2$; 10 HEPES; 5 EGTA; 0.5 $CaCl_2$; 2 Mg-ATP; 0.5 Na-GTP; 5 KCl; pH adjusted to 7.2 with KOH.

For Ca^{2+} imaging experiments cells were incubated for 40 min at 37°C in F12 medium supplemented with FBS 10% (Gibco Invitrogen, Carlsbad, CA, USA) containing fluo-3-AM (5 μM, Life Technologies, Foster City, CA, USA) followed by a 10–15 min washout period.

Chemicals

Capsaicin, HC 030031 and capsazepine were dissolved in dimethyl sulfoxide (DMSO), dithiothreitol (DTT)—in external solution. DMSO in used concentration did not change the nociceptive activity (Zakharov et al., 2015). All substances were purchased from Sigma-Aldrich (St. Louis, MO, USA). NaHS (Sigma-Aldrich, St. Louis, MO, USA) was used as a source of H_2S. In solution NaHS dissociates to give HS^- which associates with H^+ to produce H_2S. At 20°C—22.3% of total sulfide is present as H_2S (Sitdikova et al., 2014). The real-time measurements of H_2S in the chamber using amperometric sensors indicate a rapid loss of sulfide via H_2S volatilization by bubbling with about 50% H_2S loss within 3 min (Deleon et al., 2012; Sitdikova et al., 2014). In our experiments NaHS was used in a concentration of 100 μM which yields about 11 μM H_2S in the perfusion system which constantly flows to the recording chamber. Stock solutions of NaHS were prepared immediately before each experiment and kept hermetically sealed in a dark place.

Electrophysiology

TG nerve firing was recorded using a DAM 80 amplifier (band pass 0.001–3 kHz, gain 1000; World Precision Instruments, Sarasota, FL, USA). The *nervus spinosus* was placed inside the fire-polished glass recording microelectrode with a tip diameter of ∼150 μm, filled with Krebs solution. A recovery period of at least 15 min was used to obtain stable baseline conditions. Control recordings of meningeal spikes were performed for 10 min previous to drug application. Signals were digitized at 125 kHz using a data acquisition board NI PCI6221 (National Instruments, Austin, TX, USA), and WinEDR software (Strathclyde University, Glasgow, UK). Five standard deviations (SD) were used to set the threshold for spike detection.

TRPV1 receptors are predominantly expressed in small- and medium-diameter neurons, which were used in our patch clamp experiments. TRPV1 currents were recorded at a holding potential of −70 mV using the whole-cell configuration of the patch clamp technique. TRPV1 currents were evoked by local application of capsaicin in a concentration of 1 μM for 2 s using a fast perfusion system (Rapid Solution Changer, RSC-200, BioLogic Science Instruments, Grenoble, France), with a solution exchange rate of ∼20 ms. To prevent the desensitization of TRPV1 receptors, agonist was applied at intervals of 5 min. Responses to capsaicin were measured using a HEKA-10 amplifier and HEKA Patch Master Software (HEKA Electronic, Germany).

Calcium Imaging

Fluorescence signals of neurons were recorded by microscope imaging setup (TILL Photonics GmbH, Munich, Germany) with a light excitation wavelength of 488 nm using respective filters. Images were collected in a time-lapse mode (500 ms exposure time) with a 12-bit CCD camera (SensiCam, Kelheim, Germany). Fluorescent signals from single cells were quantified as $\Delta F/F0$, where $F0$ is the background subtracted baseline fluorescence and ΔF is the increment over baseline. The inhibitor of TRPV1—capsazepine (10 μM), the inhibitor of TRPA1—HC 030031 (50 μM), NaHS (100 μM, 2 s) and capsaicin (1 μM, 2 s) were applied via a fast perfusion system as indicated above, followed by application of a 50 mM KCl-containing solution to differentiate neurons. Data were analyzed off-line using Origin Pro 2015 (MicroCal, Northampton, MA, USA) software.

Statistical Analysis

For each experiment we used at least three independent replicates (animals) and n means the number of cells. Normality of sample data was evaluated with Shapiro-Wilk test and for equal variances using F-test Origin Pro 2015 (OriginLab Corp., Northampton, MA, USA). Differences were considered as statistically significant at $p < 0.05$. All values are given as mean ± SEM. Statistical significance was determined by paired Student's t-test and Mann-Whitney test.

RESULTS

NaHS Increases Firing in Meningeal Nerve Terminals

First, we tested if H_2S can induce the nociceptive effect in meningeal nerves using a technique of suction electrode recording from meningeal TG nerve fibers (Zakharov et al., 2015). The peripheral part of the TG nerve (*nervus spinosus*) was placed inside the microelectrode and the orthodromic spontaneously generated action potentials (AP; spikes) generated at the periphery were recorded (**Figure 1**). Bath application of 100 μM NaHS induced a significant increase in the frequency of nociceptive spikes during the first 5 min to 320 ± 88% of control (control 0.53 ± 0.08 s^{-1}, vs. 1.35 ± 0.13 s^{-1} in the presence of NaHS; $n = 10$, $p < 0.0001$; **Figures 1A,D**). During the next 5 min of application, the frequency of spikes did not differ significantly from control (0.95 ± 0.30 s^{-1}, $p = 0.2$) and it returned to control level after 15 min of drug application (0.40 ± 0.01 s^{-1}; $p = 0.3$; **Figure 1A**).

As previous studies suggested the action of NaHS in different tissues could be mediated either by TRPV1 or TRPA1 receptors (Trevisani et al., 2005; Andersson et al., 2012; Okubo et al., 2012; Lu et al., 2014; Hajna et al., 2016), we next examined the pro-nociceptive effect of NaHS in the presence of the specific inhibitors of these receptors. Inhibition of TRPV1 receptors by capsazepine (25 μM) did not significantly change TG nerve firing (0.40 ± 0.11 s^{-1} in control, vs. 0.32 ± 0.13 s^{-1} in capsazepine; $n = 6$, $p = 0.14$). Remarkably, the subsequent application of NaHS in the presence of capsazepine did not affect the frequency of nociceptive firing. The frequency of AP was 0.56 ± 0.30 s^{-1} ($p = 0.5$) during first 5 min of NaHS application, 0.44 ± 0.27 s^{-1} ($p = 0.85$) and 0.27 ± 0.15 s^{-1} during 10 and 15 min of application, respectively ($p = 0.25$; **Figures 1B,D**). The inhibition of TRPA1 by HC 030031 (50 μM) did not change firing itself (0.61 ± 0.11 s^{-1} in control, vs. 0.55 ± 0.09 s^{-1} in HC 030031; $n = 7$, $p = 0.51$), however, partially prevented the action of NaHS (increase to 161 ± 13%; 0.85 ± 0.13 s^{-1}; $n = 7$, $p = 0.007$; **Figures 1C,D**).

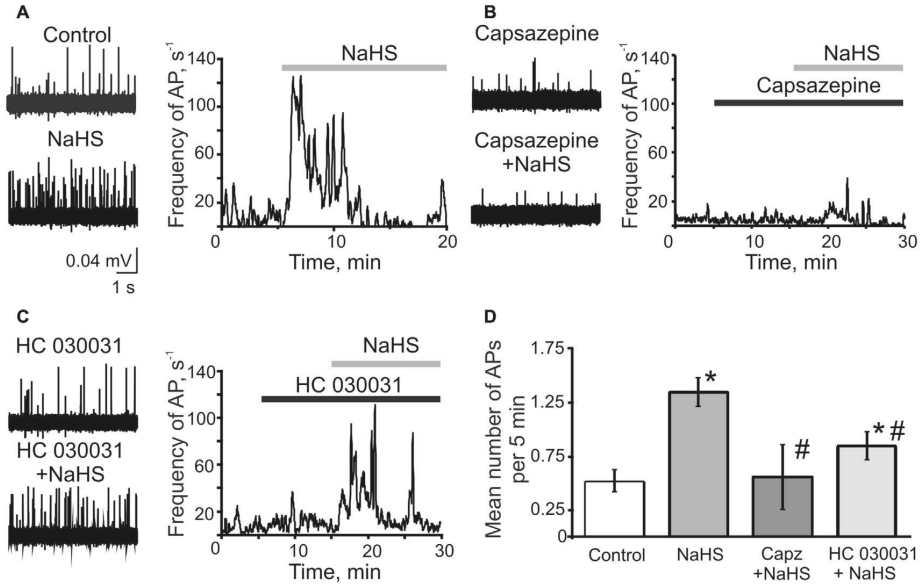

FIGURE 1 | The role of TRPV1 and TRPA1 receptors in the effects of sodium hydrosulfide (NaHS) on trigeminal (TG) nerve firing. **(A)** Representative traces of TG nerve firing in meningeal hemiskull preparation (left panel) and frequency plots of action potentials (AP) vs. time during single experiments (right panel) in control and after application of NaHS (100 μM); **(B)** after inhibition of TRPV1 receptors by capsazepine (10 μM) and capsazepine + NaHS **(C)** after inhibition of TRPA1 receptors by HC 030031 (50 μM) and HC 030031 + NaHS. The solid bars indicate the application time of NaHS, capsazepine and HC 030031. **(D)** Plot of mean number of AP per 5 min during control, after application of NaHS, capsazepine (capz) + NaHS and HC 030031 + NaHS. $^{*}p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to the effect of NaHS.

The NaHS effect after inhibition of TRPA1 was significantly lower than in control conditions. During 10 and 15 min of NaHS application the frequency of AP returned to the control level (0.70 ± 0.12 s^{-1}, $p = 0.13$ and 0.82 ± 0.19 s^{-1}, $p = 0.27$, respectively; **Figure 1C**). These results suggested the involvement of TRPV1 and TRPA1 receptors in the pro-nociceptive firing induced by NaHS in TG nerve fibers in meninges.

NaHS Induces TRPV1 Mediated Currents in Isolated TG Neurons

Next, we studied the effect of NaHS on TRPV1 receptors in single TG neurons using patch clamp recordings. The application of the TRPV1 receptor agonist capsaicin (1 μM, 2 s) induced inward currents in 22 out of 31 neurons with an average amplitude of 1345 ± 330 pA ($n = 22$; **Figures 2A,B**). In control, the repetitive application of capsaicin (interval 5 min) revealed two fractions of neurons: in one fraction, the amplitude of TRPV1 currents did not significantly change ($n = 14$, **Figure 2C**), whereas in another fraction rundown of currents was observed ($n = 8$, **Figure 2D**). These findings are consistent with an intrinsic heterogeneity of TRPV1 receptors with different rates of desensitization which was found in isolated sensory neurons previously (Akopian et al., 2007; Storti et al., 2015). We did not reveal differences in the membrane capacitance between two groups of TG neurons (36.9 ± 4.7 pF, $n = 14$ vs. 33.4 ± 7.7 pF, $n = 8$, $p = 0.67$). Superfusion with NaHS (100 μM) for 5 min revealed bidirectional effects on the fractions of cells with a different rate of desensitization. In a fraction of 14 cells with a low rate of desensitization, NaHS transiently increased currents from 1578 ± 501 pA to 2357 ± 715 pA ($n = 14$, $p = 0.0065$) followed by rundown during 10 and 15 min of NaHS application (**Figures 2A,C**). In other eight cells, we only observed a reduction of capsaicin-induced currents after 5 min exposure to NaHS from control value of 768 ± 168 pA to 414 ± 141 pA in the presence of NaHS ($n = 8$, $p = 0.016$; **Figures 2B,D**). In both cases, the effect of NaHS was not washable. The activating NaHS effects on TRPV1 currents could be explained by its reducing action on disulfide bonds of the TRPV1 channel protein (Susankova et al., 2006). Indeed, pre-application of 1 mM DTT prevented the increase of the current amplitude by NaHS. Thus, in the presence of DTT, NaHS decreased the amplitude of TRPV1 currents from 1360 ± 330 pA to 1025 ± 371 pA ($n = 11$, $p = 0.0033$) by 5 min of application with further reduction to 622 ± 171 ($p < 0.0002$) and then to 532 ± 167 pA ($p < 0.0001$), by 10 and 15 min, respectively (data not shown).

To investigate the direct effect of NaHS on TRPV1 currents, NaHS (100 μM) was applied through the fast perfusion system for 2 s. In cells responding to capsaicin, NaHS induced an

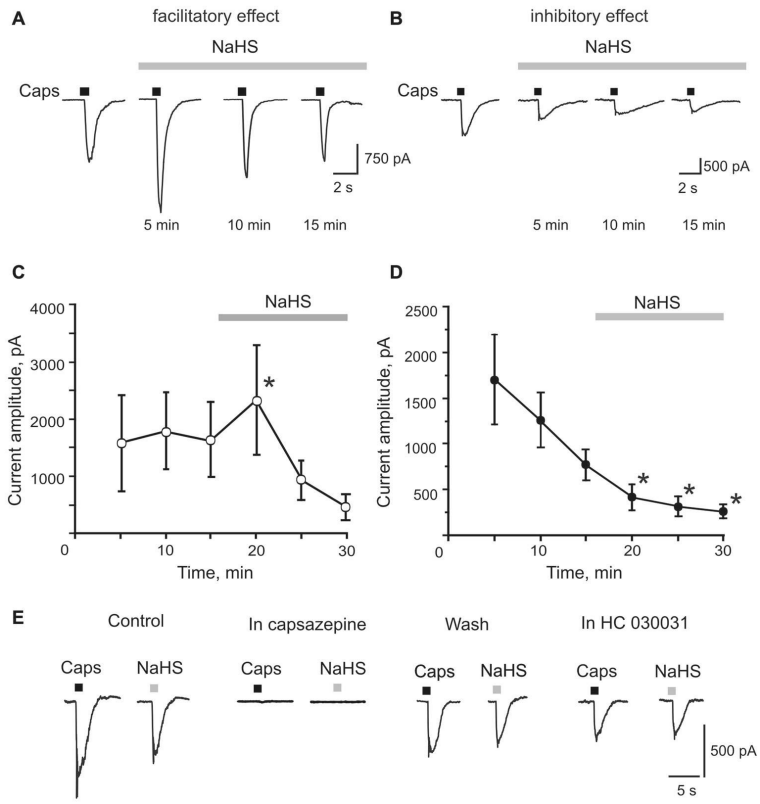

FIGURE 2 | Facilitatory and inhibitory effects of NaHS on capsaicin induced currents in rat TG neurons. **(A,B)** Representative traces of capsaicin evoked currents (Caps, 1 μM, 2 s, short bars above traces) in control and during bath application of 100 μM NaHS for 15 min (solid bar above traces). Capsaicin was applied at an interval of 5 min to prevent desensitization of TRPV1 receptors. **(C,D)** Average amplitude of TRPV1 currents in control (three subsequent capsaicin application) and during NaHS application (solid bars above traces). Notice the increase of mean amplitude of TRPV1 currents after 5 min of NaHS perfusion in **(C)** and the constant decrease of capsaicin evoked currents in **(D)**. **(E)** Representative traces of currents evoked by focal application of 1 μM capsaicin (Caps, 2 s) and 100 μM NaHS (2 s) in control and after inhibition of TRPV1 receptors by capsazepine (10 μM); after washout and after inhibition of TRPA1 receptors by HC 030031 (50 μM). Notice that NaHS and capsaicin evoked currents were completely abolished by capsazepine. $^*p < 0.05$ compared to the third application of capsaicin in control.

inward current with an average amplitude of 413 ± 114 pA (n = 12; **Figure 2E**). Application of the TRPV1 receptor antagonist capsazepine (10 μM) completely blocked currents induced by NaHS and capsaicin (n = 5, **Figure 2E**). NaHS and capsaicin-induced currents were washable from the capsazepine inhibition. Subsequent application of the TRPA1 receptor selective antagonist HC 030031 (50 μM) on the same cell did not affect currents induced by NaHS and capsaicin (n = 5, **Figure 2E**). These data suggest that NaHS can directly activate TRPV1 receptors in rat TG neurons.

NaHS Increases the Intracellular Ca^{2+} Concentration in Isolated TG Neurons

In order to explore the action of the H_2S donor on intracellular Ca^{2+} level in a large population of isolated neurons, we applied capsaicin (1 μM) and NaHS (100 μM) for 2 s before and after inhibition of TRPV1 or TRPA1 receptors by capsazepine (10 μM) or HC 030031 (50 μM), respectively. The subsequent application of KCl (50 mM) was used to distinguish neurons from glial cells (Kilic et al., 2017). As shown in **Figures 3A,B** NaHS induced Ca^{2+} transients in 41% neurons (104 of 251 cells); 43% of neurons tested were also sensitive to capsaicin (107 cells; **Figure 3B**). In 59% of cells responding to capsaicin, NaHS also induced an increase of intracellular Ca^{2+} (63 of 107 cells). Inhibition of TRPV1 receptors by capsazepine abolished Ca^{2+} responses evoked by NaHS in 37% of cells, which responded to NaHS and in 63% of cells the increase of $[Ca^{2+}]_{in}$ evoked by NaHS was still observed (**Figures 3A,C**). After inhibition of TRPA1 receptors by HC 030031, 80% of cells still showed an increase of $[Ca^{2+}]_{in}$ by NaHS application and in 20% of cells the NaHS response was eliminated (**Figures 3A,C**). In summary, in this approach

the action of NaHS was partially sensitive to TRPV1 and TRPA1 antagonists.

DISCUSSION

The main finding of our study indicates that H_2S induces an increase of firing activity in rat TG nerve and this effect is mediated by activation of both TRPV1 and TRPA1 receptors. In somas of TG neurons NaHS caused two types of effects on capsaicin evoked currents. In a fraction of neurons, NaHS induced a transient initial increase of the current amplitude followed by a subsequent decrease of responses. In the other fraction of cells, NaHS induced a progressive decline of TRPV1 currents. Moreover, H_2S when locally applied to TG neurons elicits inward currents which were inhibited by the TRPV1 antagonist capsazepine but was not sensitive to the inhibitor of the TRPA1 receptors HC 030031. Furthermore, NaHS generated Ca^{2+} transients in TG neurons which were prevented by the inhibitors of TRPV1 and TRPA1 receptors. We propose that both TRPV1 and TRPA1 receptors in peripheral nerve endings in meninges and in somas of sensory neurons are involved in the pro-nociceptive action of H_2S in the trigeminovascular system.

H_2S Increases Firing in TG Nerve by Activation of TRPV1 and TRPA1 Receptors

The TG system is directly involved in sensory and nociceptive conductance and TG nerve firing is involved in pain initiation during migraine. TRPA1 and TRPV1 receptors are widely expressed in capsaicin-sensitive sensory nerves (Huang et al., 2012). Activation of TRPV1 and TRPA1 on meningeal nerve endings induces the release of vasoactive neuropeptides CGRP and substance P and contributes to different forms of headache including migraine (Giniatullin et al., 2008; Benemei et al., 2013). Our results demonstrate that the donor of H_2S—NaHS directly increases firing in TG nerve and this effect is mainly mediated by activation of TRPV1 as

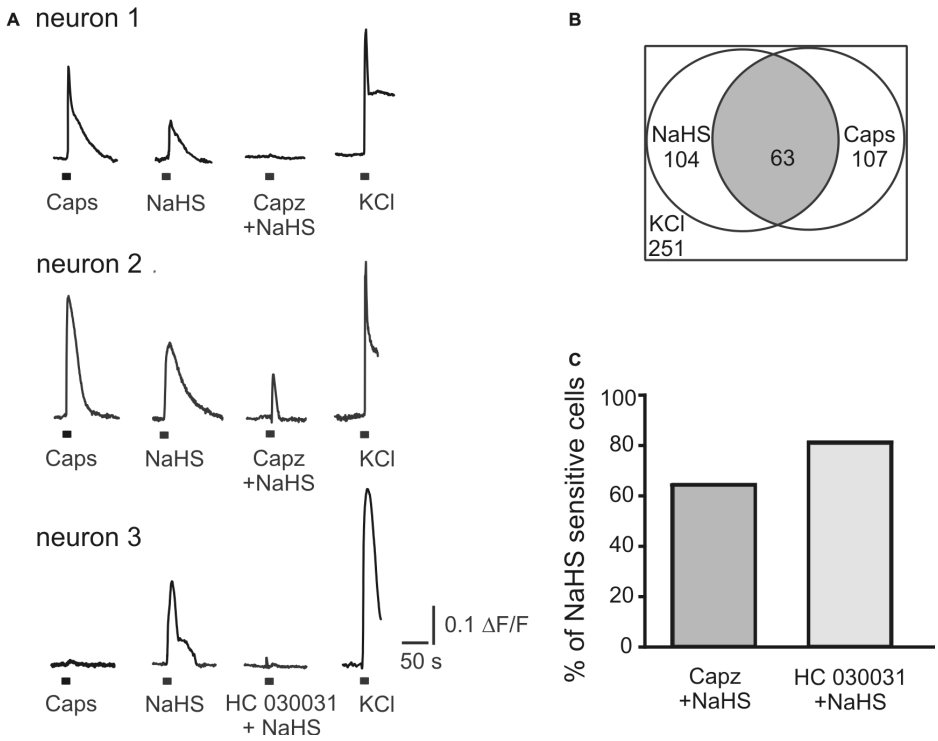

FIGURE 3 | NaHS evokes calcium transients in TG neurons. **(A)** Representative traces recorded from cultured TG neurons. NaHS (100 μM) and capsaicin (Caps, 1 μM) were applied for 2 s to TG ganglion cells loaded with fluo 3-AM (5 μM). Neurons were distinguished from other cell types due to Ca^{2+} responses induced by potassium depolarization (50 mM KCl for 2 s). In neuron 1 TRPV1 inhibitor capsazepine (capz, 10 μM) abolished NaHS induced Ca^{2+} transient; in neuron 2 capsazepine did not affect NaHS induced Ca^{2+} transient; in neuron 3 TRPA1 inhibitor HC 030031 (50 μM) abolished NaHS evoked response. **(B)** Graph indicating populations of capsaicin and NaHS sensitive TG neurons and their overlap. In a total of 251 imaged neurons (selected by their response to KCl) 104 (41%) were activated by NaHS (100 μM) and 107 (43%) by capsaicin (1 μM). In 59% of capsaicin sensitive cells NaHS also induced an increase of intracellular Ca^{2+} (63 cells from 107, gray area). **(C)** Percentage of cells responding to NaHS (100%) after inhibition of TRPV1 (Capz + NaHS) and TRPA1 (HC 030031 + NaHS) receptors.

capsazepine, TRPV1 antagonist, completely abolished the effect of NaHS. At the same time the TRPA1 antagonist HC 030031 partially prevented the increase of TG nerve firing indicating the involvement of TRPA1 receptors in the H_2S effect.

A number of indirect studies indicate the activation of TRPV1 and TRPA1 in afferent endings by H_2S. It was shown that NaHS similar to capsaicin induced the release of CGRP and substance P from sensory nerves in the airways of guinea pig which causes bronchoconstriction *in vivo* (Patacchini et al., 2004; Trevisani et al., 2005). NaHS evoked contractions of the urinary bladder by activation of capsaicin-sensitive afferents and release of sensory neuropeptides (Patacchini et al., 2004). In the guinea-pig and human colon H_2S caused mucosal Cl^- secretion (Schicho et al., 2006) and increased afferent firing in rat intestinal mesenteric nerves by activation of TRPV1 in afferent endings (Lu et al., 2014). On the other hand activation of TRPA1 by NaHS in afferent nerve fibers was reported to mediate an increased cutaneous blood flow by the release of CGRP and substance P in the mouse ear model (Hajna et al., 2016), whereas vasodilatory effects of H_2S were reduced in mice lacking TRPA1 receptors (Pozsgai et al., 2012).

H_2S Directly Activates TRPV1 Receptors and Increases Intracellular Ca^{2+} Concentrations in Isolated TG Neurons

The focal application of NaHS on TG neurons induced inward currents similar to capsaicin which were inhibited by the TRPV1 antagonist capsazepine but were not sensitive to the inhibitor of TRPA1 antagonist HC 030031, which indicates a direct activation of TRPV1 receptors by H_2S. At the same time superfusion of TG neurons with NaHS induced a bidirectional effect on capsaicin induced currents. In 63% of neurons NaHS induced an increase of the current amplitude during first minutes with subsequent desensitization, which can be explained by the reduction of disulfide bonds by H_2S. Indeed, the sulfhydryl redox agent DTT in our experiments prevented the facilitating effect of NaHS. The redox modulation of TRPV1 receptors is well-known and it was shown than DTT greatly potentiated both native and recombinant rat TRPV1 channels at extracellularly located sites (Susankova et al., 2006). H_2S is known for its reducing action which is responsible for its effects on Ca^{2+}-activated K^+ channels and NMDA-receptors (Abe and Kimura, 1996; Sitdikova et al., 2010; Kimura, 2016). In 36% of cells a constant decrease of TRPV1 currents was observed during NaHS superfusion, which may be explained by the rapid desensitization of TRPV1 receptors. This suggestion is supported by the variability in desensitization of capsaicin responses between different cell fractions. Indeed, the existence of several pools of TRPV1 receptors with slow and fast kinetics and with distinct rates of desensitization have been reported in TG neurons (Akopian et al., 2007; Storti et al., 2015; Zakharov et al., 2015) which may be determined by their lipid environment, coupling to caveolin, functional states, including redox state or phosphorylation (Storti et al., 2015). Moreover, the kinetics of TRPV1-mediated current depends on the co-expression of TRPA1 channels, with likely involvement of intracellular Ca^{2+} or other intracellular messengers affecting TRPV1 receptor desensitization (Masuoka et al., 2017). Similarly to our findings, in DRG neurons NaHS directly induced inward currents which were inhibited by capsazepine and A784168 (Lu et al., 2014). Our results suggest that H_2S is a putative agonist of TRPV1 receptors in somas of TG neurons.

Fluorescence studies demonstrate that H_2S increased the intracellular Ca^{2+} level in 41% of TG neurons cells, however, only 59% of H_2S-sensitive cells responded to capsaicin, which reflects variability of H_2S molecular targets and co-expression of other types of calcium-permeable receptors in TG neurons. Thus, in DRG neurons, the TRPV1 current densities were significantly smaller in allyl isothiocyanate (AITC)-sensitive DRG neurons than in AITC-insensitive cells and spontaneous TRPA1 channel activity inhibited the TRPV1 channels via Ca^{2+} elevation (Masuoka et al., 2017). Indeed 37% of H_2S induced Ca^{2+} transients were inhibited by capsazepine which indicates mainly activation of TRPV1 receptors. However, 20% of H_2S induced Ca^{2+} transients were also abolished by HC 030031 indicating the activation of TRPA1 receptors in a small fraction of neurons, which was supported by a number of other studies. It was shown that in TG neurons application of NaHS increased $[Ca^{2+}]_{in}$ in 20–42% of capsaicin-sensitive neurons with close correspondence between neurons that responded to NaHS and to AITC (Hajna et al., 2016). NaHS also evoked inward currents in DRG neurons and in CHO cells expressing TRPA1 receptors, which were inhibited by a TRPA1 antagonist (Miyamoto et al., 2011; Andersson et al., 2012; Ogawa et al., 2012).

However, it should be noted that high concentrations of NaHS (1–10 mM) were used in those studies which may activate TRPA1 indirectly by formation of reactive oxygen species (Andersson et al., 2008). Moreover, the main mechanism of TRPA1 activation appears to be oxidation of reactive cysteine residues whereas reducing agents induce an inhibition of TRPA1 (Macpherson et al., 2007). H_2S being a reducing agent cannot induce direct oxidation of the thiol groups of proteins (Greiner et al., 2013). Recent studies report that polysulfides generated in NaHS and Na_2S solutions or by the chemical interaction of H_2S and NO are able to activate TRPA1 receptors (Hatakeyama et al., 2015; Kimura, 2016; Miyamoto et al., 2017). It appears possible therefore that the effective molecules which activate TRPA1 receptors in our and previous studies were polysulfides. However, in our experiments relatively low concentration of NaHS (100 μM, effectively 11 μM, see "Materials and Methods" Section) were used which probably is insufficient to generate sufficient amounts of polysulfides to activate TRPA1. Moreover, TRPV1 stimulation by H_2S can cause Ca^{2+} dependent desensitization of TRPA1 receptor as TRPA1 is highly co-expressed with TRPV1 (Akopian et al., 2007; Palazzo et al., 2013).

The gating machinery of the TRPV1 receptor is complicated as different ligands are acting at distinct extra- and intracellular sites. The sites responsible for the reducing action of DTT are

located at the extracellular part of the TRPV1 protein (Susankova et al., 2006) whereas capsaicin acts from the intracellular side (Gavva et al., 2004). Thus, the S512Y and Y511A point mutations at the intracellular part of the S3 segment were able to eliminate capsaicin sensitivity (Jordt and Julius, 2002). As the effect of NaHS was inhibited by capsazepine, a competitive antagonist of the TRPV1 receptor with structural similarities to capsaicin (Pingle et al., 2007), we propose that H_2S activates the gating of the TRPV1 receptor at the same locus as capsaicin. However, the exact mechanisms of H_2S action on the TRPV1 receptor have to be determined in future experiments.

In summary, our data suggest that H_2S induces pro-nociceptive firing in the peripheral part of the TG nerve through activation of TRPV1 and TRPA1 receptors. This is consistent with the ability of H_2S to induce membrane currents and Ca^{2+} transients in cell bodies of TG neurons, which were mediated by TRPV1 and TRPA1 receptors. The endogenous H_2S producing enzyme CBS is abundantly expressed in rat TG neurons (Feng et al., 2013) and it is known that inflammation upregulates CBS expression in TG neurons at both protein and mRNA levels (Miao et al., 2014). A similar increase of CBS expression was observed also in rat DRG neurons after streptozotocin (Zhang et al., 2013) and complete Freund adjuvant treatments (Qi et al., 2013). We suggest that up-regulation of CSE or CBS during migraine related neuro-inflammation in meninges generates H_2S resulting in activation of pro-nociceptive TRPV1 and TRPA1 receptors. Associated release of neuropeptide CGRP from TRPV1/TRPA1 positive peptidergic nerve fibers should further support both neuronal sensitization (Giniatullin et al., 2008) and the long-lasting nociceptive firing underlying migraine pain. Therefore, targeting the CBS-H_2S-TRP signaling in the trigemino-vascular system might represent a novel therapeutic strategy for alleviation of TG pain.

AUTHOR CONTRIBUTIONS

KK, AM, AY, GS: experimental work and data acquisition. KK, AM, AY, GS: data analysis and preparation of figures. GS, RG, AH: study design/interpretation and drafting of manuscript. KK, AM, AY, AH, RG, GS: final approval of manuscript.

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II

Meningeal mast cells contribute to ATP-induced nociceptive firing in trigeminal nerve terminals: direct and indirect purinergic mechanisms triggering migraine pain

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Meningeal Mast Cells Contribute to ATP-Induced Nociceptive Firing in Trigeminal Nerve Terminals: Direct and Indirect Purinergic Mechanisms Triggering Migraine Pain

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Peripheral mechanisms of primary headaches such as a migraine remain unclear. Meningeal afferents surrounded by multiple mast cells have been suggested as a major source of migraine pain. Extracellular ATP released during migraine attacks is a likely candidate for activating meningeal afferents via neuronal P2X receptors. Recently, we showed that ATP also increased degranulation of resident meningeal mast cells (Nurkhametova et al., 2019). However, the contribution of ATP-induced mast cell degranulation in aggravating the migraine pain remains unknown. Here we explored the role of meningeal mast cells in the pro-nociceptive effects of extracellular ATP. The impact of mast cells on ATP mediated activation of peripheral branches of trigeminal nerves was measured electrophysiologically in the dura mater of adult wild type (WT) or mast cell deficient mice. We found that a spontaneous spiking activity in the meningeal afferents, at baseline level, did not differ in two groups. However, in WT mice, meningeal application of ATP dramatically (24.6-fold) increased nociceptive firing, peaking at frequencies around 10 Hz. In contrast, in mast cell deficient animals, ATP-induced excitation was significantly weaker (3.5-fold). Application of serotonin to meninges in WT induced strong spiking. Moreover, in WT mice, the 5-HT3 antagonist MDL-7222 inhibited not only serotonin but also the ATP induced nociceptive firing. Our data suggest that extracellular ATP activates nociceptive firing in meningeal trigeminal afferents via amplified degranulation of resident mast cells in addition to direct excitatory action on the nerve terminals. This highlights the importance of mast cell degranulation via extracellular ATP, in aggravating the migraine pain.

Keywords: ATP, 5-HT3, mast cells, pain, migraine

INTRODUCTION

Mast cells are immune cells implicated in various inflammatory diseases. Since several original studies by Theoharides et al. (1995, 2005), the role of meningeal mast cells as triggers of migraine attacks was further explored by others, showing the pro-nociceptive role of mast cell derived pro-inflammatory cytokines/chemokines (Reuter et al., 2001; Levy et al., 2007; Baun et al., 2012; Conti et al., 2019). We recently showed that serotonin appeared to be the most important neurotransmitter released by degranulated dural mast cells to activate peripheral meningeal nerve fibers (Kilinc et al., 2017). Despite several potential candidates, it remains, however, unclear which signal or chemical agent initially triggers the activation of meningeal mast cells.

In the frame of the current Research Topic, we published a recent study showing that extracellular ATP acts through the P2X7 subtype of purinergic receptors on meningeal mast cells, leading to both mast cell activation and degranulation (Nurkhametova et al., 2019). Similar results were found also in human mast cells line (Wareham and Seward, 2016). Based on these findings, we hypothesized that this mast-cell based mechanism can indirectly contribute to ATP-induced activation of meningeal afferents. Notably, it is well established that ATP directly excites trigeminal nerve terminals (Zhao and Levy, 2015; Yegutkin et al., 2016; Zakharov et al., 2016), mainly via P2X3 receptors (Yegutkin et al., 2016). Thus, ATP potentially may have a dual complementary migraine pain promoting effect. Given a plethora of pro-inflammatory and pro-nociceptive substances released from active mast cells (Conti et al., 2019) these data suggest that ATP-driven mechanisms might significantly contribute both to meningeal neuroinflammation and to prolonged pain in migraine.

Here, we set out to differentiate the indirect, mast cell-mediated, and direct actions of ATP on meningeal afferents in isolated mouse hemiskull preparations, in mice deficient of mast cells. Our data highlight the importance of ATP driven mast cell degranulation in the aggravation of nociceptive firing in migraine pain.

MATERIALS AND METHODS

Animals

Experiments were performed on 10–12-week-old male WT C57BL/6J and C57BL/6J-KitW-v/J mice provided by the Animal Facilities of the University of Eastern Finland (UEF). All procedures were approved by the Committee for the Welfare of Laboratory Animals of the University of Eastern Finland and the Provincial Government of Kuopio. Experiments were conducted according to the European Community Council guidelines (Directives 86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Toluidine Blue Staining of Meningeal Mast Cells

Toluidine Blue staining was used to identify mast cells in meningeal tissues as previously described by Shelukhina et al. (2017) and Nurkhametova et al. (2019). In short, the brains were carefully removed from the hemiskulls leaving the meninges intact on bone tissue. The hemiskulls were filled with artificial cerebrospinal fluid (ACSF) (in mM): NaCl 115, KCl 3, CaCl2 2, MgCl2 1, NaH2PO4 1, NaHCO3 25, glucose 11) for 10 min (room temperature) and oxygenated with 95% O2/ 5% CO2. The hemiskulls were then transferred to 4% paraformaldehyde and fixed overnight at 4°C followed by three washes with phosphate buffered saline (PBS). Meningeal tissues were dissected from hemiskulls and placed on glass slides (Polysine® Thermo-Scientific, United States) for staining with Toluidine Blue (Levy et al., 2007; Kilinc et al., 2017). Images were acquired with an Olympus AX-TFSM microscope (Olympus, Japan).

Electrophysiology

Isolated whole-mount mouse hemiskulls were used for spike recordings as previously described (Zakharov et al., 2015; Kilinc et al., 2017; Mikhailov et al., 2019). In short, hemiskulls were cleaned from cranial muscles, keeping the dura mater with meningeal nerves and vessels intact. The main meningeal branch of the trigeminal nerve was cleaned from surrounding tissue, cut and placed inside the glass electrode filled with the ACSF. All recordings of electrical activity from trigeminal nerves were performed from hemiskull preparations continuously perfused by ACSF oxygenated with 95% O2/ 5% CO2. Trigeminal nerve spiking activity was registered using DAM80 amplifier (World Precision Instruments, Sarasota, FL, United States). Electrical signals were digitized using a NI PCI6221 board (National Instruments, United States) stored on a PC for off-line analysis. Signals were visualized by WinEDR v.3.2.7 software (University of Strathclyde, Glasgow, United Kingdom) and analyzed with Matlab-based software (Zakharov et al., 2015). All agonists and the antagonist of 5-HT3 receptors (ATP from Sigma-Aldrich, Germany and serotonin and MDL-7222 from Tocris Bioscience, United Kingdom) were prepared immediately before usage and were applied to the receptive fields in meninges by fast perfusion (7 ml/min). ATP and serotonin were dissolved in water, while MDL-7222 was first dissolved in DMSO (stock concentration 30 mM) and then diluted to a final concentration of 10 μM in the basic solution.

Statistical Analysis

Experimental data were analyzed using Matlab (MathWorks, Inc., United States). Data are presented as mean ± SEM (standard error of mean). The data were analyzed using Student's paired *t*-test and Mann–Whitney *U*-test when appropriate, the differences accepted significant at $p \leq 0.05$.

RESULTS

ATP Induced Activation of Meningeal Afferents Reduced in Mast Cells Deficient Mice

We first verified that the mast cell deficient animals were indeed devoid of mast cells. As demonstrated in **Figures 1A,C**,

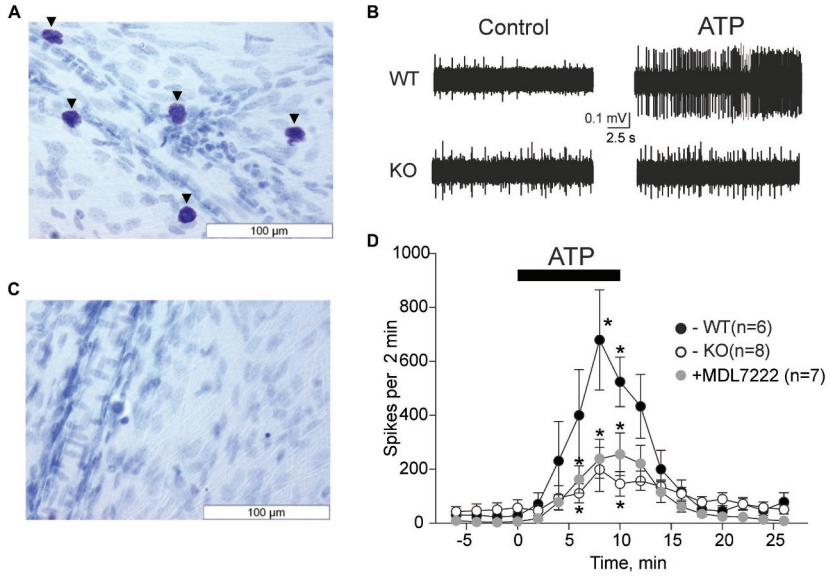

FIGURE 1 | ATP induced meningeal firing was significantly reduced in KO mice compared to WT mice and in the presence of the 5-HT3 antagonist MDL-7222. Toluidine Blue staining of meningeal tissue in the WT **(A)** and KO **(C)** mice. **(A)** Toluidine Blue-stained mast cells are shown along the middle meningeal artery (arrows) in the dura mater. **(C)** KO mice show no Toluidine Blue stained cells. **(B)** Representative traces of nociceptive firing recorded from trigeminal nerve fibers in WT and KO mice, respectively, before and after application of 100 µM ATP. **(D)** The time-course of nociceptive firing during application of 100 µM ATP in the WT (black circles, $n = 6$), KO (white circles, $n = 8$) mice, and 100 µM ATP in the presence of 10 µM MDL-7222 in WT mice (gray circles, $n = 7$). Each point represents a mean spike frequency for 2 min of recording. Mean ± SEM, nerve activation levels compared to the baseline using the Student's paired t-test, $^*p < 0.05$.

where WT meninges contained a vast amount of mast cells, there were no mast cells in the meninges of C57BL/6J-KitW-v/J mice (KO mice).

The pro-nociceptive action of ATP on trigeminal meningeal nerve fibers was electrophysiologically recorded in WT and KO mice. The baseline frequency of meningeal spikes (measured during 2 min before ATP application) was not significantly different in the two groups (27.7 ± 14.8 spikes in the WT, $n = 6$ versus 57.0 ± 29.9 spikes in KO mice, $n = 8$, $p = 0.322$). The application of ATP (100 µM) via rapid perfusion produced a pronounced firing in nerve fibers in both groups of mice (**Figure 1B**). In WT mice, the frequency of nociceptive spikes after application of ATP increased from the resting value of 27.7 spikes to 400.2 ± 169.1 spikes 6 min after ATP application ($p = 0.105$ as compared to baseline activity, $n = 6$) and to 679.2 ± 185.1 spikes 8 min after ATP application ($p = 0.024$, $n = 6$). In sharp contrast, in KO animals, ATP increased spiking activity from the resting value of 57.0 spikes only to 111 ± 35.5 spikes ($p = 0.034$, $n = 8$) by 6 min and to 199 ± 81.2 spikes ($p = 0.057$, $n = 8$) by 8 min. The detailed time-course of ATP action in WT and KO mice is shown in **Figure 1D**. Comparative analysis indicated that during the maximal effect (6–8 min of ATP action) the spike frequency in KO mice was significantly lower ($p = 0.02$) compared to the WT mice (**Figure 1D**).

MDL-7222 Inhibits ATP Mediated Nociceptive Firing

We recently showed that ATP efficiently promoted the degranulation of meningeal mast cells (Nurkhametova et al., 2019), a process which is associated with the release of multiple active mediators including serotonin. Endogenous serotonin derived from dural mast cells is a likely candidate to excite nerve fibers as it strongly promotes firing of rat meningeal afferents mainly via neuronal ligand-gated 5-HT3 receptors (Kilinc et al., 2017). Therefore, we next investigated the hypothesis that the part of the pro-nociceptive effect of ATP was mediated by endogenous serotonin via 5-HT3 receptors. To this end, we performed experiments where ATP was applied together with the 5-HT3 receptor antagonist MDL-7222. In the presence of this 5-HT3 blocker, ATP (100 µM) was still able to increase the frequency of meningeal spikes from 6.4 ± 2.8 spikes to 160.3 ± 49.9 ($p = 0.027$, $n = 7$) by 6 min, and to 235.9 ± 71 spikes ($p = 0.023$, $n = 7$) by 8 min. However, this effect was significantly ($p = 0.035$) weaker than the peak frequency induced by ATP alone (679.2 ± 185.1 spikes by 8 min, $p = 0.024$, **Figure 1D**).

Serotonin Induces Nociceptive Firing via 5-HT3 Receptors

In order to confirm that low concentrations of serotonin close to physiological levels of this monoamine (Nagata et al., 2006;

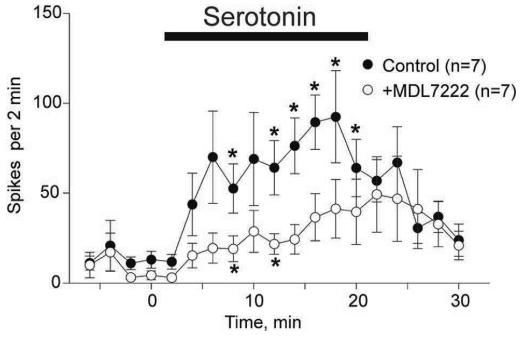

FIGURE 2 | Serotonin induced nociceptive firing and the inhibitory effect of 5-HT3 antagonist MDL-7222 on serotonin-induced activation of meningeal afferents. The time-course of nociceptive firing before and after application of 2 μM serotonin (black circles) and 2 μM serotonin in the presence of 10 μM MDL-7222 (white circles; $n = 7$ in both groups). Each point represents the mean spike frequency of a 2 min recording period. Mean ± SEM, Student's paired t-test, $^*p < 0.05$.

Ćulafic et al., 2007) are active in mice, we applied this monoamine to mouse meninges.

Application of 2 μM of serotonin increased spiking activity of trigeminal nerves in WT mice from 13 ± 4.7 spikes to 89.4 ± 15.1 spikes by 16 min ($p = 0.002$ as compared to baseline activity) and then to 92.4 ± 25.6 spikes by 18 min ($p = 0.015$) after serotonin application ($n = 7$, **Figure 2**). This excitatory action of serotonin was largely prevented in the presence of the 5-HT3 receptor antagonist MDL-7222 (10 μM) down to 36.6 ± 13.1 spikes by 16 min ($p = 0.057$, $n = 7$, **Figure 2**) and 41.3 ± 16.3 spikes by 18 min after serotonin applied together with MDL-7222 ($p = 0.084$, $n = 7$, **Figure 2**).

Comparison of the spike frequency in the period of maximal serotonin-induced activity (14–18 min) showed that the number of spikes was significantly weaker when this agonist was applied together with MDL-7222 ($p = 0.038$, $n = 7$).

Spectral Analysis of the Pro-nociceptive Effect of ATP

To compare the functional sequences of ATP induced signaling in the presence and absence of mast cells, we performed spectral analysis of firing activity in the meningeal nerves, which normally sends this information to the second order brainstem neurons (Andreou et al., 2015).

Figures 3A,B show that the pro-nociceptive effect of ATP in WT mice was characterized by high-frequency discharges. Notably, the spectral analysis revealed that in the WT mice the activity peaked at 10 Hz, which is sufficient for the temporal summation of excitatory signals at the level of secondary nociceptive neurons (Zakharov et al., 2015). In contrast, in KO mice, spectral analysis indicated a prevailing activity at 0.6 Hz (**Figure 3C**). Similar results were obtained also in the presence of MDL-7222 (**Figure 3D**). Thus, in the absence of mast cells, and when the action of serotonin was blocked, ATP-induced high frequency events were significantly reduced.

DISCUSSION

Here, we demonstrate for the first time the potent excitatory action of extracellular ATP on nociceptive firing of mouse meningeal afferents implicated in generation of migraine pain and the key role of mast cells in this phenomenon.

Despite the high prevalence of migraine, the mechanisms of pain generation in this common disorder have not been fully discovered. The trigeminovascular system of the meninges comprising trigeminal nerve fibers densely innervating dura mater blood vessels, is a well-recognized origin site of migraine pain (Messlinger, 2009; Olesen et al., 2009; Noseda and Burstein, 2013; Pietrobon and Moskowitz, 2013; Zakharov et al., 2015).

Recent evidence also suggests an important role for meningeal mast cells in triggering migraine pain. Thus, mast cells are densely present in meningeal tissues, located adjacent to both nerves and vessels (Theoharides et al., 1995, 2005; Levy et al., 2007). The contact between mast cells and nerve endings forms a neuro-immune synapse where active substances released by mast cells can activate neighboring nociceptive fibers and compounds released from active fibers, in turn, can degranulate mast cells (Dimitriadou et al., 1991). There is a long list of active substances, which can take part in the crosstalk between neurons and mast cells. Thus, degranulation of mast cells leads to release of multiple pro-inflammatory substances including enzymes, neurotrophic factors, pro-inflammatory cytokines, histamine and serotonin (Wernersson and Pejler, 2014; Conti et al., 2019). Degranulation of dural mast cells can strongly activate meningeal nerve fibers (Levy et al., 2007; Kilinc et al., 2017). Interestingly, we found that histamine is weak in excitation of meningeal nerve terminals (Kilinc et al., 2017, see also Schwenger et al., 2007). In contrast, serotonin is a powerful inducer of nociceptive firing in meningeal afferents, operating via ligand-gated 5-HT3 receptors (Kilinc et al., 2017).

One of the endogenous substances, which can activate meningeal afferents, is extracellular ATP, a powerful pro-nociceptive and pro-inflammatory agent (Giniatullin and Nistri, 2013; Burnstock et al., 2014). The purinergic hypothesis of migraine, suggesting an important role of ATP in migraine pathophysiology, was first proposed by Burnstock (1981). We previously showed in rats, that ATP induced nociceptive firing in trigeminal nerves, through ATP-gated P2X3 receptors (Yegutkin et al., 2016; Zakharov et al., 2016). The other study showed that dural topical application of ATP activated more than half of A-delta and C-fibers (Zhao and Levy, 2015). In the current study, we also found that ATP produced a huge (24.6-fold) activation of meningeal trigeminal nerve fibers in mice.

Besides this direct excitatory action on nerve terminals, extracellular ATP is also known as a substance triggering mast cell degranulation (Wareham and Seward, 2016; Nurkhametova et al., 2019). Here, we tested the hypothesis that this concomitant action of ATP contributes to activation of trigeminal fibers via degranulation of dural mast cells and the release of additional

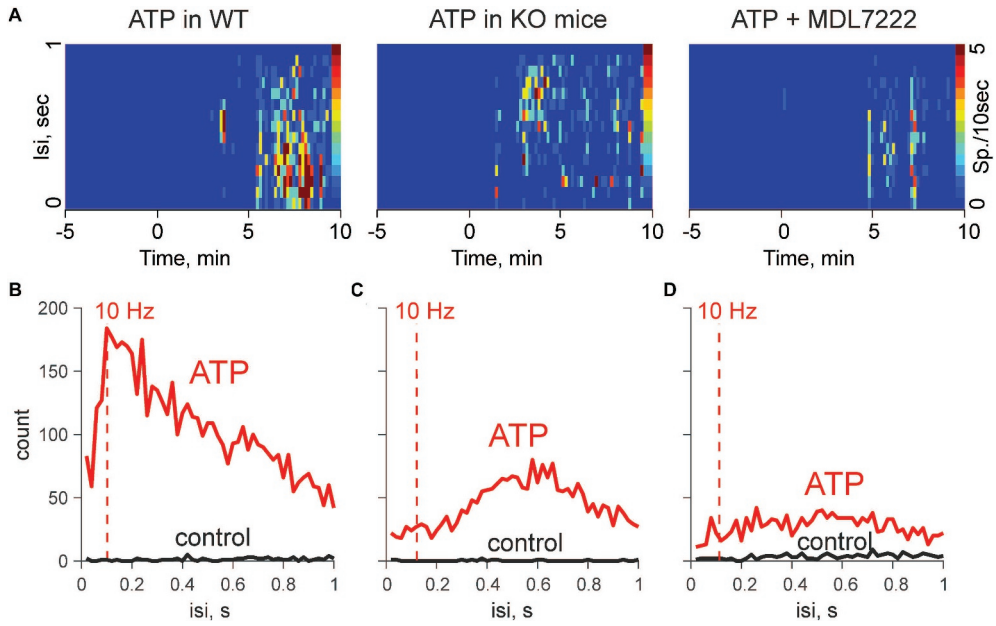

FIGURE 3 | ATP induced high-frequency discharges are reduced in KO mice and in the presence of MDL-7222. **(A)** Heat maps during application of 100 μM ATP in WT (control and in the presence of MDL-7222) and in KO mice. **(B)** Mean interspike intervals during application of 100 μM ATP in WT mice (n = 6). **(C)** Mean interspike intervals in control (black) and after application of 100 μM ATP in KO (n = 8). **(D)** Mean interspike intervals during application of 100 μM ATP in presence of MDL-7222 in WT (n = 7).

excitatory agents, such as serotonin. To test this hypothesis, we used C57BL/6J-KitW-v/J mice deficient in mast cells and found that mast cell deficient mice were significantly less sensitive to the excitatory action of extracellular ATP suggesting that mast cells provided an additional component for the pro-nociceptive action.

As serotonin is a well-known mast cell mediator stored in granules and easily released upon activation (Wernersson and Pejler, 2014), we tested its action on mouse trigeminal afferents. We found that concentrations as low as 2 μM of this biogenic amine are able to excite nerve terminals similar to ATP. Notably, like in rats (Kilinc et al., 2017), this effect of serotonin was antagonized by the specific 5-HT3 antagonist MDL-7222 demonstrating the role of the ligand-gated 5-HT3 receptor as a main target of serotonin.

Moreover, when testing the action of ATP in WT mice, ATP-induced firing was also reduced in the presence of MDL-7222 suggesting that the action of ATP is partially mediated by 5-HT3 receptors. It is worth noting that serotonin can promote release of the migraine mediator CGRP (Kilinc et al., 2017) and contributes to meningeal neuroinflammation (Buzzi and Moskowitz, 2005) which can be a reason for long-lasting pain in migraine. Thus, serotonin can be considered as the endogenous amplifier of purinergic nociception in meninges. On the other hand, at the level of 'postsynaptic' neuronal membrane, there could be the inhibitory interactions between 5-HT3 and P2X channels (Barajas-López et al., 2002), which are most significant at high agonist concentrations. This negative mechanism can limit an excessive excitation of afferents when the high level of ATP and serotonin are co-released. ATP-induced firing discharges around 10 Hz detected in the WT and missing in KO mice and in the presence of MDL-7222 may be important for the nociceptive traffic amplification at the level of second order neurons via temporal summation of input signals in the excitatory synapses in the brainstem (Zakharov et al., 2015).

In summary, we report that extracellular ATP, a powerful pro-nociceptive agent, which can be released during a migraine attack (Karatas et al., 2013), stimulates nociceptive firing in trigeminal afferents via a dual mechanism, including degranulation of resident mast cells and by the direct excitatory action on nerve terminals. ATP can be released from multiple cellular sources including astrocytes, neurons, platelets, and endothelial cells, primarily via exocytosis and/or pannexin/connexin hemichannels (Pankratov et al., 2006; Pangrsic et al., 2007; Lohman et al., 2012). Notably, ATP release could be enhanced in migraine-associated conditions such as shear stress and hypo-osmotic cell swelling (Wei et al., 2011; Burnstock and Knight, 2017) and local inflammation (Dosch et al., 2018). We suggest that ATP-driven mechanisms contribute both to excitation and to meningeal neuroinflammation in the local neuro-immune unit formed by dural mast cells and trigeminal afferent fibers.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because the raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher. Requests to access the datasets should be directed to Rashid.Giniatullin@uef.fi.

AUTHOR CONTRIBUTIONS

KK, VG, and OG contributed to data collection, analysis, interpretation, and writing the manuscript. RaisaG contributed to data collection and analysis. DN contributed to writing and editing the manuscript. OM and PL provided the KO mouse line and contributed to writing the manuscript. GS contributed to the study design and supervision of the study. TM and RashidG contributed to the study design and supervision, writing the manuscript, and the final editing. All authors approved the final version of the manuscript.

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III

Protective effects of hydrogen sulfide against the ATP-induced meningeal nociception

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Protective Effects of Hydrogen Sulfide Against the ATP-Induced Meningeal Nociception

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We previously showed that extracellular ATP and hydrogen sulfide (H_2S), a recently discovered gasotransmitter, are both triggering the nociceptive firing in trigeminal nociceptors implicated in migraine pain. ATP contributes to meningeal nociception by activating the P2X3 subunit-containing receptors whereas H_2S operates mainly *via* TRP receptors. However, H_2S was also proposed as a neuroprotective and anti-nociceptive agent. This study aimed to test the effect of H_2S on ATP-mediated nociceptive responses in rat meningeal afferents and trigeminal neurons and on ATP-induced degranulation of dural mast cells. Electrophysiological recording of trigeminal nerve activity in meninges was supplemented by patch-clamp and calcium imaging studies of isolated trigeminal neurons. The H_2S donor NaHS induced a mild activation of afferents and fully suppressed the subsequent ATP-induced firing of meningeal trigeminal nerve fibers. This anti-nociceptive effect of H_2S was specific as an even stronger effect of capsaicin did not abolish the action of ATP. In isolated trigeminal neurons, NaHS decreased the inward currents and calcium transients evoked by activation of ATP-gated P2X3 receptors. Moreover, NaHS prevented ATP-induced P2X7 receptor-mediated degranulation of meningeal mast cells which emerged as triggers of migraine pain. Finally, NaHS decreased the concentration of extracellular ATP in the meningeal preparation. Thus, H_2S exerted the multiple protective actions against the nociceptive effects of ATP. These data highlight the novel pathways to reduce purinergic mechanisms of migraine with pharmacological donors or by stimulation production of endogenous H_2S.

Keywords: migraine, trigeminal nerve, mast cells, ATP, H_2S, P2X3 receptor

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INTRODUCTION

Long-lasting migraine pain likely originating from meninges, involves local inflammation, sensitization and activation of trigeminal afferents by multiple endogenous compounds released from local vessels, somatic and parasympathetic nerves and various immune cells such as mast cells occupying this area (Bolay et al., 2002; Levy et al., 2007; Olesen et al., 2009; Burstein et al., 2015; Koroleva et al., 2019). To find new pharmacological interventions in migraine, much attention is currently paid to the substances triggering pain such as neuropeptides, most notably, calcitonin gene-related peptide (CGRP) which promote neurogenic inflammation in meninges (Ebersberger et al., 1999; Lassen et al., 2002; Schou et al., 2017)

or gaseous transmitter nitric oxide (NO) promoting pain and dilating cortical vessels (Reuter et al., 2001; Pryazhnikov et al., 2014; Messlinger et al., 2020). Less interest is given to endogenous molecules which can exhibit the anti-nociceptive and anti-inflammatory effects in migraine.

Hydrogen sulfide (H_2S) is a common gaseous transmitter that regulates many physiological and pathological processes (Kimura, 2011; Hermann et al., 2012; Wang, 2012; Paul and Snyder, 2018). In mammals, H_2S is generated from sulfur-containing amino acids, primarily L-cysteine and L-homocysteine by the enzymes cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (Kimura, 2011; Hermann et al., 2012; Wang, 2012; Paul and Snyder, 2018). CBS is the main enzyme for the synthesis of H_2S in the nervous tissue, whereas, in the cardiovascular system, liver, and kidneys, the main synthesis enzyme for H_2S is CSE (Kimura, 2011).

Emerging data suggest the participation of this endogenous gaseous transmitter in nociception (Bhatia et al., 2005; Cunha and Verri, 2007; Okubo et al., 2012; Xu et al., 2019). Immunohistochemical studies have shown expression of the H_2S synthesis enzyme, CBS in sensory DRG and trigeminal ganglia (Xu et al., 2009; Feng et al., 2013). Moreover, the level of CBS expression increases with the development of inflammation (Bhatia, 2015). Notably, inflammation in meninges essentially contributes to sensitization related to migraine pain (Strassman et al., 1996; Ebersberger et al., 1999; Waeber and Moskowitz, 2005). However, the available data on the role of H_2S in the nociceptive system is contradictory (Bhatia et al., 2005; Cunha and Verri, 2007; Kida et al., 2015; Chen et al., 2019; Xu et al., 2019). H_2S can activate several pro-nociceptive receptors such as the TRPV1, and TRPA1 receptors (Trevisani et al., 2005; Teicher et al., 2017; Pozsgai et al., 2019; Roa-Coria et al., 2019). On the other hand, H_2S activates ATP-dependent and Ca^{2+}-activated potassium channels which can reduce the neuronal excitability through membrane hyperpolarization (Sitdikova et al., 2010; Mustafina et al., 2015).

This gaseous transmitter also stabilizes mast cells (Roviezzo et al., 2015; Rodrigues et al., 2017; Matsui et al., 2019). Also, recent studies propose the antioxidant and anti-inflammatory potential of H_2S (Chen et al., 2019; Melo et al., 2019; Yakovleva et al., 2020; Yurinskaya et al., 2020).

Recently, we showed that the H_2S donor NaHS can trigger nociceptive firing in rat trigeminal afferents (Koroleva et al., 2017). However, this pro-nociceptive effect was transient as the firing quickly returned to the baseline level in the presence of H_2S.

Likewise, we have also shown that ATP stimulates the rat and mouse trigeminal afferents thus, exerting a clear pro-nociceptive and prolonged effect *via* the P2X3 subunit-containing receptors (Yegutkin et al., 2016; Koroleva et al., 2019). Moreover, ATP degranulated meningeal mast cells releasing serotonin, which indirectly supports the pro-nociceptive effect of purinergic agonists (Koroleva et al., 2019). In general, ATP activated P2X3 receptors are expressed in ∼80% of trigeminal neurons (Fabbretti et al., 2006) suggesting the leading role of ATP signaling in trigeminal nociception.

As two pro-nociceptive agents, ATP and H_2S can be endogenously generated in the trigeminovascular system, the unsolved issue remains whether they can interact. The interaction of classical transmitters with gasotransmitters is a relatively novel little-explored field. Co-expression of H_2S producing enzyme CBS and ATP-activated P2X receptors have been detected in sensory ganglia (Xu et al., 2009). This fact suggests a possible interaction between signaling cascades activated by H_2S and ATP *via* P2X receptors. However, data on the effect of H_2S on purinergic mechanisms of trigeminal nociception, in particular, in migraine are lacking.

The present study aimed to explore the modulatory action of H_2S on the pronociceptive effects of ATP in the trigeminal nerve, in isolated trigeminal neurons and on meningeal mast cells. We report that the mild and transient activation effect of H_2S is followed by almost completed suppression for ATP pro-nociceptive signaling including neurons and mast cells.

MATERIALS AND METHODS

Animals

The experiments with cultured trigeminal neurons were carried out on male P9–12 Wistar rats, whereas 4–6 weeks rats were used for testing hemiskull preparation and meningeal mast cell staining. Animals from the Animal Center of the University of Eastern Finland (Kuopio) and vivarium of Kazan Federal University were used. Rats were housed in cages with controlled temperature and humidity and a 12-h light cycle. Food and water were provided *ad libitum*. The experimental protocols complied with the ethical standards for the humane treatment of animals adopted at the Kazan Federal University and approved by the Local Ethics Committee of KFU (protocol No. 8 dated 05.05.2015), and complied with the Council of the European Union Directive of September 22, 2010 (2010/63/EEC) and were approved by the Committee for the Use of Animals of the University of Eastern Finland (licenses EKS-004-2014 and EKS-002-2017). All measures were taken to minimize the number of animals used in the experiments.

Objects of Study

Hemiskull Preparation

To study meningeal nociception, we used the isolated rat hemiskull with intact innervation of dura mater (De Col et al., 2012). This model of the peripheral nociceptive processes in the dura mater allows most directly to assess the neurochemical mechanisms of migraine pain generation (Zakharov et al., 2015). After decapitation, the rat skull was carefully cleaned from all cranial muscles. Then the skull was divided along the sagittal section into two halves (hemiskulls), whereas the brain was gently removed from the cranium. The meningeal processes of the mandibular branch of the trigeminal nerve called "*nervus spinosus*," that innervates the receptive field around the middle meningeal artery (MMA), were isolated to be placed inside the recording electrode. The hemiskulls were stabilized in artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 120 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 glucose,

24 $NaHPO_4$, 30 $NaHCO_3$ with constant oxygenation of 95% O_2/5% CO_2 for at least 30 min before starting the experiment.

Trigeminal Culture

After rat decapitation, the trigeminal ganglia were extracted, placed in a cold Ham's F12 nutrient mixture, and then were chopped. Cells dissociation was carried out in an enzymatic cocktail containing 0.25 mg/ml trypsin, 1 mg/ml collagenase and 0.2 mg/ml DNAse at shaker at a temperature of 37°C, at 1,000 rpm, for 25 min. Dissociated cells were placed on coverslips coated with poly-L-lysine and were kept in an incubator at a temperature of 37°C, in 5% CO_2 for 24 h before the start of the experiments.

Electrophysiology

The Activity of the Trigeminal Nerve Peripheral Branch

The isolated hemiskulls were fixed in the experimental chamber provided with a flow perfusion system. Drugs were applied with a speed of 6–7 ml/min. Under visual control, the peripheral process of the nervus spinosus was sucked into a glass electrode (tip diameter of ∼150 μm). The application of substances was performed to the receptive field around the area of divergence of MMA (Schueler et al., 2014). The isolated preparation was washed with ACSF under constant oxygenation with 95% O_2/5% CO_2, the pH was maintained at 7.20–7.35. Electrical signals were recorded using a DAM80 amplifier (World Precision Instruments, Sarasota, FL, USA). The signals were digitized on a PC using the NI PCI6221 board (National Instruments, Austin, TX, USA) and WinEDR v.3.2.7 software (Strathclyde University, UK). The two-phase signals, with duration in the range of 0.3–1.5 ms were considered as action potentials. Data recorded immediately before the application of the substance was used as a control.

P2X3 Receptor Responses in Trigeminal Ganglion Neurons

During the experiment, cells were constantly perfused with a solution containing (mM): 148 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 D-Glucose, pH 7.2. The intrapipette solution contained (mM): 145 KCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA, 0.5 $CaCl_2$, 2 Mg-ATP, 0.5 Na-GTP, 5 KCl, pH 7.2. The solution was supplied using a gravity-controlled perfusion system (ALA Scientific Instruments Westbury, Farmingdale, NY, USA). P2X3-induced currents were recorded using whole-cell patch-clamp with Axopatch-200B amplifiers (Axon Instruments/Molecular Devices, San Jose, CA, USA) and borosilicate glass patch pipettes (Harvard Apparatus, Holliston, MA, USA) with a resistance of 3–10 MΩ. P2X3 currents were induced by local application of the agonist α,β-meATP at a concentration of 20 μM for 2 s using a rapid perfusion system (Rapid Solution Changer 200, BioLogic Science Instruments, France), with a solution supply of ∼20 ms. To prevent desensitization of P2X3 receptors, α, β-meATP was applied at intervals of 5 min. Patch-clamp data were analyzed using the Clampfit software (Axon Instruments/Molecular Devices, San Jose, CA, USA).

Calcium Imaging in Trigeminal Cells

To visualize calcium signals trigeminal cells were loaded with Fluo4-AM fluorescent marker (2 μM) at 37°C for 30–40 min in dark following by washout with an extracellular solution for 10 min. Fluorescent visualization of stained cells was carried out using an Axio Observer D1 microscope (Carl Zeiss, Germany). Fluorescence images were recorded using an AxioCam MRm high-speed camera (Carl Zeiss, Germany). The test substances were applied using a gravity-controlled perfusion system (ALA Scientific Instruments Westbury, NY, USA). To differentiate neuronal cells from glia, a 100 mM KCl solution was applied at the end of each experiment. Image processing software (NIH, Bethesda, MD, USA) was used to process fluorescence images and estimate fluorescence intensity (in arbitrary units, a.u.). Peak amplitude was calculated using the MATLAB software package (The MathWorks, Novi, MI, USA).

Mast Cell Degranulation

To study meningeal mast cell degranulation, we stained meninges (Wistar rats P35–40) with Toluidine Blue (Guselnikova et al., 2014). Hemiskulls were filled with the studied solutions for 20 min, and then they were fixed in paraformaldehyde (4%) for 12 h. Before isolation of the meninges, the hemiskulls were washed in a phosphate-saline buffer solution of the following composition (mM): 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4. Isolated dura mater was placed on a glass slide. Staining with Toluidine Blue lasted 10 min, after three times washing with PBS the preparations were fixed with ethanol (95–99%). Pictures from stained meninges were taken at 20× magnification on Olympus AX70 microscope (Tokyo, Japan). Mast cells with inhomogeneous staining, pale cells, and cells with disfigured borders surrounding positively stained granules were ranked as degranulated (Shelukhina et al., 2017). The degranulation was evaluated in a blind manner approaching 100 cells from 10 different fields (20×) from the meningeal preparation in each experiment. The rate of degranulation was calculated as a % of the degranulated cells to the total number of cells (Pedersen et al., 2015; Shelukhina et al., 2017; Koroleva et al., 2019).

Measuring the Extracellular Level of ATP in the Dura Mater

The concentration of ATP released from the rat dura mater (Wistar rats, P35–45) was estimated using the ATP luminescence analysis kit (PerkinElmer, Waltham, MA, USA). Isolated rat hemiskulls were filled with solutions. After 20 min incubation, 100 μl of the media were taken away for analysis. The analysis was performed according to the ATP lite kit protocol using 96-well plates (Costar, Corning, USA). Luminescence was measured using a POLARstar Optima microplate reader (BMG Labtech GmbH, Germany).

Chemicals

The agonists α,β-meATP (20 μM), adenosine triphosphate (ATP, 100 μM), BzATP triethylammonium salt (BzATP, 30 μM), oxidized glutathione (GSSG, 1 mM), capsaicin (1 μM, all from Sigma–Aldrich, St. Louis, MO, USA) were used. Toluidine

Blue (Sigma–Aldrich, St. Louis, MO, USA) was employed for labeling mast cells. Sodium hydrosulfide (NaHS, Sigma–Aldrich, St. Louis, MO, USA) was used as a hydrogen sulfide donor. In solutions, NaHS dissociates into HS^- ions and binds to the hydrogen proton H^+ to form H_2S. H_2S concentration in solution depends on the temperature, pH, and ionic strength (Whitfield et al., 2008; Nagy et al., 2014). In the bath solution (pH 7.4) at 20°C, about 22% of total sulfide is expected to be as free H_2S (Sitdikova et al., 2014). Real-time measurements of H_2S concentration using amperometry indicated a fast loss of the sulfide after the volatilization of H_2S (DeLeon et al., 2012; Sitdikova et al., 2014). In our experiments, NaHS was used at a concentration of 100 μM, which provides approximately 11 μM H_2S in the perfusion system. NaHS stock solutions were prepared immediately before the experiment and they were kept tightly closed in a dark place until use.

Statistical Analysis

Statistical data processing was performed using MATLAB and Origin Pro 2015 software (OriginLab, Northampton, MA, USA). To assess reliability, the Student's *t*-test was used (for paired and independent samples). All values are indicated as the mean ± standard error of the mean (M ± SEM). In our study, *n* indicates the number of animals. Differences were considered statistically significant at $p < 0.05$.

RESULTS

NaHS Counteracted the Pro-nociceptive Effect of ATP in the Meningeal Trigeminal Nerve

First, we analyzed the effect of H_2S on the pro-nociceptive effect of ATP in the rat trigeminal meningeal nerve after the incubation of the hemiskull preparation in a solution containing the H_2S donor NaHS. In control, the application of ATP (100 μM) induced a strong and prolonged surge in the activity of the rat trigeminal nerve. Thus, the frequency of action potentials increased from 227.8 ± 38.0 spikes per 5 min in control up to 755.8 ± 129.4 spikes per 5 min after 10 min of ATP application ($n = 5$, $p < 0.05$; **Figures 1Aa,b,B**). During 20 min application of ATP, 2,725.6 ± 374.2 spikes were comparing with 588.5 ± 236.0 spikes for the same period in control ($n = 5$; $p < 0.05$; **Figure 1E**). Application of the H_2S donor NaHS (100 μM) elicited only a transient increase of trigeminal nerve spiking (from 139.6 ± 65.4 to 458.0 ± 56.1 spikes per 5 min; $n = 4$; $p < 0.05$) which further declined in the presence of NaHS to the baseline level. The subsequent application of ATP (100 μM) combined with 100 μM NaHS did not increase the frequency of action potentials which was 109.5 ± 39.9 spikes per 5 min after 10 min action of this purinergic agonist (**Figures 1Ac,C**). This activity (586.7 ± 168.5 spikes per 20 min) in the presence of NaHS+ATP did not differ from the basal frequency of action potentials ($n = 4$, $p > 0.05$; **Figure 1E**).

Next, the H_2S scavenger oxidized glutathione (GSSG; Pei et al., 2011) was used to probe that the inhibition of ATP-induced nociceptive response was mediated by H_2S. GSSG (1 mM) by itself did not affect the frequency of action potentials. Thus, there were 723.3 ± 138.1 spikes per 5 min in control and 722.5 ± 147.8 spikes per 5 min by 20 min of GSSG application ($n = 3$; **Supplementary Figure S1**). ATP (100 μM) increased action potentials frequency to 1,695.3 ± 481.4 spikes per 5 min by 10 min of application of ($n = 3$, $p < 0.05$, **Supplementary Figures S1A,C**). In the presence of 1 mM GSSG NaHS (100 μM) did not increase the frequency of action potentials (**Supplementary Figures S1B,D**). Thus, there were 810.0 ± 134.3 spikes per 5 min in control and 918.3 ± 238.9 spikes per 5 min in glutathione + NaHS. Application of ATP (100 μM) increased spiking activity up to 2,258.0 ± 558.28 spikes per 5 min ($n = 3$, $p < 0.05$) similar to the effects of ATP in control (**Supplementary Figures S1B,D**).

We have shown previously that P2X3 receptors mediated the pro-nociceptive effects of ATP in the trigeminal nerve (Yegutkin et al., 2016). Therefore, we analyzed the effects of NaHS application on the nociceptive firing induced by the P2X3 receptor agonist α,β-meATP. In control, α,β-meATP (20 μM) similarly to ATP, increased the frequency of action potentials from 545.6 ± 268.8 spikes per 5 min in control up to 957.3 ± 51.1 spikes per 5 min after 10 min ($n = 3$, $p < 0.05$). The preliminary application of NaHS (100 μM) prevented an increase of P2X3 mediated response. Thus, the frequency of action potentials was only 381.7 ± 125.2 spikes per 5 min after 10 min action of α,β-meATP compared to control (561.2 ± 283.1, $n = 3$, $p > 0.05$).

These data revealed the inhibitory action of H_2S on ATP-induced activation of P2X3 receptors in the trigeminal nerve endings in the meninges.

As we (Koroleva et al., 2017) and others (Roa-Coria et al., 2019) showed that the action of H_2S is mediated, at least, partly by TRPV1 receptors, it could be that this inhibition is due the negative crosstalk between TRPV1 and P2X3 receptors. Thus, it has been shown previously in isolated neurons that the activation of TRPV1 receptors decreased the subsequent activation of ATP-gated P2X3 receptors (Stanchev et al., 2009). We hypothesized that a similar mechanism might underlie the inhibitory action of H_2S on ATP signaling in the meninges. Therefore, the effect of ATP was analyzed in the hemiskull preparation after the preliminary activation of TRPV1 with the specific agonist capsaicin. In control, the frequency of action potentials was 85.0 ± 32.9 spikes per 5 min ($n = 4$). The application of capsaicin (1 μM) increased activity to 994.0 ± 294.9 spikes per 5 min ($n = 4$; $p < 0.05$) followed, like with NaHS, by a subsequent decline in the frequency of action potentials to values close to control (119.0 ± 12.0 spikes), likely due to desensitization of the TRPV1 receptors. However, unlike NaHS, the subsequent application of ATP (100 μM) in the presence of capsaicin efficiently increased the frequency of action potentials to 414.0 ± 117.9 spikes per 5 min ($n = 4$; $p < 0.05$) at 10 min and 527.6 ± 217.4 spikes per 5 min at 20 min ($n = 4$; $p > 0.05$; **Figures 1Ad,D**). This effect was comparable with the action of ATP alone. Thus, the application of ATP, combined with capsaicin, generated 1,969.3 ± 605.7 spikes per 20 min which were not significantly different from ATP alone (2,725.6 ± 374.2 spikes per 20 min; $p > 0.05$; **Figure 1E**).

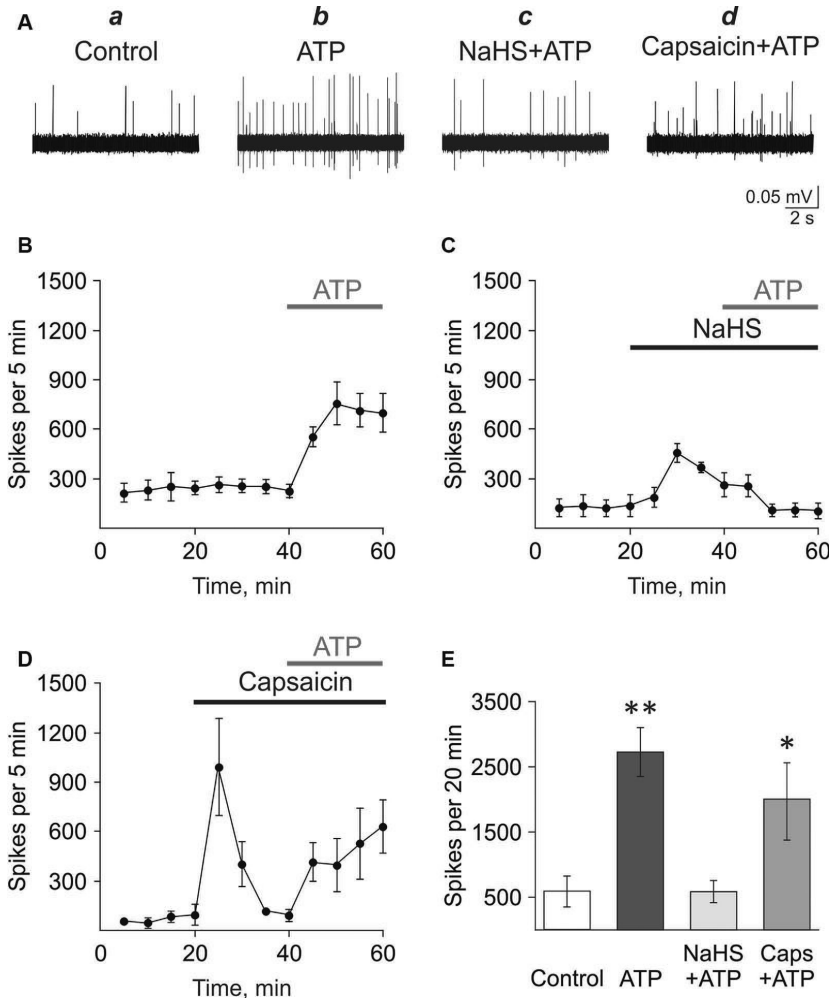

FIGURE 1 | Testing the action of hydrogen sulfide (H_2S) on the pro-nociceptive effect of ATP in trigeminal afferents. **(A)** Example traces of action potentials in the trigeminal nerve in control **(a)**, with ATP application (100 μM; **b**), ATP plus NaHS application after preincubation in NaHS (100 μM; **c**), ATP plus capsaicin application after preincubation in capsaicin (1 μM; **d**). **(B)** The frequency of action potentials during application of ATP ($n = 5$). **(C)** The frequency of action potentials during application of ATP (100 μM) in the presence of NaHS after preincubation with NaHS (100 μM; $n = 4$). **(D)** The frequency of action potentials during the application of ATP (100 μM) in the presence of capsaicin after preincubation with capsaicin (1 μM; $n = 4$). **(E)** The histograms showing the frequency of action potentials per 20 min in the trigeminal nerve in control and after application of ATP (100 μM), α,β-meATP (20 μM), in control and in the presence of NaHS or capsaicin. $^*p < 0.05$, $^{**}p < 0.01$.

These results indicated that the suppressing action of NaHS on the pro-nociceptive effect of ATP was specific and was not shared by the agonist of TRPV1 receptors capsaicin.

NaHS Decreased P2X3-Mediated Currents and Calcium Transients in Trigeminal Neurons

To further study the mechanisms of the inhibitory effect of NaHS on the pro-nociceptive properties of ATP we used the isolated trigeminal ganglion neurons. In control, the application of α,β-meATP (20 μM) agonist of the P2X3 receptor-induced fast, slow, and mixed currents, consisting of two components with fast and slow desensitization (**Figures 2Aa,Ba**). It is known that, in sensory neurons, the P2X2 and P2X3 subunits can form homomeric P2X2, homomeric P2X3, or heteromeric P2X2/3 receptors (Lewis et al., 1995; Kowalski et al., 2015). P2X3 receptors account for fast currents, whereas slow and mixed currents are mediated by heteromeric receptors

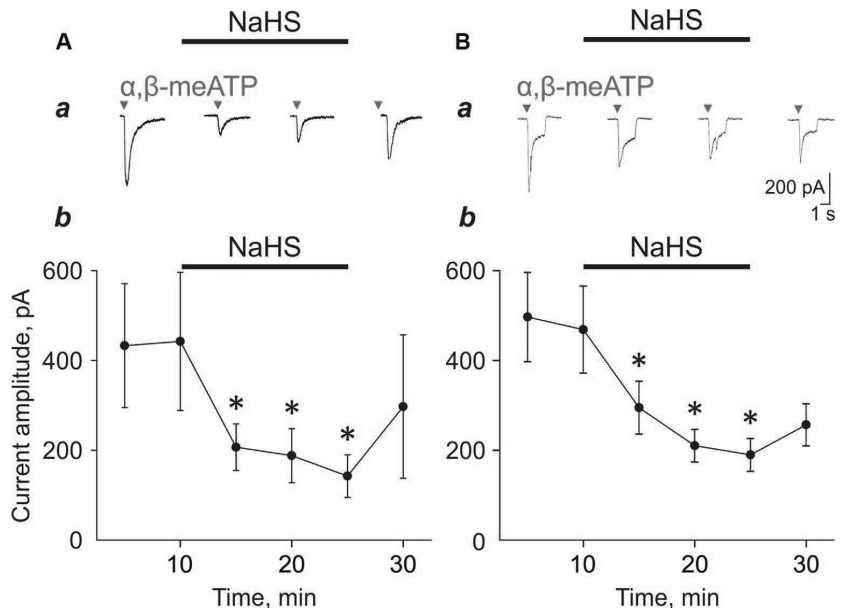

FIGURE 2 | Effect of NaHS (100 μM) on α,β-meATP induced currents in trigeminal ganglion neurons. **(A)** Examples of fast type currents in control and in the presence of NaHS **(a)**. Changes in the amplitude of the fast type currents (**b**, $n = 5$ cells). **(B)** Examples of mixed type currents in trigeminal ganglion neurons in control and in the presence of NaHS **(a)**. Changes in the amplitude of the fast component of the mixed type currents (**b**, $n = 9$ cells). *$p < 0.05$.

co-expressing P2X3 and P2X2 subunits (Lewis et al., 1995; Kowalski et al., 2015).

To test the action of H_2S on P2X3 receptors we focused on fast and mixed currents as we noted in this and in previous experiments that the slow component is relatively unstable during repetitive agonist applications. Application of NaHS (100 μM) decreased the amplitude of the fast currents from 442.4 ± 164.0 pA to 206.9 ± 51.7 pA (five cells; $n = 5$; $p < 0.05$; **Figure 2Ab**) and the amplitude of the mix currents from 468.7 ± 96.9 to 294.8 ± 58.9 (nine cells; $n = 8$; $p < 0.05$; **Figure 2Bb**) by 5 min of NaHS application. Washout only partially recovered responses. To minimize the effect possible desensitization of P2X3 receptors, we performed repeated application of α,β-meATP (20 μM) with a 5 min interval in control and did not reveal the decrease of the current amplitudes. The amplitude of currents during first application was 879.22 ± 242.92 pA, second—1,071.43 ± 329.67 pA, third—1,043.14 ± 315.22 pA, fourth—1,190.30 ± 351.9 pA and fifth—1,037.17 ± 314.88 pA (seven cells; $n = 4$, **Supplementary Figures S2A,B**).

In the next set of experiments, to test the action of H_2S on unpatched neurons, we analyzed the effects of NaHS on calcium transients induced by ATP and α,β-meATP (**Figure 3**) in isolated trigeminal neurons. ATP (100 μM) responses were observed in 70% of the whole population of cells (61/87 cells) whereas α,β-meATP triggered calcium responses were observed in 41% of cells (33/136 cells).

In control, the repeated application of ATP (100 μM) or α,β-meATP (20 μM) with an interval of 5 min did not induce significant desensitization of calcium responses (**Supplementary Figures S3A,B**). The average amplitude of the first response to ATP was 3.53 ± 0.45 a.u., 3.42 ± 0.42 a.u. of the second and 2.93 ± 0.3 a.u. of the third response (61 cells; $n = 3$; $p > 0.05$; **Supplementary Figures S3Aa,B**). The application of NaHS (100 μM) decreased the amplitude of calcium response from 3.08 ± 0.3 a.u. to 2.01 ± 0.27 a.u. (66 cell; $n = 3$; $p < 0.05$; **Figures 3Aa,B,C**) and washout did not significantly change the average amplitude of the response (1.93 ± 0.26 a.u.).

The agonist α,β-meATP (20 μM) in control elicited calcium responses with an average amplitude of 3.53 ± 0.45 a.u.—the first application, 3.42 ± 0.42 a.u.—second and 2.92 ± 0.3 a.u. to the third one (33 cells; $n = 3$; $p > 0.05$; **Supplementary Figures S3Ab,C**). The application of NaHS (100 μM) decreased the amplitude of calcium responses from 2.73 ± 0.26 a.u. to 1.69 ± 0.23 a.u. (27 cells; $n = 3$, $p < 0.05$; **Figures 3Ab,D**) and washout did not significantly change the average amplitude of the response (1.52 ± 0.2 a.u.).

NaHS Prevented Degranulation of Meningeal Mast Cells

Mast cell degranulation in the meninges plays an important role in the pro-nociceptive effect of ATP by releasing endogenous nociceptive agent serotonin (Koroleva et al., 2019). Therefore, we assessed the potential protective effect of NaHS on ATP-induced

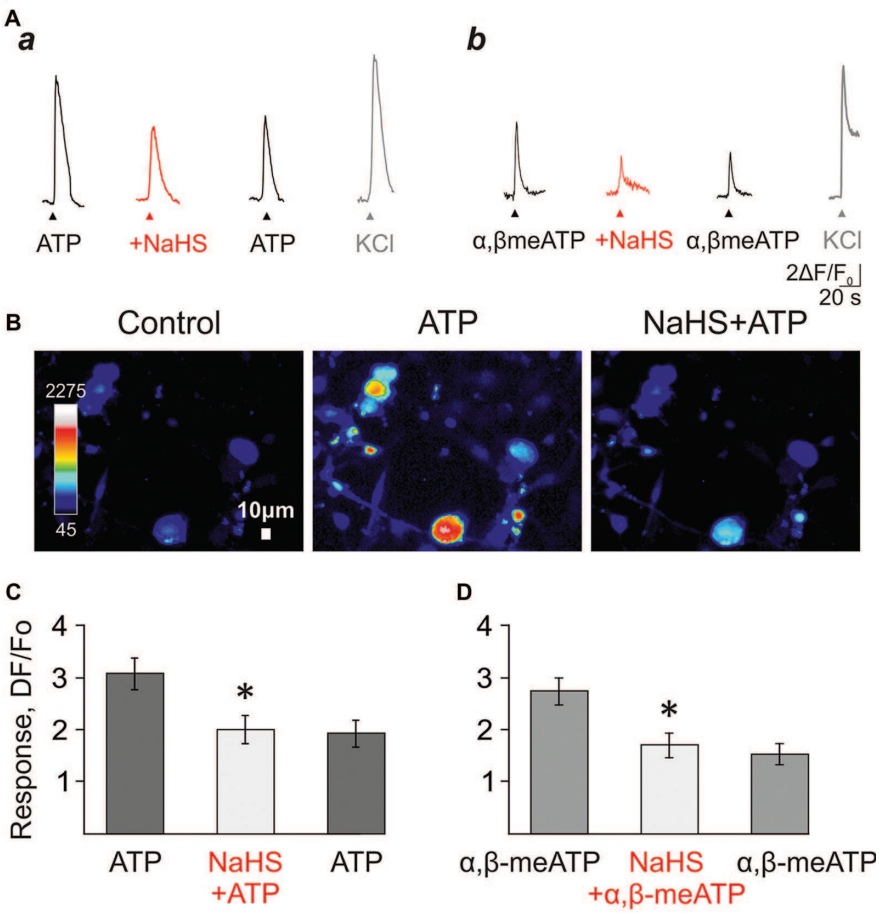

FIGURE 3 | The effect of NaHS on ATP-induced calcium signals in isolated trigeminal neurons. **(A)** Examples of calcium signals induced by application of 100 μM ATP **(a)**, or 20 μM α,β-meATP **(b)** in control, and in the presence of 100 μM NaHS. KCl was applied to distinguish neurons from trigeminal glial cells. **(B)** Pseudo-color images showing ATP-induced calcium signals in control and after the preliminary application of NaHS. **(C)** Histograms showing the amplitudes of calcium signals in response to the application of ATP (100 μM) in control, and in the presence of NaHS (n = 66 cells). **(D)** Histograms showing the amplitudes of calcium signals in response to applications of α,β-meATP (20 μM) in control, and in the presence of NaHS (n = 27 cells). *$p < 0.05$.

mast cell degranulation. ATP (100 μM) caused a significant increase in the number of degranulated cells (53.8 ± 3.7%; n = 6; $p < 0.05$; **Figures 4B,G**) compared to the control group (24.4 ± 1.8%; n = 6; $p < 0.05$; **Figures 4A,G**). Incubation of meninges in a solution containing 100 μM NaHS for 30 min did not change the functional state of mast cells, as the number of degranulated cells (31.8 ± 4.6%; n = 6) did not significantly exceed the control values (**Figures 4D,G**). Notably, the pre-incubation in NaHS for 10 min followed by addition of ATP (100 μM) to the solution for 20 min also did not increase the number of degranulated cells (34.7 ± 4.7%; n = 6) indicating that H_2S prevented mast cell degranulation by ATP (**Figures 4E,G**).

We previously showed that mast cell degranulation under the action of ATP is mediated by the activation of P2X7 receptors (Nurkhametova et al., 2019). Notably, this receptor is likely coupled to pannexin-1 channels, which can also transport ATP through the membrane (Iglesias and Spray, 2012; Kurashima et al., 2012; Wareham and Seward, 2016). Therefore, in subsequent experiments, we analyzed the ability of the P2X7 receptor agonist BzATP to activate meningeal mast cells in the presence of the H_2S donor NaHS We found that 30 μM BzATP increased the number of degranulated cells to 75.14 ± 2.8% (n = 6, $p < 0.05$; **Figures 4C,G**). Pre-incubation in NaHS (100 μM) for 10 min prevented

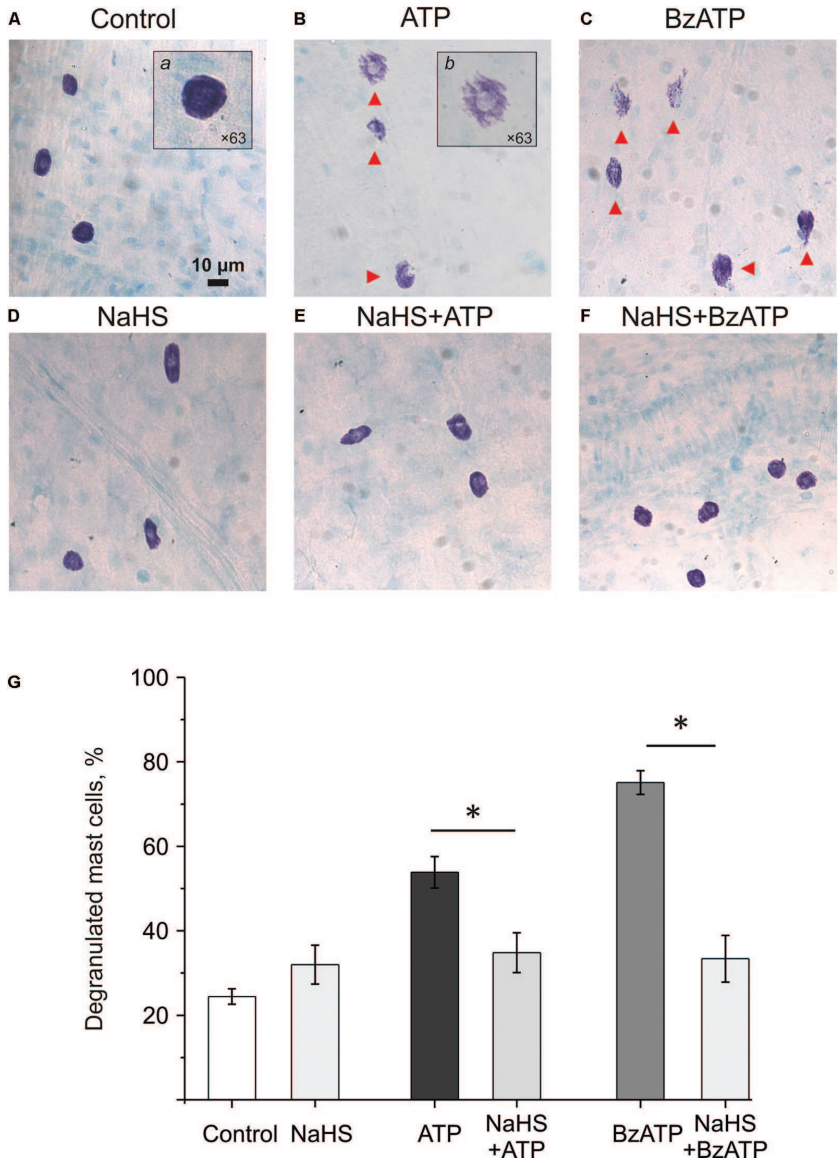

FIGURE 4 | Effect of the H_2S donor NaHS on ATP-induced mast cell degranulation. Images (×20) of Toluidin Blue stained rat meninges after incubation in basic solution **(A)** and after exposure to ATP (100 μM, **B**), BzATP (30 μM, **C**), NaHS (100 μM, **D**) or combination of NaHS+ATP **(E)** and NaHS+BzATP **(F)**. Notice red arrows indicating degranulated mast cells. Inserts **(a)** and **(b)** shows enlarged intact and degranulated mast cells (×63). **(G)** Histograms showing the percent of degranulated mast cells under various conditions ($n = 6$). *$p < 0.05$.

this P2X7 mediated degranulation of the meningeal mast cells as the number of degranulated mast cells dropped two-times to 33.4 ± 5.5%; ($n = 6$; **Figures 4F,G**). Thus, the H_2S donor prevented the ATP-induced degranulation of meningeal mast cells.

NaHS Decreased the Level of Extracellular ATP in Rat Meninges

Many cells including the meninges (Yegutkin et al., 2016) can spontaneously release ATP to provide its tone in the trigeminovascular system. Next, we analyzed the level of ATP

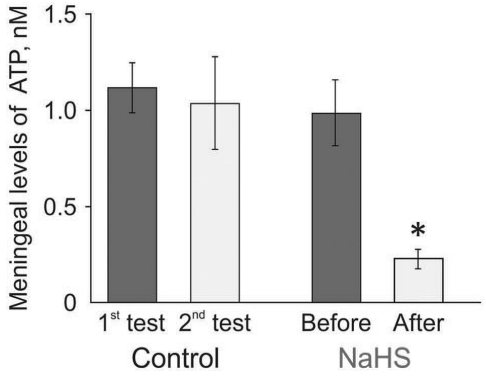

FIGURE 5 | Effect of the H_2S donor NaHS on the level of extracellular ATP in the meninges in a hemiskull preparation. The ATP level was twice determined in the basic solution in control (interval 20 min) or before and 20 min after incubation in 100 μM NaHS ($n = 8$). *$p < 0.05$.

released from rat meninges under control conditions and after incubation of the hemiskull preparation in NaHS. The ATP level in the meninges in control conditions was 1.12 ± 0.13 nM, and after 20 min this level was not significantly changed (1.04 ± 0.24 nM; $n = 8$; **Figure 5**, left). In the experimental group, the initial ATP value was 0.99 ± 0.17 nM, and after incubation in a solution containing 100 μM NaHS for 20 min, the ATP level significantly decreased to 0.23 ± 0.05 nM ($n = 8$; $p < 0.05$). Based on this data, it can be assumed that H_2S can affect the transport of ATP through the membrane and reduce the basal level of ATP in meninges.

DISCUSSION

The main finding of this study is that in meninges, the gaseous transmitter H_2S exerts the multicomponent protecting action against the powerful nociceptive agent, extracellular ATP. This finding is unusual as H_2S can itself transiently promote nociceptive firing in trigeminal afferents (Koroleva et al., 2017).

Migraine remains a largely unsolved issue due to still poorly understood pathophysiological mechanisms of headache. In particular, we still not completely characterized a large group of endogenous substances involved in triggering migraine attacks and molecules that can prevent this disabling condition. The novel members of expanding family of gaseous transmitters represent a group of such molecules which may exhibit either pro- or anti-nociceptive effects in migraine. One well-established migraine promoting gas is NO which has multiple vascular and neuronal targets in the trigeminovascular system (Messlinger et al., 2000, 2020; Marone et al., 2018). Another gaseous transmitter, carbon monoxide (CO) also recently emerged as a novel trigger of migraine (Arngrim et al., 2014). H_2S only recently attracted attention as a gas implicated in migraine (Koroleva et al., 2017; Teicher et al., 2017). According to Teicher et al. (2017), H_2S can interact with NO to produce the nociceptive effect in trigeminal neurons *via* its product nitroxyl (HNO).

The peripheral part of the trigeminal nerve in the meninges plays a key role in the pathogenesis of migraine pain (Olesen et al., 2009; Schueler et al., 2014) and represents a potential target for the action of endogenous pro- and anti-nociceptive agents. Extracellular ATP and its metabolites, mediating vasodilation of intracranial vessels were previously proposed as key players in the pathogenesis of migraine (Burnstock, 1981). In our previous study, we identified also the neuronal targets of ATP in meninges showing that ATP increased the frequency of action potentials in the trigeminal nerve fibers by direct activation of the P2X3 subunit-containing receptors (Yegutkin et al., 2016). Consistent with this, here we also showed that the P2X3 agonist α,β-meATP excited a large fraction of isolated rat trigeminal neurons in agreement with previous observations on the expression of these pain-related P2X3 receptors in sensory neurons (Wirkner et al., 2007). Moreover, our recent studies indicated that ATP causes mast cell degranulation with subsequent release of active pro-inflammatory agents, particularly serotonin, which can have its strong effect on meningeal nerve endings (Koroleva et al., 2019). Thus, the pain-producing action of ATP in meninges likely includes several direct and indirect effects involving local immune cells.

Recently discovered endogenous gasotransmitter H_2S can play both pro- and anti-nociceptive effects in different tissues, mediated through various cellular targets (Matsunami et al., 2009; Okubo et al., 2012; Di Cesare Mannelli et al., 2017; Melo et al., 2019; Xu et al., 2019). This gas is generated by several enzymes including CBS. In different types of sensory neurons, including trigeminal ganglia neurons, the high expression of CBS was detected (Xu et al., 2009; Feng et al., 2013). Further studies indicated that CBS expression increased during inflammation paralleled with enhanced neuronal excitability, mainly *via* suppression of potassium currents (Miao et al., 2014). Consistent with the pro-nociceptive action of H_2S, in our previous study, we observed the transiently increased spiking of the trigeminal nerve in response to the donor of H_2S NaHS (Koroleva et al., 2017).

The antinociceptive or pronociceptive effects of H_2S are dependent on its concentration: the low doses of this gasotransmitter contribute to the reduction of pain, whereas H_2S in high doses can exert even a pro-nociceptive action (Guo et al., 2020). Interestingly, both activating and inhibitory effects of the H_2S donor Na_2S has been shown in neurons of the spinal nucleus of the rat trigeminal nerve (Teicher et al., 2017) suggesting that this gaseous transmitter can exert both pro- and anti-nociceptive effects also in the trigeminovascular system implicated in migraine. Thus, after dural applications of Na_2S, they also found a short-lasting stimulatory (and sometimes inhibitory) effect this H_2S donor in medulla neurons and suggested TRPA1 channels for the nociceptive action of H_2S interacting with NO to produce HNO. In our previous study, by direct recordings of spikes from dural nerves, we found that the firing was preferentially mediated by activation of TRPV1 receptors (Koroleva et al., 2017). Notably, TRPV1 and TRPA1, two members of the TRP receptors family are often co-localized (Nielsen et al., 2018). Moreover, there are data that these channels can be heteromerized (Fischer et al., 2014; Weng

et al., 2015) suggesting that H_2S might act in employing these channels complex. Recently, Roa-Coria et al. (2019) proposed that not only TRPV1 and TRPA1 but also TRPC receptors were implicated in the action of H_2S.

Given that both ATP and H_2S are endogenous compounds with a clear pro-nociceptive for ATP but more controversial (either pro- or anti-nociceptive) action of H_2S, it is of interest to explore their potential crosstalk. The functional interactions in the modulation of pain signaling between ATP and NO, another gaseous transmitter implicated in migraine have already previously been shown. Thus, the selective P2X3/P2X2/3 antagonist A-317491 reduced the formation of NO by inhibiting the specific gas generating enzyme nNOS in the neuropathic mouse pain model (Ohnishi et al., 2009). However, the possible interactions between purinergic and H_2S signaling pathways were not studied yet.

In this study, we showed that the application of NaHS on meningeal afferents prevented the increase of action potentials in response to ATP application. One potential mechanism for such action might the indirect involvement of TRPV1 channels which are the target for the stimulatory action of H_2S (Koroleva et al., 2017). It has been shown that 33–58% of all trigeminal ganglia neurons co-express P2X3 and TRPV1 and both potentiating and inhibiting interactions between P2X3 and TRPV1 were shown in nociceptors (Saloman et al., 2013). Thus, in isolated neurons, the pre-activation of TRPV1 receptors decreased the subsequent activation of P2X3 receptors due to the inhibitory interactions of the C-termini of P2X3 and TRPV1 proteins (Stanchev et al., 2009; Saloman et al., 2013). Here, in a more complex structure such as the hemiskull preparation, we show that the prior stimulation of TRPV1 receptors by the specific agonist capsaicin did not prevent the nociceptive effects of ATP on meningeal afferents. These data suggest that the depressant action of H_2S on ATP nociception was not related to TRPV1-mediated inactivation of P2X3 receptors and most likely, mediated by independent signaling cascade initiated by this gaseous transmitter. To reveal the receptor mechanism of NaHS action, isolated trigeminal ganglion neurons were analyzed using electrophysiological patch-clamp methods and calcium imaging. We showed that the application of NaHS decreased fast and mixed currents activated by α,β-meATP which is consistent with data obtained in the meninges indicating the action of H_2S on P2X3 subunit-containing receptors. These data obtained in voltage-clamped neurons suggest that the H_2S donor directly affects the function of ionotropic P2X3 receptors as a plausible explanation for the prevention of the pro-nociceptive effect of ATP in peripheral trigeminal afferents.

Recently, the role of Kv7 channels in modulating neuronal excitability was proposed in pain processing (Zheng et al., 2013; Busserolles et al., 2016). Inorganic and slow-releasing H_2S donors (including the natural allyl-isothiocyanate and its derivatives) were shown to activate Kv7 channels and were effective in animal models of neuropathic pain induced by paclitaxel or oxaliplatin (Di Cesare Mannelli et al., 2017; Lucarini et al., 2018). Thus, the Kv7 channel, in our model, maybe an additional target of anti-nociceptive effects of H_2S along with inhibition of ATP mediated signaling.

Sulfur in the H_2S molecule exists in the lowest oxidation state (-2). Therefore, H_2S is rather a reductant and can be oxidized in the corresponding environment. As a result, H_2S can elicit its biological effects *via* several chemical reactions. The chemical reduction of protein disulfide bonds by H_2S was shown (Sitdikova et al., 2010; Pálinkás et al., 2015; Vasas et al., 2015). The reducing action of H_2S is responsible for its effects on Ca^{2+}-activated K^+ channels and NMDA-receptors (Abe and Kimura, 1996; Sitdikova et al., 2010; Kimura, 2016). Recently, several studies revealed that H_2S can react with protein thiol groups and form protein persulfides resulting in the functional changes of the target proteins (Mustafa et al., 2009). However, the conversion of R–SH to R–SSH is associated with oxidation, therefore, the oxidation of H_2S to per-/poly-sulfide or the oxidation of the target cysteine to sulfenic acid or disulfide is necessary for this reaction (Greiner et al., 2013).

Ionotropic P2X receptors activity can be regulated by oxidative stress. It has been shown that H_2O_2 may modulate the P2X channel function through the direct oxidation of the cysteine moieties (Coddou et al., 2009). Additionally, the reducing agent dithiothreitol applied intracellularly decreased the sensitivity of the P2X2 receptor to ATP (Nakazawa et al., 2003). Based on these data, we can suggest that the inhibitory effects of H_2S on P2X3/2 receptors are mediated by its reducing action on disulfide bonds of the channel protein, probably from the intracellular side of the membrane.

Meningeal mast cells play an important role in the pathogenesis of migraine pain due to the pro-inflammatory mediators contained in these immune cells (Levy et al., 2007; Wang et al., 2013). In particular, serotonin released in meninges after degranulation of mast cells by ATP powerfully activates nerve terminals *via* ligand-gated C-loop 5-HT3 receptors (Koroleva et al., 2019). Recent work indicated that H_2S can prevent mast cell degranulation in a mouse model of asthma (Roviezzo et al., 2015). In models of pruritus and acute skin inflammation, a donor of H_2S significantly reduced the level of histamine and attenuated C48/80-induced itching probably due to the stabilizing of mast cells (Rodrigues et al., 2017). But H_2S effects were not tested in meninges where mast cells may have region-specific properties making them different from mast cells in skin or lung tissues.

In our experiments, incubation of meninges with NaHS, resulting in a reduced number of degranulated mast cells exposed to ATP and P2X7 receptor agonist, BzATP. A possible explanation for this effect is that, like inhibitory action of P2X3 receptors, H_2S inhibits activation of P2X7 receptors thus preventing ATP-induced mast cell degranulation. P2X7 subtype was identified in meningeal mast cells as the main receptor mediating ATP-induced activation of these immune cells (Nurkhametova et al., 2019) and NaHS in our experiments, reduced the number of degranulated cells induced by P2X7 receptor agonist, BzATP. Stabilization of mast cells by H_2S should prevent serotonin release and activation of the trigeminal nerve, thus preventing this powerful mechanism of

peripheral nociceptive signaling in migraine (Kilinc et al., 2017; Koroleva et al., 2019).

Previously, the inhibitory action of H_2S on P2X7 receptors was hypothesized in a rat model of stroke associated with local inflammation. In that study, H_2S decreased the inflammatory response down-regulating P2X7 receptors in microglia (Zhao et al., 2017). NaHS could reduce the expression of the P2X7 receptor, decrease membrane permeability, and increase the cell viability in rat microglia injured by ATP. The precise mode of action of H_2S on the expression and function of the P2X7 receptor should be clarified in further studies.

Finally, among potential targets for H_2S in the meninges, we found the action of this gaseous transmitter on the level of endogenously produced extracellular ATP. Enhanced release of ATP was observed in the model of migraine with aura (Karatas et al., 2013). ATP release can take place through gap junctions or pannexins, as well as *via* P2X7 receptor channels (Dubyak, 2006). The basic level of ATP in the bulk solution in the meninges was in a nanomolar range of concentrations consistent with previous studies (Yegutkin et al., 2016). Here, we found that NaHS treatment significantly decreased the level of extracellular ATP in meninges. This finding should further contribute to the protective action of H_2S against the nociceptive signaling by endogenous ATP.

CONCLUSION

We analyzed the crosstalk in signaling between two endogenous messengers ATP and H_2S in the rat trigeminal system implicated in migraine pain. We demonstrated here that H_2S prevented ATP-induced activation of the trigeminal nerve fibers thus showing the anti-nociceptive effect. One mechanism of this effect is a decrease in membrane currents through P2X3 receptors along with suppression of ATP-induced calcium signals in trigeminal ganglion neurons. Also, H_2S reduced the ATP levels in the meninges and prevented ATP-induced mast cell degranulation. These data indicate a novel multicomponent pain-preventing role of H_2S, which is expected to be especially pronounced in conditions of neuroinflammation associated with enhanced release of ATP. The promotion of H_2S synthesis within the trigeminovascular system, might, therefore, be a novel therapeutic approach for the treatment or prevention of migraine pain.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Local Ethics Committee of Kazan Federal University (protocol No. 8 dated 05.05.2015) and the Committee for the Use of Animals of the University of Eastern Finland (licenses EKS-004-2014 and EKS-002-2017).

AUTHOR CONTRIBUTIONS

KK contributed to data collection, analysis, interpretation, and writing the manuscript. RGiniatullina, EE, and AM contributed to data collection and analysis. GS and RGiniatullin contributed to the study design and supervision, writing the manuscript, and the final editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2020.00266/full#supplementary-material.

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Migraine is a disorder with a strong and often intractable pain. This study uncovered molecular mechanisms of the direct and indirect 5-HT-mediated nociceptive action of extracellular ATP in the meninges. The endogenous gasotransmitter H_2S prevented ATP-induced activation of the trigeminal nerve fibers thus indicating the novel anti-nociceptive effect that can be used to develop pharmacological approaches for prevention of headache in migraine.

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