Modulation of noxious transmission with calcitonin gene-related peptide receptor antagonists in the thalamus

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Calcitonin gene-related peptide receptor antagonists are effective acute migraine treatments without the vascular contraindications associated with triptans. While it has been demonstrated that calcitonin gene-related peptide receptor antagonists act in the central nervous system, their effects in preclinical migraine models have been investigated in only the trigeminocervical complex. Migraine is a complex neurological disorder; sites in the brainstem and forebrain are clearly involved in its expression. We have performed electrophysiological recordings in thalamic neurons of rats responding to noxious trigeminovascular inputs and tested the effect of olcegepant, a calcitonin gene-related peptide receptor antagonist (1 mg/kg, intravenously), on cell firing. We further tested the effect of microiontophoresed calcitonin gene-related peptide and the receptor antagonists calcitonin gene-related peptide 8-37 and olcegepant on thalamic cell firing, elicited by stimulation of the superior sagittal sinus or by microiontophoretic application of L-glutamate. Additionally, we used immunofluorescent staining to demonstrate the presence of functional calcitonin gene-related peptide receptors in the ventroposteromedial thalamic nucleus by specifically co-staining for the calcitonin gene-related peptide receptor subunits calcitonin receptor-like receptor and receptor activity modifying protein 1. Intravenously administered olcegepant significantly inhibited cell firing evoked by stimulation of the superior sagittal sinus as well as the background activity. Microiontophoresis of calcitonin gene-related peptide 8-37 also showed a significant inhibition of L-glutamate-evoked cell firing and firing evoked by stimulation of the superior sagittal sinus. Immunofluorescent staining confirmed the presence of the components of a functional calcitonin gene-related peptide receptor, the calcitonin receptor-like receptor and the receptor activity modifying protein 1, within the area of the ventroposteromedial thalamic nucleus. This is the first report on the efficacy of calcitonin gene-related peptide receptor antagonists at the level of third-order neurons in the migraine pathway, showing that the central effects of calcitonin gene-related peptide receptor antagonists extend beyond the trigeminocervical complex at least to the sensory thalamus.

Keywords: migraine; calcitonin gene-related peptide; thalamus; cluster headache; trigeminovascular

Abbreviations: CALCRL = calcitonin receptor-like receptor; CGRP = calcitonin gene-related peptide; RAMP1 = receptor activity modifying protein 1
Introduction

Migraine, as defined by the criteria of the International Headache Society (Headache Classification Committee of The International Headache Society, 2004), is a disabling brain disorder (Menken et al., 2000; Goadsby et al., 2002) with a high prevalence (Lipton et al., 2001). After the early 1990s, when triptans became available as migraine-specific treatments (Ferrari et al., 2001), there had been no further developments until the demonstration of the efficacy of a calcitonin gene-related peptide (CGRP) receptor antagonist in acute migraine (Olesen et al., 2004). This new class of treatments offers the opportunity to explore the sites of action of acute anti-migraine treatments with new tools in order to understand better the pathophysiology of migraine.

The demonstration that CGRP was elevated in the extracerebral circulation of migraineurs during attacks (Goadsby et al., 1990) and that these elevated levels were reduced by triptan treatment (Goadsby and Edvinsson, 1993) suggested direct blockade of the effect of CGRP as a strategy to develop new treatments. CGRP release has been identified in many structures and pathways involved in migraine pathophysiology (Wimalawansa, 1996). It was shown to be present in trigeminal ganglion neurons innervating meningeal nerves (O’Connor and van der Kooy, 1988) and also in second-order neurons in the trigeminocephalic complex (Uddman et al., 1985). Since the effects of activation of CGRP receptors include meningeal vasodilation (Williamson et al., 1997; Akerman et al., 2002), as well as modulatory central actions (Storer et al., 2004; Fischer et al., 2005), brain effects of CGRP receptor antagonists are possible. Moreover, recent work underlines the importance of the central effect of CGRP at the level of the trigeminal nucleus (Sixt et al., 2009). The ventroposteromedial nucleus within the thalamus is the last relay before trigeminovascular input reaches the cortical level and thalamic activation certainly occurs during migraine (Afriadi et al., 2005).

Third-order neurons arising within the sensory thalamus have been successfully studied in terms of responses to nociceptive trigeminovascular inputs (Goadsby and Zagtami, 1990; Zagtami and Lambert, 1990, 1991) and to a lesser extent to characterize their pharmacology (Shields and Goadsby, 2005, 2006; Andreou et al., 2010). Here, we have sought to explore the involvement of the ventroposteromedial nucleus in the pathways involved in migraine, specifically to determine whether CGRP receptor antagonists can inhibit third-order neuronal firing, elicited by nociceptive stimulation of the superior sagittal sinus (Ray and Wolff, 1940). We used in vivo electrophysiological recordings and measured the effect of the microiontophoresed CGRP and the CGRP receptor antagonists, CGRP 8-37 and olcegepant (BIBN4096BS), on both electrical stimulation of the superior sagittal sinus and L-glutamate-evoked firing in the ventroposteromedial nucleus of the thalamus, as well as the effect of intravenous application of olcegepant on superior sagittal sinus-evoked firing in the ventroposteromedial nucleus. The studies identify third-order thalamic neurons as a possible site of action for CGRP receptor antagonists in migraine.

Materials and methods

All experiments were conducted under license of the University of California, San Francisco Institutional Animal Care and Use Committee and conforming to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

For electrophysiological experiments, male Sprague-Dawley rats (290–390 g) were anaesthetized with sodium pentobarbital (60 mg/kg i.p.). The left femoral artery and vein were cannulated for blood pressure monitoring (CT-1000 + ALM 932, CWE Inc., Ardmore, PA, USA) and further administration of anaesthetics (pentobarbital 25–30 mg/kg/h); the right femoral vein was cannulated for drug administration. Furthermore, the animals were cannulated with a tracheal tube for ventilation with oxygen enriched air, 2–3 ml, 80–100 strokes/min (small rodent ventilator—Model 683, Harvard Instruments, Kent, UK). End tidal CO₂ was monitored (Capstar-100, CWE Inc., Ardmore, PA, USA) and kept between 3.5% and 4.5%. Temperature was kept in the physiological range via a thermostatically controlled homeothermic blanket system. Animals were fixed in a controlled homeothermic blanket system. Animals were fixed in a

Superior sagittal sinus stimulation and recording in the ventroposteromedial thalamic nucleus

Two platinum wire electrodes were placed directly on the superior sagittal sinus for electrical stimulation. The area was covered with mineral oil for insulation as well as to avert dehydration of the tissue. The seven barrelled microiontophoresis combination electrodes (Carbostar-7S, Kation Scientific, Minneapolis, MN, USA) with an incorporated carbon fibre (impedances at 1 kHz: 0.4–0.8 MΩ, tip sizes ranging between 20 and 25 μm) were lowered into the thalamus using a piezoelectric motor/controller system (IW-811, Burleigh Instruments, Harpenden, UK; 8200 Controller, EXFO, Plano, TX, USA) in 5 μm steps. The signal was amplified, filtered and discriminated as previously described (Shields and Goadsby, 2005). Cells within the ventroposteromedial were selected for further recordings if they matched the following criteria: (i) response to receptive field stimulation within the V1/V2 branches of the trigeminal nerve; (ii) stable baselines of increased firing rate in response to
microiontophoresetic L-glutamate ejections; and (iii) stable baselines of increased firing rate in response to electrical stimulation of the superior sagittal sinus. The stimulation parameters were: 0.5 Hz, 100–150 μs and 10–30 V (Grass Instruments, Tujunga, CA, USA). A mean firing rate of 40% was required for the cluster of responses following electrical stimulation (Nagler et al., 1973; Armitage and Berry, 1994). The clusters had a duration of 7–10 ms with a latency of 8–10 ms.

Responses were displayed as a post-stimulus histogram of 50 repetitive stimulations. The post-stimulus histograms were collected with 1 ms bin sizes over a post-stimulus period of 100 ms. The action potentials of neurons recorded in response to microiontophoresis of L-glutamate were collected in successive 1 s bins and analysed as cumulative rate histograms. Based on the previous work (Storer et al., 2004; Shields and Goadsby, 2005, 2006), and to ensure reliability of the responses, baseline data were selected according to the study conditions. To study the effect of iontophoresically applied drugs on superior sagittal sinus stimulation, three baseline periods were collected, while to study the effect of microiontophoresed substances on L-glutamate-evoked firing, five baseline periods were collected. For studying the effect of intravenous olcegepant, four baselines, each of 50 sweeps, were evaluated prior to administration of the drug/vehicle. Further post-stimulus histograms were collected at 5, 10, 15, 20, 25, 30, 45, 60, 75 and 90 min post-administration.

**Drugs**

Microiontophoresis barrels of the combination electrode were filled with 200 mM L-glutamate monosodium, (Sigma, St Louis, MO, USA), pH 8.0; 1 mM CGRP (Sigma, St Louis, MO, USA), pH 4.0–5.5; 1 mM CGRP 8-37 (Tocris, Ellisville, MO, USA), pH 4.5–5.5; 20 mM olcegepant (Boehringer Ingelheim GmbH, Biberach, Germany), pH 5–5.5; distilled water titrated with HCl to pH 5–5.5 as a control; 2.5% pontamine skye blue (Gurr 6BX, BDH Laboratory Supplies, Poole, UK) in 100 mM sodium acetate and 200 mM NaCl for current balance (Bloom, 1974). L-Glutamate and pontamine skye blue were ionized as anions whilst CGRP, CGRP 8-37 and olcegepant were ionized as cations. Hydrogen cations were microiontophoresed as a control.

For intravenous application, olcegepant was dissolved in 0.9% NaCl solution (1 mg/ml) and administered at the dose of 1 mg/kg. An equal volume of saline was used in the control group.

**Microiontophoresis**

All ions were retained in the barrels by application of holding currents between 5 and 7 nA, with a polarity opposite to their charge (Stone, 1985). Ejection currents of the same charge as the molecule’s charge were used for ejection of CGRP, CGRP 8-37 and olcegepant, and ranged from 60 to 80 nA. The negative ejection currents for L-glutamate microiontophoresis ranged from 30 to 80 nA. The ejection current for L-glutamate for each individual cell was established by titration so a post-stimulus histogram of 50 repetitive stimulations. The post-stimulus histograms were collected with 1 ms bin sizes over a post-stimulus period of 100 ms. The action potentials of neurons recorded in response to microiontophoresis of L-glutamate were collected in successive 1 s bins and analysed as cumulative rate histograms. Based on the previous work (Storer et al., 2004; Shields and Goadsby, 2005, 2006), and to ensure reliability of the responses, baseline data were selected according to the study conditions. To study the effect of iontophoresically applied drugs on superior sagittal sinus stimulation, three baseline periods were collected, while to study the effect of microiontophoresed substances on L-glutamate-evoked firing, five baseline periods were collected. For studying the effect of intravenous olcegepant, four baselines, each of 50 sweeps, were evaluated prior to administration of the drug/vehicle. Further post-stimulus histograms were collected at 5, 10, 15, 20, 25, 30, 45, 60, 75 and 90 min post-administration.

For testing the effect of the microiontophoresed compounds on superior sagittal sinus stimulation, a baseline response out of three baseline post-stimulus histograms, separated by 5 min recovery intervals, was established. After a further 2 min, this was followed by 3 min of microiontophoresis (60–80 nA) of one of the drugs and a post-stimulus histogram at the end of the microiontophoresis episode after a further 5 min of recovery. After full recovery, this process was repeated with the other substances used. Resistances for the individual barrels ranged from 20 to 100 MΩ. In this protocol, we used three post-stimulus histograms to establish the baseline (see above), as the duration of the following procedures was much shorter than in the intravenous protocol. Upon recovery, after the ejection of each drug, a set of three baselines was recorded in order to validate the stability of the cells’ response.

Pontamine skye blue was ejected (4 μA, 10 min) at the end of the experiment for later localization of the recording sites, and for reconstruction of further recording sites in compliance with the microdrive readings. After termination of each experiment the brain tissue was collected and fixed in 10% formalin for histological processing.

**Immunohistochemistry**

Male Sprague-Dawley rats (n = 3), weighing 300–400 g, were euthanized with sodium pentobarbital i.p. (100 mg/kg) and transcardially perfused with 250 ml heparinized saline and with 500 ml of 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), followed by post-fixation of the brain in the same fixative at room temperature overnight, then cryoprotected at 4°C in a 30% sucrose solution and left until saturated. Serial coronal sections (40 μm thick) were then cut (Leica CM3050 S, Leica Microsystems Inc., Bannockburn, IL, USA) through the full rostro-caudal extent of the thalamus and collected in a free-floating state in 0.1 M phosphate buffered saline (pH 7.4). Since CGRP receptors are heterodimers of calcitonin receptor-like receptor (CALCRL) and receptor activity modifying protein 1 (RAMP1), it was necessary to double stain sections for both components with antibodies specific to CALCRL and RAMP1 (a generous gift from Merck and Co Inc., Whitehouse Station, NJ, USA). A standard wash buffer was used for washing sections as well as for diluting sera and antibodies (super sensitive wash buffer, BioGenex, San Ramon, CA, USA). For the first staining, sections were incubated for 1 h at room temperature in blocking solution containing 10% normal donkey serum (Sigma, San Louis, MO, USA) then incubated overnight at 4°C with the primary antibody specific to RAMP1 (1:100 goat anti-Ramp1 raised against QSKRTEGIV in the C-terminus of humans). This was followed by three washes of 5 min each and then a 2-h incubation with secondary antibody at room temperature (1:800 donkey anti-rabbit, conjugated with Alexa Fluor 488). For the second staining, sections were washed three times for 5 min, incubated in blocking solution containing 10% normal goat serum (Sigma, San Louis, MO, USA) then incubated overnight at 4°C with the primary antibody specific to CALCRL (1:50 rabbit anti-CALCRL, raised against SIQDIENVALKPEKMYDLV in the C-terminus of rats). This was followed by three 5 min washes and a 2-h incubation with secondary antibody at room temperature (1:800 goat anti-rabbit, conjugated with Alexa Fluor 568). Negative control samples were obtained by omitting the primary antibodies and positive controls by staining the trigeminal nucleus caudalis, which is known to contain CGRP receptors (Skofitsch and Jacobowitz, 1985). Sections were mounted and coverslipped, using a mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA) for nuclei visualization. Fluorescent staining was visualized using a Zeiss Axioplan Universal microscope (Zeiss, Jena, Germany).
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Statistical analysis

For the analysis of the effect of microiontophoresed CGRP, CGRP 8-37 and olcegepant on L-glutamate-evoked firing, baselines were calculated as a mean of five successive epochs pre-drug ejection to avoid naturally occurring variances in the response of cells to the individual L-glutamate ejection. The reliability of baselines was tested using Cronbach’s $\alpha$. For each individual agent, a mean response was calculated out of 10 successive pulses after the onset of each drug ejection. The mean firing rate of spontaneous activity over 150 s was calculated and compared with the mean spontaneous firing during and after the microiontophoresis of each drug. The resulting data were further processed using SPSS (17.0, IL, USA). For the comparison of the resulting data of background activity as well as L-glutamate-evoked firing, an ANOVA for repeated measurements was performed applying Greenhouse–Geisser corrections if the assumption of sphericity was violated. Bonferroni correction was applied for multiple comparisons. Differences found significant with the ANOVA were further explored with post hoc comparisons. Within-group comparisons were carried out using paired sample t-tests, and independent t-tests were used for between-group comparisons. Statistical significance was set at $P<0.05$. The effect size $r$ was calculated using Pearson’s correlation coefficient. The same statistical methods were applied for the evaluation of the effect of microiontophoresis of CGRP, CGRP 8-37 and olcegepant on superior sagittal sinus stimulation as well as the intravenous effects of olcegepant and control on firing rates in the ventroposteromedial.

Results

In the current study, a total of 29 cells in 20 animals in the ventroposteromedial nucleus of the thalamus were recorded (Fig. 1A and B). The cells were found within the shell region of the ventroposteromedial nucleus, adjacent to the ventroposterolateral nucleus. All cells studied matched the selection criteria as defined in the ‘Materials and methods’ section.

Electrophysiology

Glutamate responses and microiontophoresis

A total of 12 cells in seven animals were studied. All baseline responses were highly reliable with a Cronbach’s $\alpha$ value $\geq 0.98$ and no difference was seen across the responses $[F(4,148)=1.830, P=1]$. Microiontophoresis (60–80 nA) of CGRP $(n=10)$, olcegepant $(n=9)$ and control $(n=6)$ had no effect on L-glutamate-evoked firing $(P \geq 0.25)$. Ejection of CGRP 8-37 $(n=12)$ for a duration of 3 min inhibited L-glutamate-evoked cell firing by $14 \pm 4\%$ $(t_{11}=-2.173, P<0.05, r=0.48)$ compared with the control effect (Fig. 2A and B). Although microiontophoresis of CGRP had no effect on L-glutamate-evoked firing, CGRP did increase the background activity $(t_{13,1}=-2.904, P<0.05, r=0.62)$ by $25 \pm 8\%$. All other tested substances had no significant effect on background activity of the cells investigated $(P \geq 0.27)$.

Superior sagittal sinus stimulation-evoked firing and microiontophoresis

A total of 13 cells (seven animals) were studied. As with L-glutamate-evoked cell firing, the ejection of CGRP $(n=10)$, olcegepant $(n=11)$ and control $(n=10)$ had no effect on superior sagittal sinus stimulation-evoked neuronal firing $(P \geq 0.27)$. Ejection of CGRP 8-37 $(n=13; 60–80\text{nA})$ inhibited superior sagittal sinus stimulation-induced firing in the ventroposteromedial nucleus by $14 \pm 4\%$ $(t_{12}=-2.397, P<0.05, r=0.46)$ compared with the effect of control (Fig. 3A–C).

Superior sagittal sinus stimulation and intravenous application of olcegepant

For the purpose of testing the effect of olcegepant or control on superior sagittal sinus stimulation-evoked firing of third-order neurons in the thalamus, a total of 16 cells in 13 animals were tested. Injection of vehicle control $(n=8)$ had no significant effect on superior sagittal sinus stimulation-evoked firing $(P=0.71)$. Intravenous injection of olcegepant $(n=9)$ significantly inhibited the cell firing in response to superior sagittal sinus stimulation with a maximum effect of $27 \pm 8\%$ at 45 min after application [$F(8,64)=3.059, P<0.05$; Fig. 4A]. The independent t-test showed significant results at 45 min $(t_{15}=2.214, P<0.05, r=0.50)$ and 60 min $(t_{15}=2.299, P<0.05, r=0.51)$ after injection, when compared with control. Responses to superior sagittal sinus stimulation recovered to baseline values within 1.5–2 h. While control $(n=7)$ did not significantly affect background firing $(P=0.54$, mean firing rate $41 \pm 8\text{Hz}$) recorded in the ventroposteromedial nucleus, administration of olcegepant $(n=8)$ significantly inhibited the background activity $[F(7,49)=2.916, P<0.05]$ after 45 min $(t_{15}=2.216, P<0.05, r=0.52)$ and 60 min $(t_{15}=2.512, P<0.05, r=0.57)$, when compared with the control. The maximal inhibition of $27 \pm 8\%$ was recorded 45 min post-administration (Fig. 4B). No significant background activity was found during recording in one animal.

Immunohistochemistry

The qualitative analysis of the stained sections revealed CGRP receptors throughout the ventroposteromedial and ventroposterolateral nucleus of the thalamus, including the coordinates at which the recordings were made (Fig. 1A and B), demonstrated by the presence of neurons double stained for CALCRL and RAMP1 (Fig. 1C–E). These positively stained neurons were homogeneously distributed in the ventroposteromedial and ventroposterolateral nuclei, and ranged in size from 14 to 24 $\mu\text{m}$. Stained neurons were round or pear-shaped and the proximal processes were also strongly stained. It is interesting to note that not every neuron in these thalamic regions of interest contains CGRP receptors, as seen by the presence of many cells stained only with DAPI, but not with CALCRL or RAMP1.

Discussion

This is the first study to confirm the inhibitory effect of CGRP receptor antagonists at the level of third-order thalamic neurons likely to play a role in migraine. Intravenous administration of olcegepant caused a substantial decrease in spontaneous cell firing in the ventroposteromedial nucleus of the thalamus and a comparable decrease in cell firing evoked by electrical stimulation of the superior sagittal sinus. Microiontophoresis of the CGRP
receptor antagonist, CGRP 8-37, caused a significant decrease in the responses to both microiontophoresed L-glutamate and electrical stimulation of the superior sagittal sinus. Although microiontophoresis of CGRP did not modify the neuronal firing after superior sagittal sinus stimulation or L-glutamate microiontophoresis, it did facilitate the spontaneous cell firing. Using immunohistochemistry, functional CGRP receptors, identified by co-staining for the RAMP1 and the CALCRL subunits, are present in the area where the electrophysiological recordings were performed. These results add to the observations of CNS effects of CGRP receptor antagonists at the level of the trigeminocervical complex (Storer et al., 2004; Fischer et al., 2005; Sixt et al., 2009) and are consistent with the localization of CGRP receptors in the thalamus (Skofitsch and Jacobowitz, 1985; Inagaki et al., 1986; Wimalawansa and el-Kholy, 1993). Our histological observations, however, are in contrast to a previous study in which RAMP1 was not detected in the ventroposteromedial region (Oliver et al., 2001). However, we have demonstrated the presence of CGRP receptors histologically and electrophysiologically. The data from microiontophoresis should be considered alongside the finding that CGRP binding sites are found in higher densities in human thalamic areas than in rats (Wimalawansa and el-Kholy, 1993), which may have important translational implications for understanding the effect of CGRP receptor antagonists in migraine.

This study demonstrates that CGRP receptor antagonists can act at sites within the CNS beyond the second-order neurons in the trigeminocervical complex (TCC), where their effects have been previously investigated (Storer et al., 2004; Fischer et al., 2005;
When evaluating the effect of the two different CGRP receptor antagonists used in the study by microiontophoresis, it must be considered that olcegepant is designed to act on human CGRP receptors, resulting in ~200-fold lower affinity for rat CGRP receptors (Doods et al., 2000). When used in humans and tested against the peptidergic human CGRP 8-37, the non-peptidergic CGRP antagonist olcegepant was shown to have a more distinct effect on CGRP receptors (Doods et al., 2000). Mallee and colleagues (2002) demonstrated that the species-specific binding capacity of olcegepant to CGRP receptors is determined by the species-specific RAMP1 and not the CALCRL subunits, whereas CGRP 8-37 does not show species specificity (Longmore et al., 1994). The determination of the species specificity by RAMP1 is explained by the inter-species heterogeneity of the protein sequence for RAMP1 and CALCRL where humans and rats share 71% of the RAMP1 and 91% of the CALCRL sequence. For the non-peptidergic CGRP receptor antagonists, olcegepant and telcagepant, the methionine-42 on CALCRL and the tryptophan-74 on RAMP1 subunits have been identified as binding sites (Miller et al., 2010). However, it was also demonstrated that the dissociation constant for human 125I-CGRP is similar between the CGRP receptors of humans and rat (Mallee et al., 2002), an important finding underlining the relevance of studies investigating the distribution of CGRP receptors in different species prior to the findings about species-specific binding sites. Previous work demonstrates that the amount of CGRP receptors found in the ventroposteromedial nucleus is lower than what is described for the trigeminocervical complex (Skofitsch and Jacobowitz, 1985; Wimalawansa and el-Kholy, 1993). Combining these data with the data on species specificity of CGRP receptor antagonists may explain why microiontophoretic application of olcegepant onto rat ventroposteromedial neurons did not show an effect on trigeminovascular neuronal activity when compared with its action on trigeminocervical complex neurons in cat. However, the lack of a substantial effect of intrathalamic microiontophoresed CGRP on superior sagittal sinus stimulation-evoked firing is consistent with previous findings in the trigeminocervical complex (Storer et al., 2004). Nevertheless, we have demonstrated a facilitatory effect of CGRP on the spontaneous cell firing in the ventroposteromedial nucleus. The degree of third-order neuronal firing inhibition, induced by intravenous application of olcegepant, parallels the extent of inhibition observed when second-order trigeminal neurons were investigated in rat (Fischer et al., 2005). These data imply that in the rat model, the third-order response is due largely to changes to second-order transmission. As discussed for the microiontophoretic application of the compound, it must be considered that the CGRP binding site density within the human thalamus is much higher than in rats (Wimalawansa and el-Kholy, 1993). Added to that, the higher affinity of olcegepant to human RAMP1 is likely to facilitate local thalamic effects. Any conclusion concerning the importance of CGRP receptor mediated effects within a certain brain area should not be drawn solely based on its density in that region. However, the equal presence of CGRP binding sites within the human brainstem and thalamus (Wimalawansa and el-Kholy, 1993), together with the known involvement of thalamicortical changes in migraine (Coppola et al., 2005), does at least warrant consideration of the thalamus

Sixt et al., 2009). The new data demonstrate that CGRP antagonists can act on third-order neurons within the ventroposteromedial nucleus. This action probably occurs at post-synaptic sites, as cell firing was attenuated when evoked by both electrical stimulation of the superior sagittal sinus and L-glutamate. The attenuation of L-glutamate-evoked firing is likely to be due to a post-synaptic effect. Sixt and colleagues (2009) have highlighted that the amount of olcegepant needed in clinical trials was unexpectedly high (Olesen et al., 2004) in relation to its potency, perhaps due to poor blood–brain barrier permeability. Nevertheless their study did show that if sufficient doses of the drug are used, it is clearly acting in the CNS. The convincing results from CGRP receptor antagonist in clinical trials (Olesen et al., 2004; Ho et al., 2008; Connor et al., 2009) may therefore involve more than one CNS site, and may clearly include an action in the thalamus. By investigating the thalamus, a new level of drug action is described which is extremely interesting because the thalamus is the final gateway before nociceptive trigeminal inputs reach the cortex. Moreover, given the potential role of CGRP receptors in sensitization (Zhang et al., 2007), and the demonstration of allodynia outside the trigeminal territory (Burstein et al., 2000), it can reasonably be suggested that CGRP receptor antagonists may be effective in patients with allodynia, even when it has progressed out of the trigeminal distribution.

Figure 2 Effect of microiontophoresis of CGRP 8-37 and control on thalamic neuronal firing elicited by repetitive microiontophoresis of L-glutamate. (A) Summary of changes by microiontophoresis of CGRP 8-37 (60–80 nA) versus control (H+) at the same current. (B) An original example where the neuronal firing is recorded as rate histograms. Solid bars represent the ejection of L-glutamate and CGRP 8-37. Microiontophoresis of CGRP 8-37 inhibits cell firing responses due to L-glutamate. The firing rate recovered to baseline values after ~10 min. *P<0.05 significance compared with the baseline response.
among the targets for therapeutics. Based on these data and combined with the microiontophoretic data, an exclusively TCC-driven effect of CGRP receptor antagonists seems unlikely.

**Technical considerations**

For experimental use in animals, there are good data to support the dose of olcegepant used in these experiments (Fischer et al., 2005), and since the drug has already entered into clinical studies, we did not aim to do a dose response but to use the compound as a tool to explore CGRP-related mechanisms. A critical aspect of in vivo studies investigating thalamic areas is the type of anaesthesia chosen. Barbiturates are able to increase chloride influx to neurons by binding to modulation sites on GABA_A receptors. Nevertheless most clinically used anaesthetics show this potential interaction with the GABA_A receptor and therefore influence susceptibility of neuronal firing. We used pentobarbitone because it has not shown anti-nocioceptive effects (Vaught et al., 1985; Reyes-Vazquez et al., 1986) and has been used successfully to study effects of anti-migraine medicines in the thalamus (Shields and Goadsby, 2005, 2006; Andreou et al., 2010). The technique of microiontophoresis was chosen for the investigation of third-order neurons in the thalamus because it offers direct insight into the local effects of drugs in the area studied, and combines electrophysiological recording of single cell activity with the local application of compounds. The problem of local volume effects is negligible. A limitation of the technique is that due to different diffusion rates, which are influenced by a wide range of non-constant factors, it is nearly impossible to estimate the exact amount of a compound microiontophoresed (Bloom, 1974).
This makes comparisons across compounds complex (Stone, 1985). In spite of its limitations, microiontophoresis is still one of the most exact tools for investigating local neuropharmacology under in vivo conditions. We used immunohistochemical techniques to demonstrate the presence of CGRP receptors within the ventroposteromedial nucleus to complement the pharmacological work. However, as a limitation, it should be noted that staining appears most densely in the cell bodies, with some extension to the proximal processes, especially in the case of CALCRL. The conclusion of a predominant location of CGRP receptors on the cell bodies may limit their role in the thalamus given that axo-somatic synapses are less frequent than axo-dentritic ones, at least in rat (Zhang and Yang, 1999).

**Conclusion**

The data demonstrate, for the first time, inhibition of third-order neurons in the ventroposteromedial thalamus activated by nociceptive trigeminovascular stimuli by local microiontophoresis of CGRP receptor antagonists. This study demonstrates a further plausible site of action in the brain for this novel new class of anti-migraine treatments. Moreover, the data emphasize the importance of considering, exploring and understanding the complex CNS mechanisms that contribute to the expression and treatment of migraine.

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