Involvement of peptidergic Edinger-Westphal nucleus in the neurobiology of migraine and acute alcohol exposure

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Pécs, 2024

1. INTRODUCTION AND LITERATURE BACKGROUND

1.1. Edinger-Westphal nucleus

The midbrain's Edinger-Westphal nucleus (EW) comprises two distinct cell populations (Kozicz et al., 2011). One group, the preganglionic (cholinergic) division (EWpg), is part of the oculomotor complex and supplies cholinergic parasympathetic preganglionic fibers to the ciliary ganglion, regulating pupil constriction and lens accommodation. The other subdivision, known as centrally projecting Edinger-Westphal nucleus (EWcp), is peptidergic and expresses various neuropeptides including urocortin 1 (UCN1); cocaine and amphetamine-regulated transcript (CART) and cholecystokinin (CCK) among others. Intermingled with the peptidergic cells, some glutamatergic, choline acetyltransferase (CHAT) immunoreactive and tyrosine hydroxylase (TH)-containing neurons were also identified (Kozicz et al., 2003; Zuniga & Ryabinin, 2020; Li & Ryabinin, 2022; Priest et al., 2023). The EWcp sends multiple projections to a number of regions through the brain (Zuniga & Ryabinin, 2020).

UCN1 is a member of the corticotropin-releasing hormone (CRH) family, which is primarily found in the EWcp (Vaughan et al., 2007). UCN1 binds to both CRH receptors (CRH1R and CRH2R), with a higher affinity for CRH2R over CRH itself (Bale et al., 2002; And & Biol, 2002; Kozicz et al., 2007; Im et al., 2015). UCN1 plays a role in the stress response, reward, appetite control, thermoregulation, and affect the mood status (Fekete et al., 2007; Kormos & Gaszner, 2013; Zuniga & Ryabinin, 2020). CART is a neuropeptide that is known to be involved in energy metabolism (Kristensen et al., 1998.; Elias et al., 2001; Lau et al., 2018) and regulation of feeding, drug reward and addictive behaviors (Vicentic & Jones, 2007; Ong & McNally, 2020; Zuniga & Ryabinin, 2020). Interestingly, UCN1 immunoreactive neurons in the EWcp show a full co-localization with CART. Moreover, our research group previously showed that the EWcp/UCN1 positive neurons are the primary site of expression of the transient receptor potential cation channel subfamily A member 1 (TRPA1) ion channel in the brain. TRPA1 is a non-selective cation channel which upon activation leads to calcium influx. The increased intracellular calcium may lead to several types of cellular responses (Julius et al., 2013; Kádková et al., 2017; Talavera et al., 2020). TRPA1 is expressed primarily in the peripheral nervous system and plays a crucial role in several physiological and pathophysiological processes including nociception and inflammatory responses, however its role in the central nervous system (CNS) is less clear yet.

1.2. Migraine

Migraine is a neurovascular disorder (Jiang et al., 2019) of primary neuronal dysfunction (Kikkeri & Nagalli, 2022). Migraine is typically manifested as a recurring episode of headache associated with other symptoms of neurological dysfunction in varying admixtures (Goadsby et al., 2018). Episodes of migraine attacks can last for hours up to days and include multiple symptoms such as pulsating headache, nausea, vomiting, photophobia and phonophobia (Wood & Welch, 1993).

1.2.1. Neuroanatomy of migraine

The most widely accepted theory of migraine is based on the activation and sensitization of the trigeminovascular system, which consists of the trigeminal nerve and its axonal projections to the meningeal blood vessels (Ashina et al., 2019). The primary dysregulation of sensory processing will likely result in a constellation of neurological symptoms that affect our senses (Benemei et al., 2017). The trigeminovascular system is comprised by the meningeal blood vessels and type C as well as type Aδ dendritic axons of first order sensory neurons located in the trigeminal ganglion (TRG) providing nociceptive innervation for intracranial meninges (Ashina et al., 2019). The central (axonic) axon of the pseudounipolar nociceptive TRG cells transmit the pain signals to the spinal trigeminal nucleus (STN) by the fast-acting excitatory neurotransmitter, glutamate. At the

same time, when activated, TRG neurons release a variety of neuropeptides from their peripheral and central terminals that are known to play a role in the pathogenesis of migraine. These include substance P (SP), pituitary adenylate cyclase-activating polypeptide (PACAP), and calcitonin gene-related peptide (CGRP) (Durham et al., 2016; Spekker et al., 2023).

The STN is responsible for relaying the protopathic sensory modalities that include perception of pain, temperature and crude touch. Considering that the facial skin, the orbital structures, the nasal and oral cavities are innervated mainly by the trigeminal nerve, the main input arrives from here to the STN. The upper part of forehead, skin of skalp and, importantly, the meningeal nociception projects to the caudal division of the STN, located in the caudal most medulla oblongata, as well as the first and second cervical spinal cord segments. The second order STN neurons convey the nociceptive signal to the contralateral ventral posteromedial nucleus of the thalamus *via* the trigeminal lemniscus. Third order neurons in the thalamus relay the nociceptive signal through the superior thalamic radiation to the primary somatosensory cortex. The cortical representation area of the meninges is located in the lateral inferior part of the postcentral gyrus in the parietal lobe (a review for neuroanatomy: Edvinsson et al., 2020.) It has to be noted that the STN sends direct connections to multiple brainstem centers also. Fibers to the rostral ventromedial medulla, and locus coeruleus may control the perception of pain during migraine including the autonomic, endocrine, cognitive and affective symptoms. The afferents to the ventrolateral periaqueductal gray, nucleus raphe magnus, are particularly important, because these are centers that contribute to the control of the descending antinociceptive serotonergic and noradrenergic systems. The dorsal raphe nucleus (DRN) is also part of the serotonergic midline nuclear complex and its role in pain modulation *via* STN is well-known (Li et al., 1993, Andrade et al., 2013, Rattanawong et al., 2022; Shibata et al., 2022).

The connection of the STN to the CGRP and PACAP-expressing parabrachial nucleus, provides an input of pain information into the higher-order limbic centres including the amygdala, nucleus accumbens among others, that provide the affective value of pain (Almeida et al., 2004).

It's important to note that probably also due to the above summarized anatomical complexity, the exact cause and pathophysiology of migraine are still the subject of ongoing research, and no single mechanism can explain all aspects of this complex condition, highlighting the importance of further research in this area.

1.2.2. Calcitonin gene-related peptide

CGRP is a 37-amino-acid neuropeptide, primarily released from sensory nerves, commonly known for its potent cerebral vasodilative activity (Brain & Grant, 2004). The dilatory effect is facilitated by activation of CGRP receptors, G-protein-coupled heterodimers composed of calcitonin receptor-like receptor (CLR), and receptor activity modifying protein-1 (RAMP1) (McLatchie et al., 1998). The role of CGRP in the pathogenesis of migraine is supported by the large body of preclinical and clinical research results. CGRP is abundant in nociceptive fibers of TRG neurons and can act on CGRP receptors of second-order neurons in the trigeminal nucleus caudalis when released at central nerve endings, providing a connection between the periphery and the central nervous system (Bartsch et al., 2003; Iyengar et al., 2019). CGRP binds with equal affinity to CLR and calcitonin receptor (CTR, encoded by calcitonin receptor mRNA (*Calcr*) gene) when co-expressed with RAMP1. The CLR/RAMP1 complex forms CGRP receptor and CTR/RAMP1 complex creates amylin receptor (AMY1) (Salvatore et al., 2006). CGRP receptor component protein (CRCP) is not necessary for the receptor's activity, but it enhances the efficacy of CGRP (Dickerson et al., 2013).

Activation of the trigeminal system leads to an elevated level of CGRP during migraine attacks (Goadsby et al., 1990), which can be restored to baseline by administration of 5-HT receptor

agonists (triptans), such as sumatriptan and dihydroergotamine (Goadsby & Edvinsson, 1993), concurrent with pain relief (Juhasz et al., 2005).

Although it is generally known that CGRP cannot penetrate the walls of cerebral blood vessels (Edvinsson et al., 2007), several studies have demonstrated that both peripheral (intravenous (i.v.) and intraperitoneal (i.p.)) and central (intracerebroventricular (i.c.v.)) administration of CGRP-induced many migraine-like symptoms in rodents. Additionally, the CGRP-induced migraine-like symptoms were reversed by concomitant administration of sumatriptan or anti-CGRP monoclonal antibodies (Kaiser et al., 2014; Mason et al., 2017; Rea et al., 2018). Therefore, it was reasoned that CGRP can act in both the CNS and peripheral nerves (Raddant & Russo, 2011).

Human studies have shown that intravenous administration of CGRP can cause migraine headaches in migraineurs (Asghar et al., 2011; Kuburas & Russo, 2023), which can be reversed by systemic intravenous infusion of the CGRP receptor antagonist olcegepant (Olesen et al., 2004). In addition, elevated concentrations of CGRP have been detected in the peripheral blood outside migraine attacks, determining CGRP as a possible biomarker in the diagnosis of chronic migraine (Cernuda-Morollon et al., 2013). Three anti-CGRP antibodies (eptinezumab, fremantezumab and galcanezumab) and one anti-CGRP receptor antibody (erenumab) have been shown to be therapeutically effective in migraine prevention (Tepper et al., 2018; de Vries & MaassenVanDenBrink, 2019; Schiano di Cola et al., 2023). Their inability to pass the human brain-blood barrier (BBB) suggest a site of action outside the CNS (Edvinsson et al., 2015; Ashina et al., 2017). Potential targets may be the trigeminal system, cerebral blood vessels, and dura mater (Henson et al., 2020). Small molecule CGRP receptor antagonists (gepants), such as atogepant and remegepant, are promising candidates for the treatment of acute migraine attacks (Hargreaves & Olesen, 2019) due to their minor molecule nature (Negro & Martelletti, 2019; Henson et al., 2020). They are on the way to becoming the newest class of drugs approved for the treatment of migraine (Dubowchik et al., 2020).

1.2.3. Possible role of EWcp in migraine

The involvement of the CRH-related neuropeptide, UCN1-containing division of the EWcp in acute pain was shown. It is known that the EWcp is activated and expresses more *Ucn1* mRNA in acute pain (Kozicz et al., 2001; Rouwette et al., 2011). Moreover, EWcp sends multiple projections to various pain-sensitive centers in the brain, implicated in the pathophysiology of migraine, including the DRN, STN and lateral hypothalamus (lH) (Zuniga & Ryabinin, 2020). Among EWcp's projection areas the DRN plays a crucial role in the pathophysiology of migraine by regulating central 5-HT levels (Rattanawong et al., 2022; Shibata et al., 2022). 5-HT contribute to the pathomechanism of migraine possibly by regulating CGRP levels and by its direct vascular action (Puledda et al., 2023). Variations in the gene responsible for the production of the rate-limiting enzyme in 5-HT synthesis, known as tryptophan hydroxylase 2 (TPH2), could potentially increase individuals' susceptibility to migraine (Marziniak et al., 2009; Jung et al., 2010). The DRN expresses both CRH1R and CRH2R, with a relatively high CRH2R expression compared to other brain regions (Chalmers et al., 1995). As UCN1 is an endogenous ligand of these receptors, the presence of CRH receptors in migraine-related brain areas suggests a potential regulatory role of EWcp in migraine.

This hypothesis is supported by prior studies demonstrating the presence of several migraine-related neurotransmitters, neuromodulators, and receptors in the EWcp, including PACAP and its specific receptor (PAC1) (Markovics et al., 2012; Fehér et al., 2023; Priest et al., 2023). Additionally, neuronal nitric oxide synthase (nNOS), substance P, TRPA1 ion channel (Kormos et al., 2022) and CGRP-immunoreactive nerve fibers have been identified in the EWcp (Maciewicz et al., 1983; Smith et al., 1994; Spina et al., 2004).

There is a large body of literature data on the role of peripheral TRPA1 ion channel in migraine pathogenesis (Souza Monteiro de Araujo et al., 2020; Shibata et al., 2021; Iannone et al., 2022a, 2022b; Spekker et al., 2022; Fila et al., 2023; Masood et al., 2023). Several substances identified as TRPA1 agonists, including cigarette smoke, ammonium chloride, formaldehyde, chlorine, garlic, and others (Bautista et al., 2005; McNamara et al., 2007; Andrè et al., 2008; Bessac et al., 2008; Fujita et al., 2008) are known triggering factors of migraine attacks (Courteau et al., 1994; Peatfield et al., 1995; Wantke et al., 2000; Irlbacher & Meyer, 2002; Kelman et al., 2007). Collectively, these studies indicate that compounds capable of desensitizing TRPA1 and TRPA1-expressing nerve endings may be considered as new therapeutic targets in the treatment of migraine (Benemei & Dussor, 2019). Our recent finding, that the peptidergic EWcp cells exhibit high *Trpa1* mRNA expression while in other brain areas the expression was relatively low, suggests that the investigation of the possible role of EWcp/TRPA1 in migraine is relevant.
In line with this idea, the EWcp also plays a role in stress adaptation (Kormos & Gaszner, 2013) is influenced by circadian rhythm (Gaszner et al., 2009) and ovarian hormones changes (Derks et al., 2007, 2010), which are known triggers of migraine attacks.
Based on all these, we decided to examine the potential role for EWcp in the neurobiology of migraine.

1.3. Alcohol addiction

Ethanol is a toxic and psychoactive chemical with strong addictive and dependence-producing properties (World Health Organization 2022). Misuse of alcohol is an important public health problem as well as a significant risk factor for disability and death worldwide (Ferraguti et al., 2015), as it is associated with more than 200 kinds of diseases (Ilhan & Yapar, 2020; Shield et al., 2013). Alcohol use disorders are responsible for 3 million deaths worldwide each year, accounting for 5.3% of all deaths. Adverse consequences of addiction include mental and behavioral changes, mood disorders, and depression, highlighting the importance of this research topic.

1.3.1. Role of EWcp in alcohol consumption

Many research groups have provided evidence on the role of EWcp UCN1 and CART neuropeptides in actions of alcohol and other addictive drugs (Ong & McNally, 2020; Zuniga & Ryabinin, 2020).

Several genetic studies have indicated that higher alcohol preference in various strains of mice and rats was associated with increased UCN1 levels (Turek et al., 2005; Bachtell et al., 2003; Fonareva et al., 2009). Additionally, lesions of the rodent EW nucleus significantly reduce ethanol preference (Bachtell et al., 2004; Ryabinin & Weitemier, 2006). UCN1/CART positive neurons exhibit a strong FOS (marker of acute neuronal activity) response, when exposed to both passive and self-administered ethanol acutely, as demonstrated in several studies (Bachtell et al., 1999; Ryabinin et al., 2001; Weitemier et al., 2001; Zuniga & Ryabinin, 2020). The increased neuronal activity was positively correlated to the amount of consumed alcohol, suggesting a dose-dependent response (Sharpe et al., 2005; Giardino et al., 2017). Similarly, previous studies reported increased FOSB activity (marker of chronic neuronal activation) of EWcp UCN1/CART-expressing neurons in response to chronic alcohol exposure in mice (Bachtell et al., 1999; Ozburn et al., 2012).

While numerous studies have explored the significance of CART in addiction (Bakhtazad et al., 2016; Kuhar et al., 2016), there is a limited body of research concerning the involvement of EWcp/CART in the regulation of alcohol consumption. For instance, CART knockout (KO) mice exhibited significantly lower alcohol preference compared to WT mice in a free choice alcohol consumption model (Salinas et al., 2014). Another study reported that in low alcohol preference DBA/2J mice there is a decrease in CART expression at both the mRNA and peptide levels within the EWcp when compared to high alcohol-preferring C57BL6J mice (Giardino et al., 2017). The

full co-localization of UCN1 and CART within the EWcp, along with their elevated peptide and mRNA levels in mice with higher alcohol preference, implies a shared and significant role in controlling alcohol consumption and associated behaviors.

The EWcp UCN1 and CART co-expressing neurons project to several addiction- and reward-related-, moreover CRH receptor-expressing brain areas, among which I would like to highlight the ventral tegmental area (VTA) and dorsal raphe nucleus (DRN) (Schreiber & Gilpin, 2018; Zuniga & Ryabinin, 2020).

The DRN contains approximately one third of all serotonergic neurons in the brain (Huang et al., 2019). The brain's serotonergic system plays a crucial role in controlling reinforcement. 5-HT is known to be involved in the regulation of emotional response to reward (Ren et al., 2018; Liu et al., 2020).

The VTA is a part of the mesocorticolimbic pathway, regulating reward and addiction behaviors through mediating dopamine release (Cai et al., 2022).

1.3.2. Possible role of TRPA1 ion channel in alcohol addiction

Komatsu and colleagues (2012) found that ethanol activates TRPA1, as measured by calcium imaging in human embryonic kidney-derived 293 (HEK293) cells expressing human TRPA1.
Alcohol is metabolized by alcohol dehydrogenase into the reactive and toxic intermediate product acetaldehyde, which is rapidly converted into acetic acid (Cederbaum et al., 2012). Acetaldehyde is considered as the major contributor of the detrimental effects by acute and chronic alcohol consumption including flushing, headache, cirrhosis, and cancer (Eriksson et al., 2001).
Bang and colleagues (2007) found in pain models that human and mouse TRPA1 are activated by acetaldehyde, in the HEK293T cell heterologous expression system and in cultured mouse trigeminal neurons. Acetaldehyde failed to activate other temperature sensitive transient receptor potential ion channels (TRP) expressed in sensory neurons.

TRPA1 antagonists, camphor, gadolinium, and a general TRP blocker ruthenium red inhibited the TRPA1 activation by acetaldehyde. Another research group showed that Schwann cells express TRPA1 that orchestrates ethanol-evoked neuropathic pain in mice (De Logu et al. 2019). Most recently Landini (2023) found that acetaldehyde *via* CGRP receptor and TRPA1 in Schwann cells mediates ethanol-evoked periorbital mechanical allodynia in mice.
Wang's research group (2011) found that the final metabolite of alcohol, acetic acid also can activate the TRPA1 in trigeminal neurons based on patch clamp recordings and Ca^{2+} microfluorometry.
As we most recently showed that urocortinergic neurons in EWcp uniquely express significant amount of TRPA1 in the mouse CNS (Kormos et al. 2022) and because a) the CRH system, more particularly UCN1 is involved in acute and chronic alcohol consumption (Schreiber & Gilpin, 2018; Zuniga & Ryabinin, 2020), moreover, b) alcohol and all the above-described metabolites efficiently pass through the blood brain barrier, the question arises if they directly act on TRPA1 receptors in the EWcp. The activation of TRPA1 leads to calcium influx which triggers several intracellular pathways, which may contribute to the regulation of UCN1 and/or CART peptide release.
Based on this, we decided to examine the role of TRPA1 in the UCN1 and CART co-expressing EWcp neurons response to acute alcohol exposure in mice.

2. AIMS

2.1. Investigation of EWcp in migraine

We aimed to investigate the involvement of EWcp urocortinergic neurons in the neurobiology of migraine. We hypothesized that EWcp urocortinergic neurons may be involved in the regulation of migraine induction or in the endogenous response to migraine through direct neuroanatomical connection with migraine-related brain areas.

I. Neuroanatomical and qualitative morphological examination of migraine-related targets

To support our hypothesis on the involvement of EWcp urocortinergic neurons in the neurobiology of migraine, we aimed to investigate the expression of CGRP receptor components in mouse and human EWcp and DRN, as well as mouse STN. We also aimed to examine a possible urocortinergic projection from EWcp to *Crhr1* and *Crhr2* positive neurons in the STN.

II. Investigation of the EWcp in a nitroglycerine (NTG)-induced migraine mouse model

Our goal was here to assess the functional and morphological changes in the EWcp in the NTG-induced migraine model. In this study, we hypothesized that NTG may modulate the EWcp function by inducing a migraine-like state.

III. Investigation of the EWcp and its migraine-related projection areas in a CGRP-induced migraine model

In this project we aimed at investigating the functional-morphological changes in the urocotinergic EWcp and its in migraine-related projection areas (such as DRN and STN) in the CGRP-induced migraine-like state. Here, our expectation was that CGRP influences the function of EWcp, DRN and STN.

IV. Targeted ablation of the EWcp urocortinergic neurons with leptin-conjugated saporin in mice

To confirm the role of EWcp/UCN1 neurons in migraine, upon selective ablation of EWcp/UCN1 neurons we examined the migraine-related behaviors in response to CGRP treatment. Our hypothesis was that the selective ablation of EWcp/UCN1 neurons will influence the migraine-related behaviors induced by CGRP.

V. Mapping of human EW's functional connectivity by fMRI

To provide human data supporting our hypothesis that EW can influence the function of migraine-related areas through a direct anatomical connection, we aimed to examine the functional connectivity matrix of EW in control humans, with a special focus on the STN and DRN. Then, to compare it with interictal migraineurs' functional connectivity matrix. Finally, we also aimed to examine the association between migraine frequency and the functional connectivity of the EW. In this study, we anticipated that there is a positive functional connectivity between the EW nucleus and the STN as well as the DRN.

2.2. Examination of EWcp/TRPA1 in acute alcohol exposure model

In this project we aimed to investigate the involvement of EWcp/TRPA1 in a mouse model of acute alcohol exposure. We hypothesized that alcohol and its metabolites, may influence the function of EWcp urocortinergic neurons by activating TRPA1 ion channels.

I. Investigation of the functional activity of TRPA1 in the mouse EWcp

Because we did not have evidence for the occurrence of TRPA1 at protein level due to the lack of reliable antibodies, our first aim in this project was to provide evidence for the functional activity

of the TRPA1 in acute mouse EWcp slices by electrophysiology. Here we expected that TRPA1 in the EWcp is functionally active.

II. Examination of the expression of *TRPA1* mRNA in human EWcp

To support the translational relevance of our previous findings and due to the lack of literature data on the expression of TRPA1 ion channel in human EW, our objective was to examine the expression of *TRPA1* mRNA in human EWcp urocortinergic neurons. In this experiment we hypothesized that, like the mouse EWcp/UCN1 neurons, human EWcp/UCN1 neurons also expresses *TRPA1* mRNA.

III. Investigation of the *Trpa1*-expressing EWcp in a mouse model of acute alcohol exposure

Here, we aimed to test weather TRPA1 ion channels may contribute to the recruitment of EWcp urocortinergic neurons in response to acute alcohol exposure involving *Trpa1* KO mice. Here, our hypothesis was that alcohol may regulate UCN1 and/or CART peptide levels in the EWcp by modulating the function of TRPA1 ion channel.

3. MATERIAALS AND METHODS

3.1. Animal studies

3.1.1. Investigation of EWcp in migraine

3.1.1.1. Neuroanatomical and qualitative morphological examination of migraine-related targets

Naïve C57Bl6/J mice (n=6) were used to examine the expression of AMY1 and CGRP receptor components in the EWcp, DRN and STN as well as *Crhr1*, *Crhr2* mRNA in the STN.
Immunofluorescence targeting CLR and RNAscope *in situ* hybridization (ISH) targeting *Ramp1*, *Calcr* and *Crcp* mRNA was combined with a) UCN1 immunofluorescence in the EWcp to assess the co-localization with urocortinergic neurons; b) TPH2 or 5-HT immunostaining in the DRN as a marker of the serotonergic neurons; c) a neuronal nuclear marker (NeuN) immunofluorescence in the STN to visualize the dorsal horn neurons.
To examine the possible urocortinergic connection between the EWcp and STN, the mouse EWcp (n=6) was injected with an anterograde tracer, adeno-associated virus serotype 8 (AAV8) containing enhanced green fluorescent protein gene (EGFP) (AAV8 Syn EGFP). For the mouse STN, the dorsal horn of the C1 spinal segment (n=6) was injected by a retrograde tracer, cholera toxin subunit B (CTB). To validate the anatomical localization of the site of injections, CTB and green fluorescent protein (GFP) immunofluorescence was performed in the C1 segment of spinal cord and in the EWcp, respectively. For anterograde tracing, UCN1 and GFP double immunostaining was applied on C1 spinal cord sections. For the retrograde tracing, UCN1 and CTB double immunofluorescence was performed on EWcp slices.

Finally, we investigated the expression of the receptor targets of UCN1, *Crhr1* and *Crhr2* in the I-III laminae of the STN. For this purpose, RNAscope ISH targeting *Crhr1*, *Crhr2* and *NeuN* as a neuronal marker, was combined with UCN1 immunofluorescence in the STN. Our goal was here to identify urocortinergic afferentation innervating *Crhr1*- and *Crhr2*-positive neurons in the STN.

3.1.1.2. Investigation of the EWcp in NTG-induced migraine model

C57Bl6/J mice (n=6/group) were assigned into three experimental groups: NTG as treatment group, vehicle and saline as control groups. Mice were intraperitoneally injected by NTG (10

mg/kg) or by the vehicle (saline-based solution containing 6% ethanol and 16% propylene glycol) or by saline, respectively. Mice were euthanized 4 hours after the treatment for the morphological studies. Immunohistochemistry (IHC) for the acute neuronal marker (FOS) with diaminobenzidine (DAB) was performed in the EWcp slices.

3.1.1.3. Investigation of the EWcp and its migraine-related projection areas in a CGRP-induced migraine model

C57Bl6/J mice (n=11-15/group) were handled for two weeks and assigned into two experimental groups: saline as control and CGRP-treated groups. Upon i.p. injection of 0.1 mg/kg CGRP (α-CGRP mouse, rat (CRB), Cat. No.: crb1000889) or saline, light aversion was measured 30 minutes after the injection using light-dark box (LDB) test to assess photophobia associated with migraine-like state. Another cohort of mice (n=13) was used to assess periorbital hyperalgesia using von Frey filaments in the same model, 30 minutes after the treatment.
Because the EWcp is known to be sensitive to various stressors (Gaszner et al., 2004, 2012), we had to avoid the acute stress-related changes in the EWcp caused by LDB or von Frey tests. Therefore, we used an independent cohort of mice for the functional-morphological studies. Two experimental groups were created: saline (n=6) as a control and CGRP-treated (n=6) groups. After two weeks handling and habituation to i.p. injections, mice were treated with 0.1 mg/kg CGRP or saline, respectively. Mice were euthanized 4 hours after the treatment. Immunohistochemistry for the neuronal activation marker FOS was performed in the EWcp, lateral periaqueductal gray matter (lPAG) and laminae I-III of STN. Additionally, we performed immunofluorescence for the alternative neuronal activity marker phosphorylated cAMP-responsive element binding protein (P-CREB) (Priest et al., 2023) in the STN. Immunohistochemistry for FOS with DAB was performed in the EWcp, lPAG and STN. Whole mount FOS immunofluorescence was applied in the TRGs to assess the acute neuronal activation in response to CGRP treatment. UCN1 and FOS immunofluorescent staining was applied to prove that the activated neurons in the EWcp were urocortinergic. *Ucn1* RNAscope ISH was combined with UCN1 immunofluorescence to assess the mRNA's and peptide's density in the EWcp. 5-HT and TPH2 double staining was performed to assess 5-HT and TPH2 signal density in the DRN.

3.1.1.4. Targeted ablation of the EWcp urocortinergic neurons

We performed stereotactic surgery to induce selective UCN1 neuron ablation using leptin-conjugated saporin (ribosome-inactivating protein) in C57Bl6/J mice (n=13). Saporin is a neurotoxin that enters neurons only if it is conjugated to a substance that is internalized by receptor-mediated endocytosis and irreversibly inhibits the cells' protein synthesis (Wiley et al., 2000). Given that in the EWcp only UCN1 immunoreactive neurons express leptin receptor, leptin-conjugated saporin injection provides a reliable tool to perform selective UCN1 neuron ablation (Ujvári et al., 2022, Xu et al., 2022). Periorbital hyperalgesia in response to intraperitoneal injection of 0.1 mg/kg CGRP or saline were assessed before and 2 weeks after the surgery. EWcp/UCN1 positive cells were counted to evaluate the saporin-induced urocortinergic neuronal loss using DAB immunohistochemistry for UCN1.

3.1.2. Examination of EWcp/TRPA1 in acute alcohol exposure model

3.1.2.1. Examination of the functional activity of TRPA1 in the mouse EWcp

We performed patch clamp electrophysiological examinations to test the functional activity of the TRPA1 in acute mouse EWcp slices by electrophysiology, using JT010, a selective and potent TRPA1 agonist.

3.1.2.2. Investigation of the TRPA1-expressing EWcp in a mouse model of acute alcohol exposure

9-12 weeks-old male *Trpa1* KO mice and their WT counterparts were assigned to four experimental groups (n=6-8/group): *Trpa1* KO and WT mice received i.p. injection of 6% ethanol (D=1g/Kg), another set of *Trpa1* KO and WT mice received i.p. injection of equivalent volume of physiological saline as control. Mice were euthanized 2 hours after the treatment for the morphological studies, where we examined the FOS, UCN1 and CART peptide immunoreactivities, moreover *Trpa1*, *Ucn1* and *Cart* mRNA expression.

3.2. Human studies

3.2.1. Qualitative morphological studies

To examine the expression of *TRPA1* mRNA in the EWcp, AMY1 and CGRP receptor components in the EWcp and DRN, we used human EW and DRN samples from subjects who died suddenly from extracranial disease and did not show any brain neuropathologies. RNAscope ISH targeting *TRPA1* mRNA was combined with UCN1 immunofluorescence in the EWcp to assess the co-localization with urocortinergic neurons. Immunofluorescence targeting CLR and RNAscope ISH targeting *Ramp1*, *Calcr* and *Crcp* mRNA was combined with a) UCN1 immunofluorescence in the EWcp to assess the co-localization with urocortinergic neurons; b) TPH2 or 5-HT immunostaining in the DRN as a marker of the serotonergic neurons.

3.2.2. Mapping of human EW's functional connectivity profile

We analysed the functional connectivity matrix of EW in control humans with a special focus on the STN and DRN by fMRI. Then, we compared it with the functional connectivity matrix of interictal migraineurs. Finally, we examined the association between migraine frequency and the functional connectivity of EW.

4. RESULTS

4.1. Investigation of EWcp in migraine

4.1.1. Neuroanatomical and qualitative morphological examination of migraine-related targets

4.1.1.1. Expression of the CGRP receptor target components in the EWcp, DRN and STN

To prove that central CGRP may directly affect the urocortinergic EWcp neurons, we performed RNAscope ISH for *Calcr*, *Ramp1* and *Crcp* mRNA moreover immunofluorescence for CLR and UCN1. We proved that *Ramp1* and *Calcr* mRNAs coding for AMY1 components are co-expressed both in mouse and human EWcp neurons. Notably, the expression pattern of *Calcr* mRNA in the EWcp suggests a substantial co-localization with UCN1 immunoreactive neurons. Moreover, almost all EWcp/UCN1 neurons were found to contain CLR and *Crcp* both in mice and humans. In the DRN, RNAscope ISH in combination with immunofluorescence for *Calcr*, *Ramp1* and CLR as well as *Crcp* mRNAs were found to be expressed in TPH2-immunoreactive serotonergic and non-serotonergic neurons.

We detected *Calcr*, *Ramp1* and *Crcp* mRNAs in both neuronal and glial cells in the mouse STN laminae I-III. Additionally, in line with an earlier study, the expression of CGRP receptor component CLR was confirmed in both neurons (Miller et al., 2016) and astrocytes.

4.1.1.2. Urocortinergic afferentation of the *Crh1r* and *Crh2r*-expressing neurons in the mouse STN

Anterograde and retrograde tracing studies were performed to investigate a possible urocortinergic projection from the EWcp to the STN. The accurate anatomical position of the injection site was approved by CTB and GFP immunostaining on C1 spinal cord and EWcp sections, respectively. CTB and UCN1 double positive neurons were detected in EWcp after a CTB injection into the STN. Moreover, GFP and UCN1 double positive fibers were observed in the STN in samples of mice injected with AAV8-EGFP injection into the EWcp.
Next, we examined the expression of UCN1 receptor targets, *Crhr1* and *Crhr2*, in the I-III lamina of the STN and we tested whether they are approached by UCN1-immunoreactive fibers. RNAscope ISH for *Crh1r, Crh2r* and *NeuN* mRNAs was combined with UCN1 immunofluorescence. In laminae I-III of the STN, the neurons were seen to express both *Crhr1* and *Crhr2* mRNAs and they received urocortinergic fibers.

4.1.2. Investigation of the EWcp in a nitroglycerin-induced migraine mouse model

To examine the neuronal activity in the EWcp, we performed FOS immunohistochemistry with DAB. Both the vehicle and NTG treatment significantly increased the number of FOS positive neurons in the EWcp with a strong main effect of the treatment in one-way ANOVA, compared to saline. However, there was no significant difference between the vehicle- and NTG-treated groups.

4.1.3. Investigation of the EWcp and its migraine-related projection areas in a CGRP-induced migraine model

4.1.3.1. Model validation

To assess photophobia associated with migraine-like headache, we performed LDB test. CGRP-treated mice spent significantly increased ratio of time in the dark compartment compared to the controls.

Von Frey test was performed to assess periorbital pain associated with migraine-like headache. CGRP treatment significantly reduced the periorbital withdrawal threshold compared to the saline.

FOS immunofluorescent labelling was performed to assess the activation of the TRG neurons in the trigeminovascular nociceptive pathway. CGRP treatment resulted in significantly increased number of FOS positive neurons in the TRG, compared to the controls.

In the STN, although CGRP treatment did not affect the FOS positive neuron count, it increased the number of P-CREB in laminae I-III.

FOS immunohistochemistry was performed in the lPAG to assess the activation of the antinociceptive pathway in a migraine-like state. We found about two-fold increase in the number of activated lPAG nuclei in the CGRP-treated group, compared to the control.

4.1.3.2. Functional and morphological changes of the EWcp in the CGRP model

FOS immunohistochemistry was performed to assess the acute neuronal activity of the EWcp. CGRP treatment resulted in an approximately two-fold rise in the number of FOS positive neurons, compared to the control.

Using a double-label immunofluorescence, we found that most of the FOS-immunoreactive nuclei were localized to UCN1 neurons (91.79%) suggesting that the urocortinergic EWcp was activated.

To assess the effect of CGRP treatment on *Ucn1* mRNA expression and UCN1 peptide content in the EWcp neurons, RNAscope ISH was performed, combined with immunofluorescence. In

CGRP-treated animals, significantly higher *Ucn1* mRNA expression and UCN1 peptide immunosignal was found in the EWcp neurons, compared to the controls.

In order to assess how migraine-like state affects the number of *Trpa1* mRNA transcripts in the EWcp/UCN1 cells, we performed RNAscope ISH to detect *Trpa1* mRNA combined with UCN1 immunostaining. Semi-quantification of the staining showed that CGRP treatment increased nearly two-fold the number of *Trpa1* transcripts in UCN1 neurons.

4.1.3.3. Morphological changes of the DRN in the CGRP model

In the DRN, double immunostaining targeting 5-HT and TPH2 was performed. Both 5-HT and TPH2 content of the serotonergic neurons were significantly decreased in response to CGRP treatment in the DRN.

4.1.4. Targeted ablation of the EWcp urocortinergic neurons with leptin-conjugated saporin in mice

UCN1 immunohistochemistry was performed to confirm EWcp/UCN1 neuron loss. Leptin-conjugated saporin treatment significantly reduced the number of UCN1 immunoreactive neurons in the EWcp compared to the naïve mice. Von Frey test was performed to assess periorbital pain associated with migraine-like headache. Before the ablation of EWcp/UCN1 neurons CGRP treatment significantly reduced the periorbital withdrawal threshold compared to saline. Interestingly, after the ablation of EWcp/UCN1 neurons, the saline treatment significantly reduced periorbital withdrawal threshold compared to non-ablated controls, and CGRP treatment did not change it further.

4.2. Human fMRI study

4.2.1. Functional connectivity of the human Edinger-Westphal nucleus

Extensive positive functional connectivity of the EW nucleus with frontal and temporal gyri, cerebellum, caudate and midbrain were identified in the whole population. In accordance, significant positive functional connectivity was found between the EW and DRN, as well as STN in whole group analysis. No significant negative connections were detected.

4.2.2. Comparison of Edinger-Westphal nucleus functional connectivity between interictal migrainours and control groups

There was no significant difference between interictal migraine patients and healthy controls in the functional connectivity of EW nucleus after correction for multiple testing.

4.2.3. Association between migraine frequency and functional connectivity of Edinger-Westphal nucleus

We identified a positive correlation between migraine frequency and the functional connectivity of EW nucleus with two clusters. One cluster contained the angular gyrus and superior temporal gyrus, while the other cluster contained the middle frontal, and triangular part of inferior frontal gyri.

4.3. Examination of EWcp/TRPA1 in acute alcohol exposure model

4.3.1. Investigation of the functional activity of TRPA1 in mouse EWcp

As we aimed to examine the importance on TRPA1 in EWcp/UCN1 neurons, we first wanted to see whether our mRNA studies would be confirmed by a functional tool. This was important for

us, because the lack of reliable TRPA1 antibody precluded the opportunity to provide evidence at protein level.
To test the functional activity of TRPA1 ion channel in the urocortinergic neurons of EWcp, we performed patch clamp experiment in whole cell configuration on acute brain slices. All patched neurons were filled with biocytin and tested for UCN1 immunoreactivity *post hoc*. Only UCN1-immunoreactive cells were used in the statistical analysis. UCN1-immunoreactive neurons were tonically active at resting membrane potential as it was shown previously (Topilko et al., 2022). We used JT010, a potent and selective, covalently binding agonist (Takaya et al., 2015) to activate the TRPA1 in current clamp mode upon application of JT010. Resting membrane potential of recorded cells were adjusted *via* the amplifier to keep the cells just below the threshold for AP firing (few AP still occurred). This strategy prevented spontaneous firing, however even a moderate membrane potential depolarization -by the activation of TRPA1- resulted in high frequency firing. Firing frequency was significantly increased during JT010 application in UCN1-immunoreactive neurons. AP frequency was 0.14 ±0.07 Hz at baseline, 0.87 ± 0.17 Hz during drug application and 0.22 ± 0.07 Hz after washing out the drug (n=9/4 mice). Notably UCN1-immunonegative neurons in the EW region showed no change in firing frequency or in membrane potential upon the application of JT010.

4.3.2. Examination of *TRPA1* mRNA expression in the human EWcp

To confirm the expression of *TRPA1* mRNA in human EWcp urocortinergic neurons also, RNAscope ISH targeting *TRPA1* mRNA was combined with UCN1 immunofluorescence. UCN1 positive neurons in human EWcp were found to co-express *TRPA1* mRNA.

4.3.3. Investigation of the *Trpa1*-expressing EWcp in a mouse model of acute alcohol exposure

4.3.3.1. UAC measurement

To confirm the absorption of i.p. administered ethanol, the urine alcohol concentration (UAC) was measured using headspace gas chromatography. As expected, in the saline-treated groups no ethanol was detected in the urine. We detected similar ethanol concentration in the urine of both alcohol-treated WT and *Trpa1* KO animals.

4.3.3.2. FOS immunohistochemistry

FOS immunohistochemistry was performed to assess the acute neuronal activity in EWcp. Alcohol treatment significantly increased the number of FOS-positive neurons in the EWcp of both WT and *Trpa1* KO mice, compared to the respective controls without the main effect of genotype.

4.3.3.3. *Trpa1* RNAscope *in situ* hybridization

RNAscope ISH was performed to examine the effect of ethanol on the number of *Trpa1* mRNA transcripts in the EWcp/UCN1 neurons of WT animals. *Trpa1* mRNA showed a full co-localization with the UCN1 peptide immunosignal in the EWcp. Ethanol treatment significantly decreased the number of *Trpa1* transcripts compared to the control.

4.3.3.4. Dynamics of UCN1 mRNA and peptide upon alcohol treatment

To examine the *Ucn1* mRNA expression and UCN1 peptide content in the EWcp neurons in response to alcohol treatment, RNAscope ISH and immunofluorescence were performed, respectively. We detected a main effect of the genotype on *Ucn1* mRNA expression. In control

groups, no difference was detected, however a lower *Ucn1* mRNA expression was observed in KO animals upon alcohol treatment.
We also observed a strong effect of genotype × treatment interaction on UCN1 at peptide level. The basal UCN1 content of the EWcp neurons was significantly lower in *Trpa1* KO mice compared to WTs. Moreover, alcohol treatment differentially regulated the UCN1 peptide content in the EWcp. The UCN1 peptide content of EWcp was significantly decreased in WT mice and significantly increased in KO mice, in response to alcohol treatment.

4.3.3.5. Dynamics of CART mRNA and peptide upon alcohol treatment

To examine the *Cart* mRNA expression and CART peptide content in the EWcp neurons in response to alcohol treatment, we performed RNAscope ISH and immunofluorescence, respectively.
We observed a main effect of the genotype on *Cart* mRNA expression. A lower expression of *Cart* was observed in *Trpa1* KO mice regardless the treatment condition. There was no main effect of alcohol treatment nor that of genotype on CART peptide content.

5. DISCUSSION

5.1. Investigation of EWcp in migraine

In our preliminary pilot study, the NTG administration did not cause significant activation in the EWcp in comparison to the vehicle control. In the view that the vehicle injection elicited a strong neuronal activation in comparison to the saline control, we concluded that the ethanol content of the NTG preparation caused the activation and not the NTG effect proper. Indeed the EWcp was shown to be very sensitive to ethanol (Zuniga & Ryabinin, 2020; Al-Omari et al., 2023) and because all available NTG preparations contain high concentrations of alcohol as vehicle (Bates et al., 2010; Farkas et al., 2016; Kim et al., 2018), we decided to use the intraperitoneal injection of CGRP as a model of migraine according to Mason et al. (2017).

The central distribution of CGRP and that of its receptor has been described at protein level in rats, mice and humans (Ma et al., 2003; Edvinsson et al., 2020; Huang et al., 2021). However, CGRP receptors AMY1 and CGRP receptor components expression in EWcp has not been studied yet. Here we have confirmed the expression of *Ramp1*, *Calcr* and *Crcp* mRNA as well as CLR peptide in EWcp/UCN1 neurons using the highly sensitive and specific RNAscope ISH technique combined with immunofluorescence (Wang et al., 2012) in both mice and humans. This supports the translational relevance of our animal work and confirms findings of earlier mRNA studies (Ma et al., 2003; Edvinsson et al., 2020). Because AMY1 and CGRP receptor components were also detected in the migraine-related EWcp projection areas such as DRN and STN, we assume that the CGRP may activate the DRN and STN directly or indirectly *via* AMY1 and CGRP receptor-expressing EWcp/UCN1 neurons. Indeed, both our anterograde and retrograde tracing proved the direct projection from the EWcp to the STN. Since urocortinergic fibers were detected in the close proximity of STN neurons which expressed *Crh1r* and *Crh2r* mRNAs we provide neuroanatomical evidence for a direct urocortinergic EWcp projection to STN neurons with a possible functional significance.

Next, we investigated the response of EWcp in a migraine model. Several well-validated *in vivo* models were described, including direct electrical stimulation of trigeminal neurons, administration of inflammatory substances to the meninges and the use of algogenic substances (e.g. NTG, CGRP) (Harriott et al., 2019). Importantly, the CGRP signaling is involved in all these models (Wattiez et al., 2019). Electrical stimulation of TRG and meningeal administration of

inflammatory substances are invasive and considering the acute pain sensitivity of EWcp Kozicz et al., 2001; Rouwette et al., 2011) these models were not suitable. Intraperitoneal administration of NTG and CGRP successfully resembled many aspects of acute and chronic migraine (Kim et al., 2018; Harriott et al., 2019) in rodents that were alleviated by anti-migraine drugs like sumatriptan (Bates et al., 2010; Farkas et al., 2016; Mason et al., 2017; Rea et al., 2018; Wattiez et al., 2019). In our project, the use of NTG would have been unreliable because all available preparations contain ethanol as vehicle (Bates et al., 2010; Farkas et al., 2016; Kim et al., 2018) and the EWcp is highly alcohol sensitive (Zuniga & Ryabinin, 2020; Al-Omari et al., 2023). Therefore, we decided to use the intraperitoneal CGRP treatment model of migraine described by Mason et al. (2017). Several migraine-related symptoms have been observed upon central and peripheral CGRP administration in humans and rodents (Lassen et al., 2002.; Kaiser, 2014; Mason et al., 2017), suggesting both peripheral and central site of action, this is in line with the wide distribution of CGRP and AMY1 receptors in the CNS and periphery (Iyengar et al., 2017; Edvinsson et al., 2020). TRG neurons express CGRP and its receptor, TRG provide anatomical connection between peripheral and CNS. Activation of CGRP receptors in trigeminal neurons stimulate CGRP release from a) peripheral nerve endings of afferent terminals which innervate meninges, b) TRG cell bodies and c) terminals of central processes in the STN (Durham et al., 2016). CGRP release from central terminals promote central sensitization *via* CGRP receptors on second-order nociceptive STN cells sensitization (Durham et al., 2016; Iyengar et al., 2019), which may ultimately lead to further central CGRP release from CGRP-expressing brain areas. This is one mechanism for central sensitization mediated by peripheral action of CGRP, but several others may also contribute (Mason et al., 2017; Iyengar et al., 2017, 2019).

The CGRP model (Mason et al., 2017) was validated by the periorbital hyperalgesia and light aversion behavior. According to Mason et al, CGRP treatment did not induce anxiety in mice, thus, the LDB behaviour can be considered as photophobia, referring to a migraine-like state. Further, CGRP-induced light aversion did not correlate with the blood pressure excluding vasomotor mechanisms (Mason et al., 2020).

The elevated TRG/FOS and STN/P-CREB immunosignals upon CGRP treatment refers to the activation of these key players in migraine pathogenesis (Iyengar et al., 2017; Edvinsson et al., 2020). Increased lPAG/FOS immunosignal in CGRP-treated mice suggests the activation of the descending antinociceptive pathways further reinforcing that a migraine-like headache has occurred.

The neuromodulatory role of PAG in migraine is well known (Napadow et al., 2019; Vila-Pueyo et al., 2019) but no studies have reported the contribution of EWcp to migraine, even though it is located within the PAG. The two-fold increase in the number of FOS positive EWcp/UCN1 neurons upon CGRP treatment suggests increased neuronal activation that requires adaptation at the level of gene expression (Al-Omari et al., 2023; Gaszner et al., 2012). This was concomitant with increased *Ucn1* mRNA and UCN1 peptide content in response to CGRP administration suggesting higher UCN1 peptide release. We propose that peripherally administered CGRP may activate the EWcp/UCN1 neurons directly by centrally released CGRP or by indirect manner as a consequence of central sensitization. The presumably increased UCN1 release may directly influence the function of the STN *via* CRH1R and CRH2Rs, as supported by increased P-CREB immunosignal in the STN. In line with earlier studies (Bhatt et al., 2014, 2015), no FOS activation was observed in the STN upon CGRP treatment.

As the *Trpa1* mRNA is expressed at the highest level in the EWcp in mouse brain (Kormos et al., 2022), moreover, literature has linked TRPA1 to migraine (Souza Monteiro de Araujo et al., 2020; Shibata et al., 2021; Iannone et al., 2022a, 2022b; Spekker et al., 2022; Fila et al., 2023; Masood et al., 2023), we aimed to examine the *Trpa1* mRNA expression of EWcp in our migraine model. Using RNAscope ISH we showed that urocorinergic neurons in the EWcp of the CGRP-treated

group exhibited significantly higher number of *Trpa1* mRNA transcripts compared to the control. We assume, that the upregulation of *Trpa1*, a cation channel may lead to higher influx of calcium, hence causing an increase in the release of UCN1. This expectation is supported by our findings suggesting increased urocortinergic activity, although this awaits experimental confirmation.

Beyond the STN, the DRN is also a brain area known to be activated in migraine (Shibata et al., 2022). The EWcp/UCN1 neurons innervate the DRN (Dos Santos Júnior et al., 2015; van der Doelen et al., 2017; Zuniga & Ryabinin et, 2020), where both CRH receptors are expressed (Chalmers et al., 1995; Ma et al., 2003; Valentino et al., 2010; van der Doelen et al., 2017). Activation of CRH1R in DRN reduces 5-HT release, whereas CRH2R signaling has opposite effect (Lukkes et al., 2011; Fox & Lowry, 2013). The decreased DRN/5-HT content in our CGRP-treated mice suggests 5-HT release upon CGRP administration. Supporting this, elevated CNS 5-HT levels were found during migraine attacks attack (Drummond & Drummond, 2006; Sakai et al., 2008; Deen et al., 2018; Razeghi et al., 2019). We propose that CGRP increased the UCN1 release in the DRN that *via* CRH2Rs of DRN/5-HT neurons ultimately elevated 5-HT release. Nevertheless, we cannot exclude a direct CGRP effect on serotonergic neurons as they also express CGRP receptors (Ma et al., 2003).

CGRP treatment decreased the TPH2 enzyme level in DRN. TPH2 is the rate-limiting enzyme of central 5-HT synthesis (Walther et al., 2003) and its gene polymorphisms have been linked to migraine (Marziniak et al., 2009; Jung et al., 2010). Chronic central UCN1 microinjection elevated the *Tph2* mRNA expression in the caudal and dorsal subdivision of the DRN, while an opposite effect was observed in the ventrolateral part (Donner et al., 2020). This finding is in contrast with our results as we saw a rise in UCN1 levels but decreased the TPH2 protein content upon CGRP treatment. This discrepancy may be explained by methodological differences as in our study we examined the TPH2 at protein level. On the other hand, we applied a single CGRP treatment, which elevated the UCN1 level, while Donner et al (2021) applied chronic UCN1 administration. Thirdly, a DRN subdivision-specific difference in CRH2R expression, moreover a stress exposure-dependent change in the CRH1R and CRH2R receptor trafficking and consequent ligand availability in the plasma membrane (Waselus et al., 2009) may explain the difference in the outcome of these studies. All in all, the complex action of UCN1 on DRN serotonergic neurons may reflect the proclaimed abnormalities in central 5-HT turnover in migraine (Drummond & Drummond, 2006; Razeghi et al., 2019).

Given that the CART and EWcp/UCN1 show full co-localization (Zuniga & Ryabinin, 2020; Priest et al., 2023), decreased periorbital withdrawal threshold following EWcp/UCN1 ablation aligns with earlier study result where increased the activity of EWcp/CART resulted in increased paw withdrawal threshold, suggesting that these neurons modulate pain (Priest et al., 2023).

The ability of EWcp to influence migraine-related areas through direct neuroanatomical connection was further supported by the significant positive functional connectivity between EW, DRN and STN. To the best of our knowledge this is the first study to describe the functional connectivity of EW in the human brain. Moreover, our fMRI study revealed a strong positive correlation between the frequency of migraine attacks and the functional connectivity of EW with brain areas that are known to be part of the affective pain pathway including angular gyrus, superior temporal gyrus, middle frontal and the triangular part of inferior frontal gyri (Jia & Yu, 2017; Ong et al., 2019; Wang et al., 2019), supporting previous findings that changes in these brain areas may predispose a person to pain conditions including migraine (Jia & Yu, 2017).

5.2. Examination of EWcp/TRPA1 in acute alcohol exposure model

Our research group previously proved the presence of the *Trpa1* mRNA in mouse and human UCN1-immunoreactive neurons in the EWcp (Kormos et al., 2022). Here, we used an

electrophysiological tool to prove the existence of the functionally active TRPA1 channel in the EWcp.

Early studies suggested that pharmacological blockade of TRPA1 channel can be protective in granule cell degeneration (Koch et al., 2011) however this study did not prove the presence of the channel by histological experiments. Recently, we have shown using that *Trpa1* transcript is present in urocortinergic neurons of the EWcp. Here, we suggest that TRPA1 is functionally active in these neurons. UCN1-immunoreactive neurons are spontaneously active and fire APs at resting membrane potential. Our hypothesis was that activation of TRPA1 in these neurons will result in Ca^{2+} influx and subsequent membrane potential depolarization which in turn will increase the frequency of spontaneous firing. Indeed, application of JT010, a selective and potent TRPA1 agonist, significantly increased the spontaneous firing frequency of UCN1-immunoreactive neurons while it was ineffective in neighboring neurons lacking UCN1. To our knowledge, this is the first evidence suggesting the functional role of TRPA1 in neurons of the mouse brain.

In our model for acute alcohol exposure, the measurement of urine alcohol concentration proved the reliability of the model as the absorption of ethanol was identical in WT and *Trpa1* KO mice. Ethanol and all its metabolites efficiently pass through the blood-brain barrier (Nurmi et al., 1999; Quertemont & Tambour, 2004), and they were also shown to activate the TRPA1 *in vitro* (Bang et al., 2007; Wang et al., 2011; Komatsu et al., 2012) therefore, we propose that they may directly act on TRPA1 receptors in the EWcp. Increased FOS expression upon alcohol treatment further supports that alcohol could activate the EWcp urocortinergic neurons in WTs, which is consistent with the literature (Bachtell et al., 1999; Ryabinin et al., 2001; Weitemier et al., 2001; Zuniga & Ryabinin, 2020). Our present finding, that the FOS activation was observed in alcohol-treated *Trpa1* KO mice also, suggests that besides the TRPA1 other receptors/ion channels may contribute to the alcohol-induced activation of urocortinergic cells. In line with this assumption, Bachtell et al. (2002) proposed that the alcohol-induced FOS response in EWcp is a result of signaling *via* GABA-A receptors, modified by α2A/D-adrenoceptors and dopamine receptors (Bachtell et al. 2002). Another possibility is that the alcohol-induced FOS activation in the EWcp is at least in part orchestrated through a TRPA1-independent mechanism by another alcohol-responsive brain area that innervates to the urocortinergic cells of the EWcp (Ryabinin et al., 1997; da Silva et al., 2013).

Because of the lack of genotype effect in the FOS cell counts, our data do not suggest unequivocally the role of TRPA1 in the EWcp, the reduced *Trpa1* mRNA expression in WT mice upon ethanol treatment, provides a further support for this assumption. Indeed, it is well-known that the effect of an agonist may downregulate their target (Ann R Finch et al., 2009). The fact that the *Trpa1* transcripts were restricted to the cells with UCN1 signal in both groups, on one hand replicated our recent finding that exclusively urocortinergic cells of the EWcp express the *Trpa1* (Kormos et al. 2022) on the other hand this indicates that acute alcohol treatment does not induce the transcription of *Trpa1* mRNA in non-urocortinergic EWcp cells.

The UCN1 peptide content in EWcp differed between the two genotypes in saline-treated groups. The UCN1 content was much higher in WTs, compared to *Trpa1* KO mice. The comparison of the changes of UCN1 peptide content upon alcohol treatment revealed an opposite dynamics, with a decrease in WT mice, and an increase in *Trpa1* KO animals. This suggests that in WT mice the UCN1 is released from EWcp/UCN1 neurons in response to ethanol, while in *Trpa1* KO mice, increased UCN1 peptide content was observed suggesting the accumulation of the UCN1 peptide, possibly due to a reduced release. This was in part further supported by the RNAscope ISH, where we found lower *Ucn1* mRNA expression in the alcohol-treated *Trpa1* KO mice, compared to the WTs. This suggests that the peptide accumulation was associated with lower mRNA production due to the slower turnover (Gaszner et al., 2009).

Both control and alcohol-treated *Trpa1* KO animals showed lower *Cart* mRNA expression than the WT counterparts. Because the lower *Cart* mRNA expression is associated with reduced alcohol preference (Giardino et al., 2012), in our ongoing experiments we test if this is indeed

characteristic for *Trpa1* KO mice. Neither the *Cart* mRNA expression, nor the CART peptide content of EWcp/UCN1 neurons was altered by acute alcohol treatment in any genotypes, suggesting that acute alcohol exposure does not have a deep impact on EWcp/CART. Based on the known role CART in addiction (Vicentic and Jones, 2007, Zuniga & Ryabinin 2020; Zhi Yi Ong & Gavan P. McNally, 2020) we predict that, a chronic alcohol exposure model could prove its recruitment in alcohol abuse.

These above discussed observations together suggest that TRPA1 signaling may be involved in both the storage and release of UCN1 peptide from EWcp/UCN1 neurons. In our previous study we also found that the lack of functionally active TRPA1 affected the UCN1 content both in models of depression (Kormos et al., 2022) and posttraumatic stress disorder (Konkoly et al., submitted). In these models, we detected a genotype-related difference in the basal *Ucn1* mRNA (but not peptide) content in naïve control animals (Kormos et al., 2022, Konkoly et al., 2022). In contrast, in the present study saline-injected controls did not show a genotype difference in *Ucn1* mRNA expression but the peptide did. The discrepancy may be explained by the high acute stress sensitivity of the nucleus (Gaszner et al., 2004. Kormos et al., 2016) and by the stress effect of the ip. injection procedure.

The activation of TRPA1 in the membrane leads to calcium influx, triggering several intracellular pathways (Song et al., 2019). Indeed, our electrophysiological experiments showed that activation of TRPA1 increased the excitability and the rate of spontaneous firing of UCN1-expressing neurons. The increased intracellular calcium may cause exocytosis of the neuropeptide containing vesicles. Further experiments using pharmacological tools and electrophysiological recordings are required to the determine how exactly TRPA1 signaling contributes to the content and release of the UCN1 peptide. Considering the fact that lack of TRPA1 affected only UCN1 but not CART content of EWcp neurons regardless their co-localization (Kozicz, 2003; Priest et al., 2023, Li and Ryabinin 2022), which we also confirmed here, it will be important to investigate the mechanism of UCN1-specific regulatory role of the TRPA1 channel.

6. SUMMARY OF THE NEW FINDINGS

- We proved the expression of CGRP receptor targets in the EWcp and DRN in mice and humans.
- We identified a direct urocortinergic projection arising from the EWcp to the STN.
- We demonstrated the expression of UCN1 receptors (CRH1R and CRH2R) in the I-III laminae of the STN dorsal horn.
- We showed behavioral and morphological evidences to support the validity of the CGRP mouse model of migraine.
- We proved the activation of the EWcp/UCN1 expressing neurons in the mouse CGRP model of migraine, with increased UCN1 peptide content and *Ucn1*, as well as *Trpa1* mRNA expression in the EWcp, moreover, decreased 5-HT and TPH2 content in the DRN.
- We showed that the selective ablation of the EWcp/UCN1 neurons reduces the periorbital pain threshold.
- We provided evidence on a positive functional connectivity between the EWcp and the STN as well as the DRN in humans by fMRI.
- We proved the functional activity of TRPA1 ion channel in the mouse EWcp/UCN1 neurons by electrophysiological tools.
- We demonstrated the expression of *TRPA1* mRNA in human EWcp/UCN1 neurons.
- We showed the activation of EWcp/UCN1 expressing neurons in a mouse model of acute alcohol exposure with decreased *Trpa1* mRNA expression and UCN1 peptide content.

7. CONCLUSION

As a conclusion, our findings strongly suggest a regulatory role of EWcp/UCN1 neurons in migraine with high translational value and the involvement of TRPA1 ion channels in the regulation of UCN1 storage and release, which could influence the alcohol consumption.

8. PUBLICATIONS

The thesis is based on the following publications:

Al-Omari A, Kecskés M, Gaszner B, Biró-Sütő T, Fazekas B, Berta G, Kuzma M, Pintér E, Kormos V. Functionally active TRPA1 ion channel is downregulated in peptidergic neurons of the Edinger-Westphal nucleus upon acute alcohol exposure. **Frontiers in Cell and Developmental Biology** 2023 Jan 10;10:1046559. doi: 10.3389/fcell.2022.1046559. PMID: 36704197; PMCID: PMC9872022. **IF: 4.6; Q1**

Al-Omari A, Gaszner B, Zelena D, Gecse K, Berta G, Biró-Sütő T, Szocsics P, Maglóczky Z, Gombás P, Pintér E, Juhász G, Kormos V. Neuroanatomical evidence and a mouse calcitonin gene-related peptide model in line with human functional magnetic resonance imaging data support the involvement of peptidergic Edinger-Westphal nucleus in migraine. **Pain**. 2024 Jun 14. doi: 10.1097/j.pain.0000000000003294. Epub ahead of print. PMID: 38875125. **IF: 5.9; Q1; D1**

Other publications of the author:

Konkoly J, Kormos V, Gaszner B, Sándor Z, Kecskés A, **Al-Omari A**, Szilágyi A, Szilágyi B, Zelena D, Pintér E. The Role of TRPA1 Channels in the Central Processing of Odours Contributing to the Behavioral Responses of Mice. **Pharmaceuticals** (Basel). 2021 Dec 20;14(12):1336. doi: 10.3390/ph14121336. PMID: 34959735; PMCID: PMC8703823. **IF: 5,21; Q1**

Kormos V, Kecskés A, Farkas J, Gaszner T, Csernus V, **Al-Omari A**, Hegedüs D, Renner É, Palkovits M, Zelena D, Helyes Z, Pintér E, Gaszner B. Peptidergic neurons of the Edinger-Westphal nucleus express TRPA1 ion channel that is downregulated both upon chronic variable mild stress in male mice and in humans who died by suicide. **Journal of Psychiatry and Neuroscience** 2022 May 4;47(3):E162-E175. doi: 10.1503/jpn.210187. PMID: 35508327; PMCID: PMC9074809. **IF: 4.3; Q1**

Orján EM, Kormányos ES, Fűr GM, Dombi Á, Bálint ER, Balla Z, Balog BA, Dágó Á, Totonji A, Bátai ZI, Jurányi EP, Ditrói T, **Al-Omari A**, Pozsgai G, Kormos V, Nagy P, Pintér E, Rakonczay Z Jr, Kiss L. The anti-inflammatory effect of dimethyl trisulfide in experimental acute pancreatitis. **Scientific Reports** 2023 Oct 5;13(1):16813. doi: 10.1038/s41598-023-43692-9. PMID: 37798377; PMCID: PMC10556037. **IF: 3.8; Q1; D1**

Kormos V, Kriszta G, **Al-Omari A**, Kovács-Rozmer K, Konkoly J, Pozsgai, Pintér E. TRP Channels as Therapeutic Targets. Chapter: TRP channels as potential target molecules for pharmacotherapy of neurological diseases. Elsevier, ISBN: 9780443186530 (accepted, in press)

Cumulative IF: 23.81
MTMT ID: 10082296

9. ACKNOWLEDGEMENTS

First and foremost, I express my deepest gratitude to my supervisor, Dr. Gasznerné Dr. Kormos Viktória, whose expertise, patience, mentorship and limitless support have been indispensable. Her insightful feedback and constructive criticism have shaped my academic progress. I am fortunate to have had the opportunity to work under her guidance.

I am grateful to Prof. Dr. Erika Pintér, Head of the Doctoral School and Head of our Institute, for her scientific advice and support throughout my studies. I extend my deepest appreciation to Dr. Balázs Gaszner for his exceptional contributions and profound insights that have greatly enriched every facet of this project. This work would have not been possible without their contribution.

I extend my sincere appreciation to my thesis committee members, for their insightful feedback, constructive criticism.

I would like to thank Tünde Biró-Sütő for her substantial dedication and assistance in the laboratory work, Dóra Zelena for performing the stereotaxic surgery, Miklós Kecskés for the electrophysiological experiments, Zsófia Maglóczky, Péter Gombás and Péter Szocsics for providing us with the human brain samples, Gabriella Juhász and Kinga Gecse for performing the fMRI study, Gergely Berta for helping with the confocal microscopy, Mónika Kuzma for conducting the urine alcohol concentration measurement and Balázs Fazekas for his TDK work. I am grateful for the staff and resources at the University of Pécs, Medical School, Department of Pharmacology and Pharmacotherapy who have facilitated a conducive research environment.

I would like to express my heartfelt gratitude and appreciation to my family for their unwavering love, steadfast support, and enduring encouragement, illuminating even the darkest moments though out this journey.

Finally, I acknowledge the countless individuals, friends, and colleagues who have supported me through any acts of kindness. Your contributions, no matter how small, have not gone unnoticed and are deeply appreciated.

This thesis is a testament to the collective efforts of all those mentioned above, and I am profoundly grateful for the role each one of you.