PAC₁ receptor blockade reduces central nociceptive activity: new approach for primary headache?

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Abstract

Pituitary adenylate cyclase activating polypeptide-38 (PACAP38) may play an important role in primary headaches. Preclinical evidence suggests that PACAP38 modulates trigeminal nociceptive activity mainly through PAC₁ receptors while clinical studies report that plasma concentrations of PACAP38 are elevated in spontaneous attacks of cluster headache and migraine and normalize after treatment with sumatriptan. Intravenous infusion of PACAP38 induces migraine-like attacks in migraineurs and cluster-like attacks in cluster headache patients. A rodent-specific PAC₁ receptor antibody Ab181 was developed, and its effect on nociceptive neuronal activity in the trigeminocervical complex was investigated in vivo in an electrophysiological model relevant to primary headaches. Ab181 is potent and selective at the rat PAC₁ receptor and provides near-maximum target coverage at 10 mg/kg for more than 48 hours. Without affecting spontaneous neuronal activity, Ab181 effectively inhibits stimulus-evoked activity in the trigeminocervical complex. Immunohistochemical analysis revealed its binding in the trigeminal ganglion and sphenopalatine ganglion but not within the central nervous system suggesting a peripheral site of action. The pharmacological approach using a specific PAC₁ receptor antibody could provide a novel mechanism with a potential clinical efficacy in the treatment of primary headaches.

Keywords: Pituitary adenylate cyclase activating peptide, Sumatriptan, Headache, Migraine, Cluster headache, Trigeminal activation, Monoclonal antibody, PAC₁ receptor

1. Introduction

Migraine and cluster headache are highly disabling disorders[50] involving activation of the trigeminovascular system, which includes the perivascular meningeal afferents and the trigeminal ganglion in the peripheral nervous system, and the trigeminocervical complex (TCC) as well as higher brain structures including the hypothalamus, thalamus, and periaqueductal gray in the central nervous system (CNS).[18,33]

Pituitary adenylate cyclase activating polypeptide-38 (PACAP38) has received increasing attention in the context of migraine and cluster headache. PACAP38 is a 38-amino acid neuropeptide that is structurally and functionally related to vasoactive intestinal peptide (VIP).[26,39] Both neuropeptides act on the same set of receptors, namely PAC₁, VPAC₁, and VPAC₂ receptors, which belong to the G-protein-coupled receptors of the secretin family.[26] PACAP38[43] and VIP[22] have vasodilatory properties and play a major role in parasympathetic communication.[7,26,35] In cluster headache, both neuropeptides are released during a spontaneous attack.[15,49] In migraine, despite some shared biology, both molecules have important differences. During a spontaneous migraine attack, PACAP38 is released into the cranial circulation regardless of the presence of autonomic symptoms[48,52] while VIP is only released if cranial autonomic symptoms accompany the attack.[17] The infusion of PACAP38,[2,25,43] but less reliably VIP,[42] induces migraine-like attacks, which can be effectively treated with sumatriptan. Sumatriptan normalizes elevated levels of PACAP38 during migraine.[52] These findings strikingly resemble the preclinical and clinical observations made with calcitonin gene-related peptide (CGRP),[14,16,17,36,54] which plays a prominent role in the pathophysiology of migraine and cluster headache and has been proven to be a validated target in the treatment of both disorders.[13,18,23,29,38,44]

A large body of preclinical evidence has supported and begun to dissect the mechanisms behind these clinical observations. The fact that PACAP38 and VIP share similar affinities to the VPAC₁ and VPAC₂ receptors but that the PAC₁ receptor has a 100- to 1000-fold higher affinity to PACAP38[9] has led to the conclusion that the relevant action of PACAP38 is likely to be mediated mainly through the PAC₁ receptor.[52] These findings are supported by in vivo studies that demonstrate the release of

PACAP38 into the cranial circulation upon peripheral trigeminal activation[52,53] and the facilitation of nociceptive neuronal transmission in the TCC that is reversible by the administration of a PAC_1 receptor antagonist.[1]

These observations suggest that targeting the PAC_1 receptor may offer an effective and highly selective approach to reduce trigeminal activation and offer a novel target for the treatment of primary headaches. We therefore developed a potent and selective monoclonal mouse anti-rat PAC_1 antibody (Ab181), fully characterized the pharmacologic and pharmacokinetic properties of the agent, and studied its effect on nociceptive neuronal activity within the TCC in an in vivo model that has been proven to be highly predictive for clinical efficacy in primary headaches.[19–21,47] Preliminary results have been presented at the fifth European Headache and Migraine Trust International Congress[31] and the International Headache Congress.[32]

2. Methods

The generation and in vitro characterization of Ab181, the pharmacodynamic and pharmacokinetic analyses as well as the immunohistochemistry were conducted between 2009 and 2013 in the Amgen laboratories, Thousand Oaks, CA. The electrophysiological studies were conducted between 2012 and 2013 in the laboratory of the Headache Group at the Department of Neurology, University of California, San Francisco, CA.

2.1. The generation of the Ab181

Mice anti-rat PAC_1-specific monoclonal antibodies were generated using a conventional immunization method. Five 4- to 6-week-old hybrid 129xC57BL/6 mice (Charles River Laboratories, Hollister, CA) received 3 rounds of immunizations with soluble rat PAC_1, chemically conjugated to Padre Peptides (Pan DR Helper T-cell epitopes). Mice were immunized SQ/IP with up to 50 μg of antigen-mixed or emulsified in either Freund's complete adjuvant (Cat# 77140; Pierce, Thermo Fisher Scientific, Waltham, MA), RIBI (Cat# S6322; Sigma-Aldrich, St. Louis, MO), or Poly I:C/CpG. Soluble PAC_1 receptor polypeptides containing the N-terminal extracellular domains of rat PAC_1 (amino acids 1-135 of GenBank accession no. NM133511.1) were generated by transiently cotransfecting 293-6E cells. All mice were maintained according to the regulations of the Amgen Institutional Animal Care and Use Committees in Thousand Oaks, CA.

Mice with the highest detected FACS titer to rat PAC_1 expressed on CHO AMID cells were selected for fusion. Four days before spleen harvest, mice selected for fusion were given a final IP boost of 50-μg soluble rat PAC_1 in phosphate-buffered saline (PBS) (Cat# 14040; GIBCO, Thermo Fisher Scientific, Waltham, MA). B-cell hybridomas were obtained by fusing immune splenocytes with nonsecreting murine myeloma cells, Sp2/0-Ag14 (American Type Culture Collection), at a ratio of 2.5:1 by electrofusion. Ab181 is identified through screening assays including binding competition, functional blocking, and receptor selectivity against the rat PAC_1 receptor.

2.2. In vitro characterization of Ab181

Potency and selectivity of Ab181 were analyzed in vitro cell-based functional assay.

2.2.1. Cell culture

In house developed stable $rPAC_1$/CHO cells were grown in Ham's F12 nutrient mixture (Cat# 11765; GIBCO, Thermo Fisher Scientific), 10% fetal bovine serum (Cat# 10099; GIBCO, Thermo Fisher Scientific), 1X Penicillin–Streptomycin–Glutamine (Cat# 10378; GIBCO, Thermo Fisher Scientific), 400-μg/mL G418 (Cat# 10131; GIBCO, Thermo Fisher Scientific), and 250-μg/mL Zeocin (Cat# R250-01; Invitrogen, Thermo Fisher Scientific). All cell flasks were maintained in incubators at 37°C with 5% CO_2. U2OS (Cat# HTB-96; ATCC, Manassas, VA) cells were grown in the medium of McCoy's 5A (Cat# 16600; GIBCO, Thermo Fisher Scientific) containing 10% fetal bovine serum (Cat# 10099; GIBCO, Thermo Fisher Scientific), 1X L-glutamine (Cat# 25030; GIBCO, Thermo Fisher Scientific), 1X Penicillin–Streptomycin–Glutamine, and 1X MEM nonessential amino acids (Cat# 11140, GIBCO; Thermo Fisher Scientific). All cell flasks were maintained in incubator at 37°C with 5% CO_2.

2.2.2. BacMam virus construct

The $rVPAC_1$ and $rVPAC_2$ BacMam virus constructs were prepared in house. The titer of $rVPAC_1$ BacMam virus is 7.01×10^8 IU/mL, and the titer of $rVPAC_1$ BacMam virus is 5.75×10^8 IU/mL.

2.2.3. Preparation of BacMam virus transduced $rVPAC_1$ and $rVAPC_2$ cells

U2OS cells were cultured in T-75 flask until the cell density reaches to 70% to 80% confluent before transduction. Cell medium was removed from flask, and the cells were rinsed with 1X PBS (Cat# 14040; GIBCO, Thermo Fisher Scientific) once, and then Versene (Cat# 15040; GIBCO, Thermo Fisher Scientific) was added to detach the cells. 3×10^6 U2OS cells were resuspended with culture medium and mixed with $rVPAC_1$ or $rVPAC_2$ BacMam virus at a concentration of 50 multiplicity of infection/cell. The cell mixture was further incubated overnight for assay.

2.2.4. Cell-based functional assay

The cAMP assay was performed by using LANCE cAMP ultra assay kit (Cat # TRF0263; PerkinElmer, Inc, Waltham, MA) to determine the activity of Ab181. Assay buffer contain Ham's F12 nutrient mixture (Cat# 11765; GIBCO, Thermo Fisher Scientific), 0.1% bovine serum albumin (Cat# CR84-100; PerkinElmer, Inc, Waltham, MA), and 1 mM IBMX (Cat# I5879, Sigma-Aldrich, St. Louis, MO). Agonist dose–response curve was first run to determine the appropriate concentration to be used in subsequent antagonism studies (data not shown) (**Table 1**).

The antagonist activity of Ab181 was performed by using EC_{80} of PACAP38 (Cat# H-8430; Bachem, Bubendorf, Switzerland) or maxadilan (Cat# H-6734; Bachem, Bubendorf, Switzerland). PACAP6-38 (Cat# H-2734; Bachem), a PAC_1 receptor antagonist, was used as a positive control. Ab181 (0.5 pM-1 μM) was preincubated with the rPAC1-CHO cell suspension (2000 cells/well) at room temperature for 30 minutes before the addition of agonists. PACAP38 or maxadilan was then added and incubated with the mixture for additional 15 minutes at room temperature. The reaction was stopped by adding detection mix of Eu-cAMP tracer and ULight-anti-cAMP (Cat# TRF0263; PerkinElmer, Inc) to all wells. After a 45-minute incubation at room temperature, the assay plate was read on an EnVision instrument (Cat# 2105-0100; PerkinElmer, Inc).

Same assay method was used to determine the activity of Ab181 on $rVPAC_1$ and $rVPAC_2$ receptors. The activity of VIP (Cat#: H-3775; Bachem), a $rVPAC_1$ and $rVPAC_2$ receptor agonist, was first evaluated, and the EC_{80} concentration of VIP was used in the assay to determine the activity of Ab181 on these receptors.

Table 1

Antagonist activities (IC_{50}) of Ab181.

Agonist	rPAC$_1$		rVPAC$_1$	rVPAC$_2$
	PACAP38	Maxadilan	VIP	VIP
Ab181 (IC_{50}, nM)	20 ± 3.3 (n = 3)	4.5 ± 0.1 (n = 2)	>1000 (n = 2)	>1000 (n = 2)
PACAP6-38 (IC_{50}, nM)	266 ± 77 (n = 3)	2.9 ± 1.5 (n = 2)	430 ± 79 (n = 2)	38 ± 4 (n = 2)

The antagonist activity of Ab181 at the rat PAC$_1$ receptor (rPAC$_1$) was performed by measuring the potency of Ab181 in inhibiting EC_{80} of agonist PACAP38 or maxadilan-stimulated cAMP production. PACAP6-38, a PAC$_1$ receptor antagonist, was used as a positive control.
Similar assay was used to determine the activity of Ab181 at rat VPAC$_1$ (rVPAC$_1$) and rat VPAC$_2$ (rVPAC$_2$) receptors against the EC_{80} of agonist VIP.
IC_{50} values from these experiments (Table 1) are expressed as mean ± SD, and "n" represents the number of separate experiments.
VIP, vasoactive intestinal peptide.

All results were analyzed using GraphPad Prism software's nonlinear regression curve fit (GraphPad Software, Inc, La Jolla, CA), and data are presented as mean ± SD. Data from the agonist dose–response curves were used to calculate the half maximal effective concentration (EC_{50}) and the half maximal inhibitory concentration (IC_{50}) values for agonist and antagonist studies, respectively.

2.3. Pharmacokinetic and pharmacodynamic analysis

Male naïve Sprague–Dawley rats, 6 to 12 weeks from either Taconic Farms, Inc (Oxnard, CA) or Harlan Laboratories (Indianapolis, IN), at the average age of initiation of treatment were used, and all procedures in this report were conducted in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. Animals were group-housed in nonsterile, ventilated microisolator housing on corn cob bedding in Amgen's Assessment and Accreditation of Laboratory Animal Committee (AAALAC)-accredited facility with controlled temperature (70 ± 5° F), relative humidity (50 ± 20%), and 12-hour light/dark cycles (06:00-18:00). Animals had ad libitum access to pelleted feed (Harlan Teklad 2020X, Indianapolis, IN) and water (onsite-generated reverse osmosis) through automatic watering system.

2.3.1. Test and control materials

Ab181 was generated from Amgen and diluted to a series of concentrations in A5Su (10 mM sodium acetate, 9% sucrose, pH = 5.0). An isotype control was used as a dummy antibody in A5Su at a concentration of 5 mg/mL. Maxadilan (trifluoroacetate salt, Bachem) was used, and a dosing solution was freshly prepared daily by dissolving maxadilan in 1X Dulbecco's PBS (DPBS) (Sigma-Aldrich) at a final concentration of 0.5 μg/mL.

2.3.2. Laser Doppler imaging

A laser Doppler imager (Moor Instruments, Ltd, Wilmington, DE) was used to measure dermal blood flow (DBF) on the shaved skin of the rat abdomen.

After anesthetic with propofol on the test day, the rat's abdominal area was shaved, and each animal was placed in a supine position on a temperature-controlled circulating warm-water heating pad to help maintain a stable body temperature during the study. After a 10- to 15-minute stabilization period, a black rubber O-ring (0.925-cm inner diameter; O-Rings West, Seattle, WA) was placed on the rat abdomen without directly positioning it over a visible blood vessel. After placement of an O-ring on the selected area, a baseline (BL) DBF measurement was taken. After the BL scan, the maxadilan solution prepared fresh daily in 20-μL vehicle (DPBS) was injected intradermally at the center of the O-ring. The postmaxadilan DBF was measured either every 15 minutes over a 60-minute period or at specified time points such as 15 and 30 minutes. The O-ring serves as an area of interest in which the DBF will be analyzed within the O-ring. Ab181 was prepared in A5Su at different concentrations depending on the dose range and given in a single bolus intravenous injection. In this report, MIIBF was measured and expressed as % change from the baseline [100 × (individual postagonist flux − individual baseline flux)/individual baseline flux] or as % inhibition [(mean of % change from BL from vehicle-treated animals − individual % change from BL from drug-treated animals)/mean of % change from BL from vehicle-treated animals].

2.3.2.1. Dose–response study at 48 hours after Ab181 treatment

Ab181 was administered through penile vein or tail vein at various doses (0.1, 0.3, 1, and 10 mg/kg). Forty-eight hours later, rats received an intradermal maxadilan injection (10 ng in 20-μL DPBS) followed by postmaxadilan DBF scans every 15 minutes over a 60-minute period. After the final DBF scan, 3 rats that had been pretreated with 10 mg/kg of Ab181 were allowed to recover from the propofol anesthesia, returned to their home cages, and underwent a second DBF measurement at 168 hours after drug administration. Serum PK samples were taken through tail vein immediately before the first maxadilan challenge at 48 and 168 hours after Ab181 treatment. The post-MIIBF scan time point for the later studies was determined based on the postmaxadilan DBF response from 15 to 60 minutes.

2.3.2.2. Dose–response study at 3.25 hours after Ab181 treatment

Ab181 was administered through the penile vein at various doses (0.1, 0.3, 1, 3, and 10 mg/kg). Three hours later, rats received an intradermal maxadilan injection (10 ng of maxadilan in 20-μL DPBS) followed by a post-MIIBF scan 15 minutes later. Serum PK samples were taken through tail vein immediately after the DBF scan (ie, 3.25 hours after Ab181 treatment). The time point was chosen to confirm that the receptor occupancy of Ab181 had reached and stayed at the desired pharmacodynamic measures when performing the electrophysiological studies.

2.3.2.3. Time-course study of Ab181 at a dose of 10 mg/kg

Ab181 was dosed intravenously at 10 mg/kg through either rat tail vein or penile vein at various pretreatment times (0.5, 3, 6.25 hours) before maxadilan challenge. The full time-course study comprised several experiments, including the 48 and 168 hours after Ab181 treatment studies described above.

2.3.2.4. Statistical analysis of laser Doppler flow experiments

All DBF results were expressed as the mean ± SEM. A one-way analysis of variance followed by Dunnett's multiple comparison

test was used to assess the statistical significance of Ab181 effects relative to either vehicle or the control antibody. A $P < 0.05$ was used to determine significance between any 2 groups. ED_{50} values were calculated in GraphPad Prism after logarithmic transformation and nonlinear fit of data using a sigmoidal dose–response model with variable slope and with the top constrained to the resulting mean percent change in the corresponding control group (vehicle or control antibody-treated group) and bottom constrained to zero.

2.4. Electrophysiological recordings

All experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Experiments were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals, the ARRIVE guidelines, and the guidelines of the Committee for Research and Ethical Issues of the International Association of the Study of Pain.

2.4.1. General surgical preparation

Twenty-four male Sprague–Dawley rats (Charles River Laboratories) were used in the experiments. In each animal, only one experiment was conducted, and recordings were performed from one site. The animals were anesthetized by an induction with a single dose of pentobarbital (60 mg/kg intraperitoneally; Nembutal, Lundbeck, Deerfield, IL) followed by a continuous infusion of propofol (20-25 mg/kg/h intravenously; Propoflo, Abbott, Abbott Park, IL) for maintenance throughout the entire experiment. For the administration of the anesthetic and drugs, both femoral veins were cannulated. The left femoral artery was cannulated for the continuous monitoring of arterial blood pressure.

2.4.2. Physiological monitoring

Rats were placed on a self-regulating homeothermic blanket system with a rectal probe (Harvard Apparatus, Holliston, MA), and core body temperature was maintained at 37 $\pm$ 0.5℃. Arterial blood pressure was monitored from the femoral artery using a transducer (DTX Plus DT-XX; Becton Dickinson, Sandy, UT) connected to an amplifier (PM-1000; CWE, Ardmore, PA). Following a tracheostomy, animals were mechanically ventilated (3-5 mL/min, 75-90 strokes/min; 7025, Ugo Basile, Comerio, VA, Italy) with oxygen-enriched air, and end-expiratory CO_2 was kept between 3.5% and 4.5%. Data on arterial blood pressure and CO_2 concentration were continuously displayed and fed into a data acquisition system (Power 1401; Cambridge Electronic Design-CED, Hertfordshire, Cambridge, United Kingdom) and saved on a hard disk.

2.4.3. Recording preparation

The rat's heads were fixed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). A craniotomy was performed over the parietal cortex with a dental burr using constant irrigation to reduce heat production. With this procedure, the middle meningeal artery (MMA) was exposed without lesioning the dura mater. For electrical stimulation, a bipolar stimulating electrode (NE200; Rhodes Medical Instruments, Summerland, CA) was placed above the MMA touching the dura mater at either side of the blood vessel. The electrode was connected to a stimulus isolation unit (SIU5A; Grass Instruments, Quincy, MA).

For the extracellular recording of neuronal activity in the TCC, a C1 partial hemilaminectomy was performed, and the spinal dura mater was removed. A tungsten electrode with a nominal impedance of 1 MΩ (TM31A10; World Precision Instruments, Sarasota, FL) was then introduced in the TCC near the dorsal root entry zone. For the localization of optimal site for the extracellular recording, the electrode was advanced or retracted in 5-μm steps with a piezoelectric motor-driven micromanipulator. Wide dynamic range neurons with convergent input from the dura mater and the facial skin were identified by their responsiveness to electrical stimulation of the perivascular afferents surrounding the MMA as well as innocuous brush and noxious pinch of the skin innervated by the first branch of the trigeminal nerve.

2.4.4. Stimulation of meningeal afferents and recording in the trigeminocervical complex

Electrical stimulation of the perivascular meningeal afferents was performed applying electrical square wave pulses (10-18 V, 0.1-0.2 ms, 0.5 Hz, 20 sweeps) (S88; Grass Instruments).

The stimulus-evoked neuronal signal and the neuronal background activity were acquired by the recording electrode placed in the TCC. The electrical signal was fed into a headstage amplifier (NL100AK; Neurolog, Digitimer, Welwyn Garden City, Hertfordshire, United Kingdom) and passed to an AC preamplifier (NL104, Neurolog, Digitimer) set to a gain of 1000×. The signal was then passed through a band-pass filter (bandwidth 300 Hz-10 kHz) (NL125/126; Neurolog, Digitimer) and a 60-Hz noise eliminator (Humbug; Quest Scientific, Vancouver, BC, Canada) before further amplification by an AC-DC amplifier (NL106; Neurolog, Digitimer). This signal was fed to a gated amplitude discriminator (NL201; Neurolog, Digitimer) and a data acquisition system (Power 1401; Cambridge Electronic Design-CED). Data were collected, analyzed, and stored using Spike 5.2 software (Cambridge Electronic Design-CED). The output of the gated amplitude discriminator was also fed into an audio amplifier (NL120; Neurolog, Digitimer) and loudspeaker as well as an oscilloscope to assist spike discrimination from background activity. For the analysis of stimulus-evoked neuronal activity, poststimulus histograms were produced online. Background activity gated through the amplitude discriminator was collected into successive bins.

2.4.5. Experimental protocol and drug administration

After completing the surgical procedure, the animals had a resting period of 30 minutes. After this period, baseline recordings were obtained. These were obtained by calculating the mean of 3 series of poststimulus histograms, each consisting of 20 electrical stimuli.

After the assessment of the baseline values, Ab181 (10 mg/kg) or its vehicle (A5Su) were administered intravenously over 1 minute. Because of the pharmacological properties of the antibody, the animals underwent a second resting period of 2.5 hours to allow sufficient time for the antibody to bind its target. After this second resting, sumatriptan (10 mg/kg) or vehicle was administered intravenously over 1 minute followed by another resting period of 30 minutes. Poststimulus histograms were then established 180, 185, 190, 195, 200, 205, 210, and 225 minutes after the administration of the first pharmacological intervention (Ab181 or vehicle) (**Fig. 1**). Based on the treatments described above, animals were divided in 3 treatment groups, group 1 receiving the Ab181 (intervention 1) and sumatriptan vehicle (intervention 2), group 2 receiving vehicle (intervention 1) and
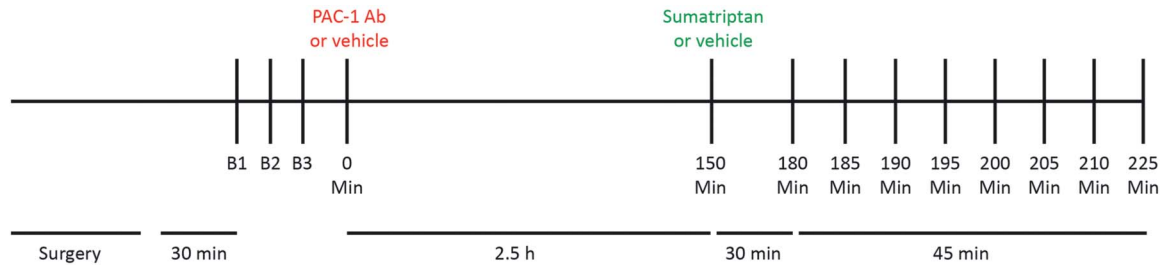
Figure 1. Timeline of the electrophysiological in vivo experiments. After the surgical procedure and the recording of baseline activity, either Ab181 (10 mg/kg) or vehicle (A5Su) was injected intravenously. After a resting period of 2.5 hours to allow for the distribution of the antibody throughout the circulation and the relevant tissues, sumatriptan or its vehicle was administered intravenously. Following another resting period of 30 minutes to allow sumatriptan to act, stimulus-evoked and spontaneous neuronal activity was recorded in the TCC. TCC, trigeminocervical complex.

sumatriptan (intervention 2), and group 3 receiving vehicle at both interventions (**Fig. 1**).

2.4.6. Statistical analysis

Statistical analysis was performed using SPSS 22.0 software (IBM Corporation, Armonk, NY). Data are expressed as percentages of baseline values with SEs of the mean (±SEM). Effects within a treatment group were calculated using the analysis of variance with repeated measures applying the Greenhouse–Geisser correction of the assumption of sphericity was violated. Bonferroni correction was applied for multiple comparisons. Statistical significance was assumed at $P < 0.05$. For a detailed comparison of individual data points with the baseline value within one treatment group, the dependent t test was used.

2.5. Immunohistochemistry

Adult male Sprague–Dawley rats (n = 3 per group) were injected intravenously with Ab181 or a monoclonal mouse isotype control antibody against an unrelated target or vehicle. After 3.5 hours, animals were terminally anesthetized by FatalPlus (Vortech Pharmaceuticals, Dearborn, MI) and perfused with ice-cold PBS at pH 7.4 (Cat# 14040; GIBCO, Thermo Fisher Scientific) followed by 4% paraformaldehyde (Cat# P6148; Sigma-Aldrich) in PBS (Cat# 14040; GIBCO, Thermo Fisher Scientific). The sphenopalatine ganglia, TG, and brains were dissected and cryoprotected with 30% sucrose in PBS. Twelve-micrometer sections were cut, washed in PBS, and incubated in 3% normal

goat serum and 0.3% TritonX-100 in PBS for 1 hour at room temperature. Sections were then washed in PBS and incubated with AlexaFluor488 goat anti-mouse IgG (Invitrogen, Thermo Fisher Scientific) for 1 hour at room temperature. After final washes in PBS (Cat# 14040; GIBCO, Thermo Fisher Scientific), sections were cover slipped with Vectashield mounting medium with DAPI (Cat# H-1200; Vector Labs, Burlingame, CA). The following additional controls were conducted: A set of sections from Ab181-dosed rats was incubated with secondary antibodies against rabbit (AlexaFluor488 goat anti-rabbit IgG; Invitrogen, Thermo Fisher Scientific) as negative control. As positive control for PAC$_1$ staining, a set of vehicle sections was incubated with 10-μg/mL Ab181 in blocking solution at 4°C for 48 hours before further processing with AlexaFluor488 goat anti-mouse IgG (Invitrogen, Thermo Fisher Scientific) as described above.

3. Results

3.1. In vitro potency of the PAC$_1$ receptor antibody Ab181

Ab181 is a full antagonist of the rat PAC$_1$ receptor. It dose-dependently inhibited PAC$_1$ receptor agonist PACAP38 or maxadilan, a PAC$_1$ selective receptor agonist, induced cAMP accumulation in CHO cells expressing rat PAC$_1$ receptors with IC$_{50}$ of 20 ± 3.3 nM (n = 3) and 4.5 ± 0.1 nM (n = 2), respectively (**Fig. 2** and **Table 1**). Ab181 is selective to the PAC$_1$ receptor. IC$_{50}$ at rat VPAC$_1$ and rat VPAC$_2$ receptors against the agonist VIP is greater than 100 nM, the highest concentration tested.
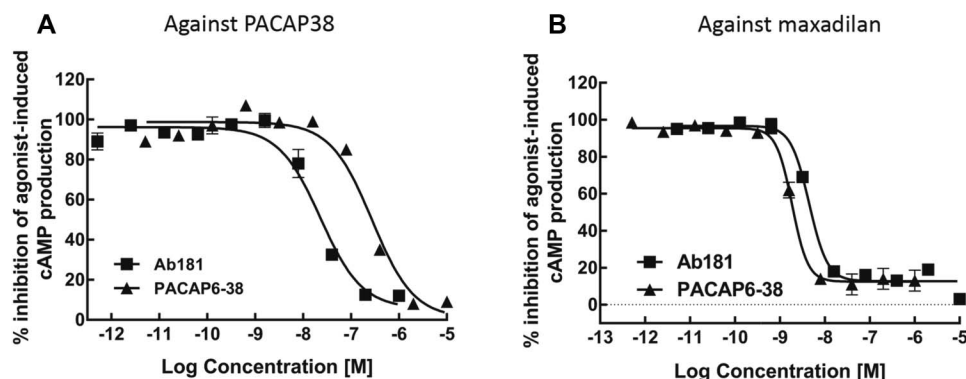

Figure 2. Antagonist activity of Ab181 at rat rPAC1 CHO cell (rPAC1-CHO) against PACAP38 or maxadilan. The antagonist activity of Ab181 at the rat PAC$_1$ receptor was performed by measuring the potency of Ab181 in inhibiting EC$_{80}$ of agonist PACAP38 or maxadilan-stimulated cAMP production. PACAP6-38, a PAC$_1$ receptor antagonist, was used as a positive control. Full antagonist activity of Ab181 against both PACAP38 (A) and maxadilan (B) induced cAMP accumulation was observed in rPAC1-CHO cells with IC$_{50}$ of 20 ± 3.3 nM (n = 3) and 4.5 ± 0.1 nM (n = 2) against respective agonist.

3.2. Pharmacokinetic and pharmacodynamic effect of Ab181

3.2.1. Dose–response study at 48 hours after Ab181 treatment

Pretreatment of Ab181 48 hours before maxadilan challenge prevented the maxadilan-induced increase in DBF (MIIDBF). The change in DBF over 60 minutes after maxadilan is depicted in **Figure 3A**. There was a statistically significant difference ($P < 0.001$) between the vehicle-treated group and each of 3 Ab181 dose groups (0.3, 1, and 10 mg/kg) at 15 minutes after maxadilan injection with a calculated ED_{50} of 0.16 mg/kg ($CI_{95\%}$ = 0.10-0.26 mg/kg). Serum concentration of Ab181 is plotted on the Y-axis of **Figure 3B**.

3.2.2. Dose–response study at 3.25 hours after Ab181 treatment

A shorter pretreatment of Ab181 at various doses was also evaluated. A 15-minute postmaxadilan measurement was used in this study. The change in MIIDBF after 3.25 hours after Ab181 is depicted in **Figure 4**. There was a statistically significant inhibition of the MIIDBF following dose of 1, 3, and 10 mg/kg at 3.25 hours after treatment ($P < 0.01$), resulting in a calculated ED_{50} of 0.95 mg/kg ($CI_{95\%}$ = 0.59-1.51 mg/kg). Serum concentration of Ab181 is plotted on the Y-axis of **Figure 4**.

3.2.3. Time-course study of Ab181 at a dose of 10 mg/kg

A 15-minute postmaxadilan measurement was used in this study. Ab181 at 10 mg/kg produced a statistically significant inhibition of the MIIDBF starting ($P < 0.05$) from a pretreatment time of 0.75 hours and lasting through the last measured 168-hour time point (**Fig. 5**). The resulting mean serum concentration at 1, 3, 6, 48, and 168 hours after drug treatment is plotted on the Y-axis in **Figure 5**.

3.3. Electrophysiological recordings

3.3.1. Ab181 inhibits stimulus-evoked responses in the trigeminocervical complex

The intravenous administration of Ab181 (**Fig. 1**) induced a long-lasting inhibition of stimulus-evoked nociceptive neuronal activity within the TCC ($F_{1.48, 5.91}$ = 8.43, $P = 0.022$). Compared with the baseline value, the effect was significant at all investigated time points throughout the entire observational period. Maximum inhibition reached $-43 \pm 13\%$ ($t_7 = 3.41$, $P = 0.011$) compared with baseline.

Sumatriptan, which was administered as a positive control, significantly reduced stimulus-evoked responses in the TCC ($F_{2.21, 15.49}$ = 4.97, $P = 0.019$). Similar to the Ab181 group, the inhibiting effect of sumatriptan was significant at all investigated time points reaching a maximum inhibition of $-42 \pm 15\%$ (t_7 = 2.78, $P = 0.027$) compared to baseline without recovering until the end of the observational period.

By contrast, the intravenous administration of the vehicle did not affect stimulus-evoked neuronal activity ($F_{2.21, 15.48}$ = 2.45, $P = 0.115$; **Figs. 6A, B**).

3.3.2. Neuronal background activity in the trigeminocervical complex is not affected by Ab181

In contrast to the observed effect on stimulus-evoked neuronal activity, intravenous administration of Ab181 does not attenuate unspecific neuronal background activity within the TCC ($F_{1.32, 5.27}$ = 0.767, $P = 0.456$). The same lack of effect on background activity was observed in animals treated with sumatriptan ($F_{2.33, 16.28}$ = 0.821, $P = 0.474$) and those treated with vehicle ($F_{1.68, 11.78}$ = 0.99, $P = 0.385$; **Fig. 6C**).

3.3.3. Arterial blood pressure is unaffected by Ab181

Intravenous administration of Ab181 does not affect arterial blood pressure ($F_{1.69, 6.78}$ = 0.656, $P = 0.525$). By contrast, sumatriptan induced a significant decrease in arterial blood pressure ($F_{2.55, 17.84}$ = 5.82, $P = 0.008$) throughout the entire observational period, reaching a maximum reduction of $-13 \pm 3\%$ (t_7 = 4.89, $P = 0.002$) compared to baseline without recovering until the end of the experiment. In the vehicle control group, arterial blood pressure was unaffected ($F_{2.29, 16.02}$ = 0.93, $P = 0.426$; **Fig. 6D**).

3.4. Immunohistochemistry

To investigate whether Ab181 or the control antibody distributed into tissues of interest after intravenous administration, we treated
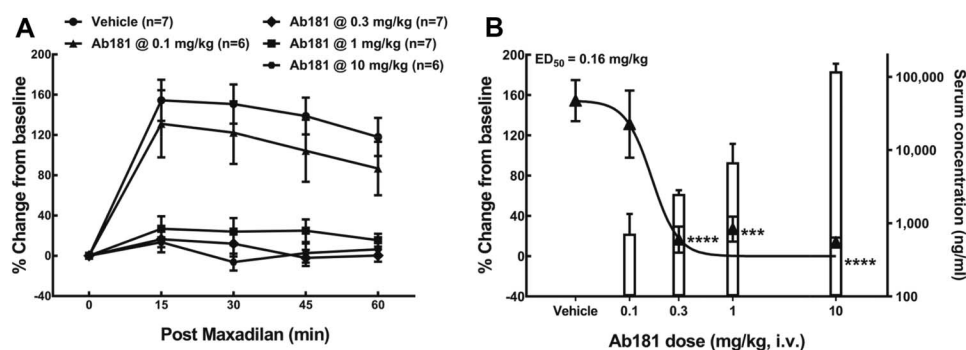

Figure 3. Prophylactic effect of Ab181 on maxadilan-induced increase in DBF in rats 48 hours after drug treatment. Rats were given intravenous bolus injection of Ab181 (0.1, 0.3, 1, and 10 mg/kg, n = 6-7 rats/group). Forty-eight hours later, after obtaining a postdrug baseline (BL), maxadilan at a dose of 10 ng (in 20-μL DPBS) was injected intradermally. The dermal blood flow (DBF) was measured by a laser Doppler imager. (A) The increase in DBF (change from BL) caused by maxadilan injection over 60 minutes was expressed as % change from BL = [100 × (individual postmaxadilan flux − individual BL flux)/individual BL flux]. (B) % change from BL (left-axis) vs resulting serum concentrations (right axis) at 15 minutes after maxadilan; solid triangle represents % change from BL at 48 hours after drug treatment (left axis, mean ± SEM), and open bar represents serum concentration at 48 hours after drug treatment (right axis, mean ± SD). ***$P < 0.001$, ****$P < 0.0001$ compared with vehicle-treated group by one-way ANOVA followed by Dunnett's MCT. ANOVA, analysis of variance; DBPS, Dulbecco's phosphate-buffered saline; MCT, multiple comparison test.
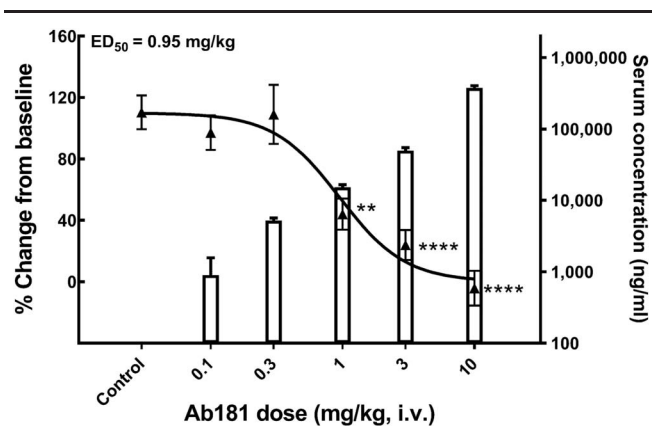
Figure 4. Effect of Ab181 on a maxadilan-induced increase in DBF in rats 3.25 hours after treatment. Rats were given intravenous (i.v.) bolus injection of Ab181 (0.1, 0.3, 1, 3, and 10 mg/kg, n = 7-8 rats/group). Three hours later, after obtaining a postdrug baseline (BL), maxadilan at a dose of 10 ng (in 20-μL DPBS) was injected intradermally. The DBF was measured by a laser Doppler imager. Solid triangle represents % change from baseline (mean ± SEM, left axis), and open bar represents resulting serum concentration (mean ± SD, right axis). Control: Dummy antibody + vehicle (n = 13 rats). $**P < 0.01$, $****P < 0.0001$ compared with the control group by one-way ANOVA followed by Dunnett's MCT. ANOVA, analysis of variance; DBF, dermal blood flow; DBPS, Dulbecco's phosphate-buffered saline; MCT, multiple comparison test.
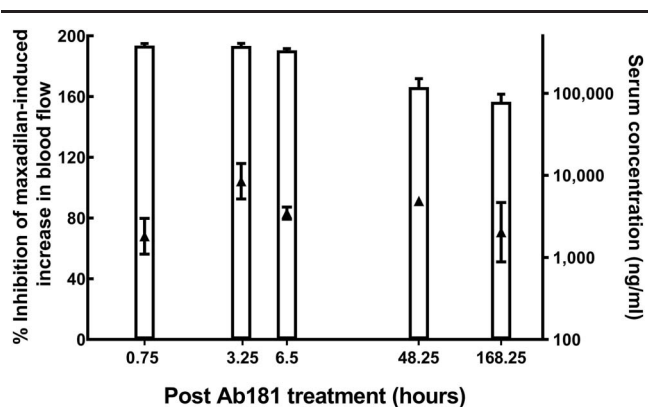

Figure 5. Time course of effect of Ab181 (at 10 mg/kg) on a maxadilan-induced increase in DBF in rats. Rats were given intravenous bolus injection of Ab181 at 10 mg/kg with pretreatment time at 0.5, 3, 6.25, 48, and 168 hours before maxadilan challenge (n = 3-8 rats/group). After obtaining a postdrug baseline (BL), maxadilan at a dose of 10 ng (in 20 μL DPBS) was injected intradermally. The DBF was measured by a laser Doppler imager. Solid triangle represents % inhibition (mean ± SEM, left axis), and open bar represents resulting serum concentration (mean ± SD, right axis). DBF, dermal blood flow; DBPS, Dulbecco's phosphate-buffered saline.

satellite rats from the electrophysiology study but dissected and fixed tissues of interest at the 3.5-hour time point after injection. Using fluorescently labeled secondary antibodies against the Fc portion of the antibodies, we were able to detect immunoreactivity in dura, trigeminal ganglion (TG), and SPG of Ab181-dosed rats, but not in rats dosed with control antibody (**Fig. 7**). By contrast, no labeling above background was detected in spinal trigeminal nucleus or superior salivary nucleus of the brainstem as well as in the hypothalamus and thalamus indicating that Ab181 did not cross the blood–brain barrier, or the amount of antibodies that entered the CNS after intravenous injection was below the detection threshold of the immunohistochemical method (**Fig. 7**).

4. Discussion

PACAP38, first described in 1989,[39] is one of the key neurotransmitters of the parasympathetic system. Soon after its discovery, preclinical evidence suggested effects beyond its parasympathetic role, in particular in specific pain pathways related to headache.[53] Neuroanatomical and functional data obtained since strongly suggest a prominent role in primary headaches including cluster headache and migraine. Our data may provide a plausible underpinning biology for a potential clinical effect of PAC_1 blockade in these disorders.

First, PACAP receptors (PAC_1, $VPAC_1$, and $VPAC_2$) are located in several key areas along the trigeminovascular system. Within the central part of the pain pathways processing trigeminal pain, they have been identified in the thalamus,[27] hypothalamus,[27] and the TCC,[34] while in the peripheral part, they are located in the TG[34] and the meningeal vasculature.[5,6] Beyond that, they are found in the SPG[8] highlighting the relevance of PACAP38 in the parasympathetic system, as well as its potential role in the functional interaction between the trigeminal and parasympathetic systems, namely the trigeminoautonomic reflex.

Second, from a functional perspective, PACAP38 may play a role in cluster headache and migraine since its plasma concentration is elevated in spontaneous attacks[49,52] and normalizes after effective attack abortion with sumatriptan.[52] In addition, PACAP38 triggers migraine-like attacks in migraineurs,[25,43] most likely through an activation of the PAC_1 receptor.[52] In the context of migraine, the ability to trigger attacks clearly distinguishes PACAP38 from VIP. These clinical observations can now be explored in specific animal models. For example, electrical stimulation of the superior sagittal sinus of the cat induces the release of PACAP38 into the cranial circulation.[52,53] In line, the administration of PACAP38 facilitates nociceptive neuronal activity in the TCC.[1] These functional features of PACAP38 strikingly resemble the preclinical[47] and clinical observations[16,17] made with CGRP, which predicted clinical efficacy of 6 small-molecule CGRP-receptor antagonists[10,28,30,37,41,51] and CGRP/CGRP-receptor antibodies[11,23,45] in the acute and preventive treatment of migraine as well as a CGRP antibody in the preventive treatment of episodic cluster headache.[13]

Third, the parasympathetic and trigeminal sensory systems connect and interact with each other. From an anatomic perspective, CGRP-positive sensory fibers project from the TG to the SPG,[8] and in vivo data demonstrates that PACAP38 induces the release of CGRP from the TCC.[34] Nevertheless, the detailed molecular mechanisms behind the attack-triggering capability of PACAP38, and in particular, the clinical relevance of the above-described mechanism remain to be fully elucidated. Interestingly, the administration of PACAP38, despite creating an activation of the trigeminovascular system with the clinical picture of a migraine-like attack, does not induce the release of CGRP in a human model of migraine.[25] These results suggest that the PACAP38-induced effects on trigeminal activation are likely to be the result of a direct effect on nociceptive trigeminal neurons rather than a CGRP-mediated mechanism, although both mechanisms do not exclude each other. In the case of a direct activation of either of the PACAP receptors, PACAP38 stimulates the cAMP/PKA pathway thereby increasing neuronal excitability.[46] In addition, all PACAP receptors may be modulated in their expression profile under chronic pain conditions.[55] It may be speculated that these upregulating and downregulating properties and the ability to produce neuronal sensitization may even play a role in the chronification of migraine. Based on this large body of evidence, we set out to develop a specific antibody
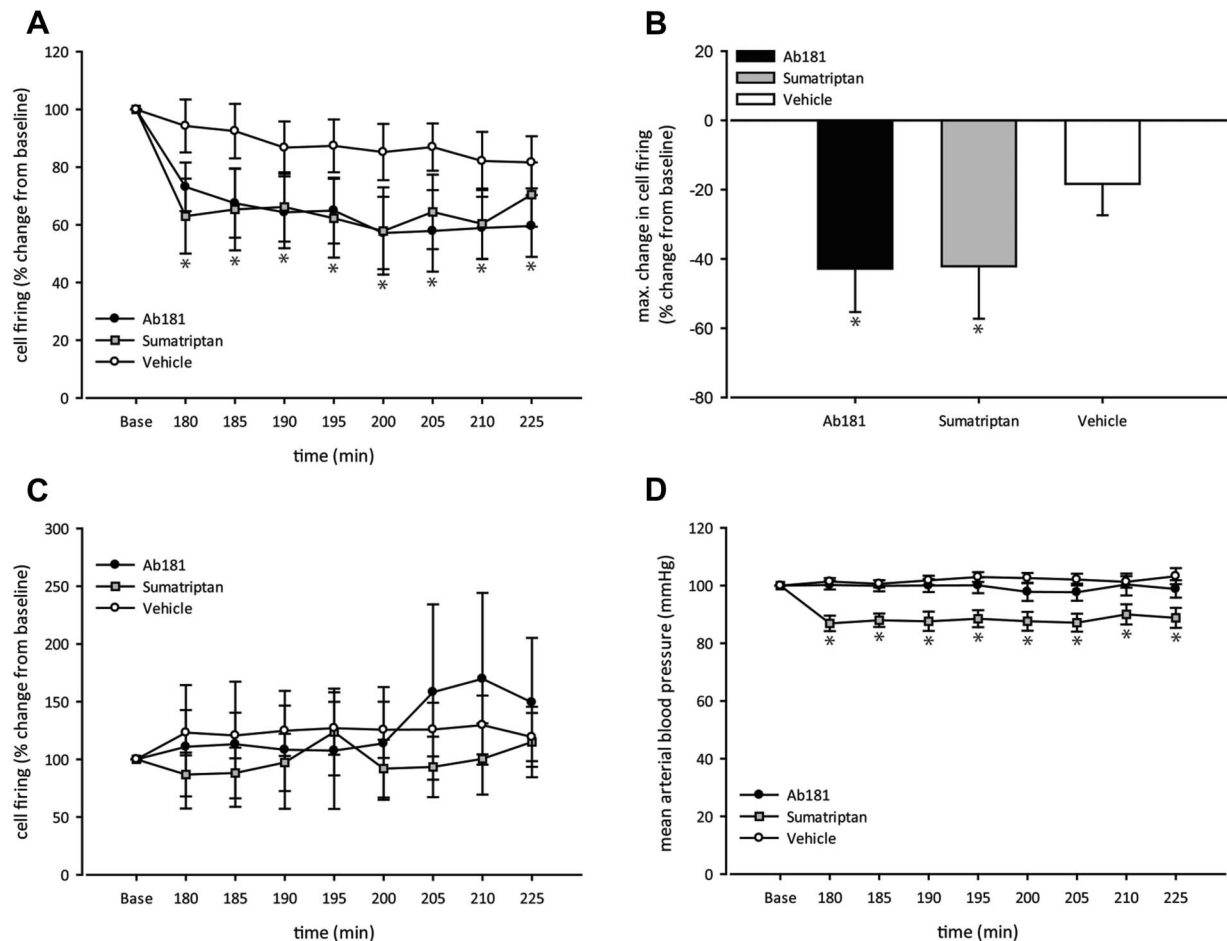
Figure 6. Effect of Ab181 on neuronal activity in the TCC. Ab181 effectively inhibited stimulus-evoked neuronal activity in the TCC (A). The maximum inhibition was very similar to the one observed after the intravenous administration of sumatriptan (B). By contrast, an inhibiting effect on spontaneous background activity was not observed (C). Ab181 did not affect arterial blood pressure throughout the entire course of the experiment (D). TCC, trigeminocervical complex.

against the PAC_1 receptor because this receptor, as outlined above, is most likely the most relevant PACAP receptor in mediating the attack-triggering effect of PACAP38 and to test its effects on a model system that represents significant elements of cluster headache and migraine pathophysiology.[4]

PACAP38 is a nonselective agonist at all the PACAP receptors, and VIP is only mildly selective to the $VPAC_1$ and $VPAC_2$ receptors.[26] Maxadilan, on the other hand, is the most selective agonist at the PAC_1 receptor,[40] which allowed us to measure pharmacodynamic activities specifically mediated through the PAC_1 receptor. Although some years have passed since PACAP38 was discovered,[39] selective antagonists to the individual receptors in the PACAP receptor family are still lacking. Several peptide antagonists have been reported with mild selectivity between different receptors,[5,9,24] but their pharmacokinetic properties, especially their very short plasma half-life, hindered their utility in *in vivo* pharmacology studies. Therefore, Ab181 was developed. As demonstrated in the Results section, it is a potent and selective antagonist at the PAC_1 receptor, with no activity at the VPAC receptors. It is also a potent and selective inhibitor of maxadilan-induced increases in DBF in a time- and dose-dependent manner. Through the in vitro and in vivo profiling, Ab181 demonstrated full-target coverage of the PAC_1 receptor at 3.5 hours after intravenous administration and maintained sustained plasma concentration throughout the study duration. It is therefore the first ideal tool for

the studying of selective PAC_1 pharmacology in preclinical species.

The new results of the study show that the intravenous administration of Ab181 inhibits stimulus-evoked nociceptive activity in the TCC. Unlike conventional small molecular agents whose molecule weight (MW) are normally <500, the much larger sized antibodies (MW > 145KD) generally have a slower tissue distribution rate. As a result, it requires a substantial equilibrium period between the administration of the antibody and the initiation of the experiment before the assessment of its influence on stimulus-evoked activity. Therefore, the experimental design resembles more of a short-term preventive scenario than an acute reversal of nociceptive trigeminal activation. The results show that the extent of neuronal inhibition was almost identical to that observed with sumatriptan, suggesting a robust effect. The fact that we did not observe an effect on nonspecific background activity reflects the specificity of the effect on nociceptive neuronal transmission.

The exact site of action of PAC_1 remains speculative. Literature suggested that agonist PACAP38, with MW >4000, triggers migraine-like attacks without crossing the blood–brain barrier.[12] However, passage of PACAP27 and PACAP38 across the blood–brain barrier has been suggested.[3] Here, immunohistochemical analysis of Ab181 found a minimum presence in CNS, which suggests that the site of the modulating action on nociceptive activity is likely to be located outside of the CNS.
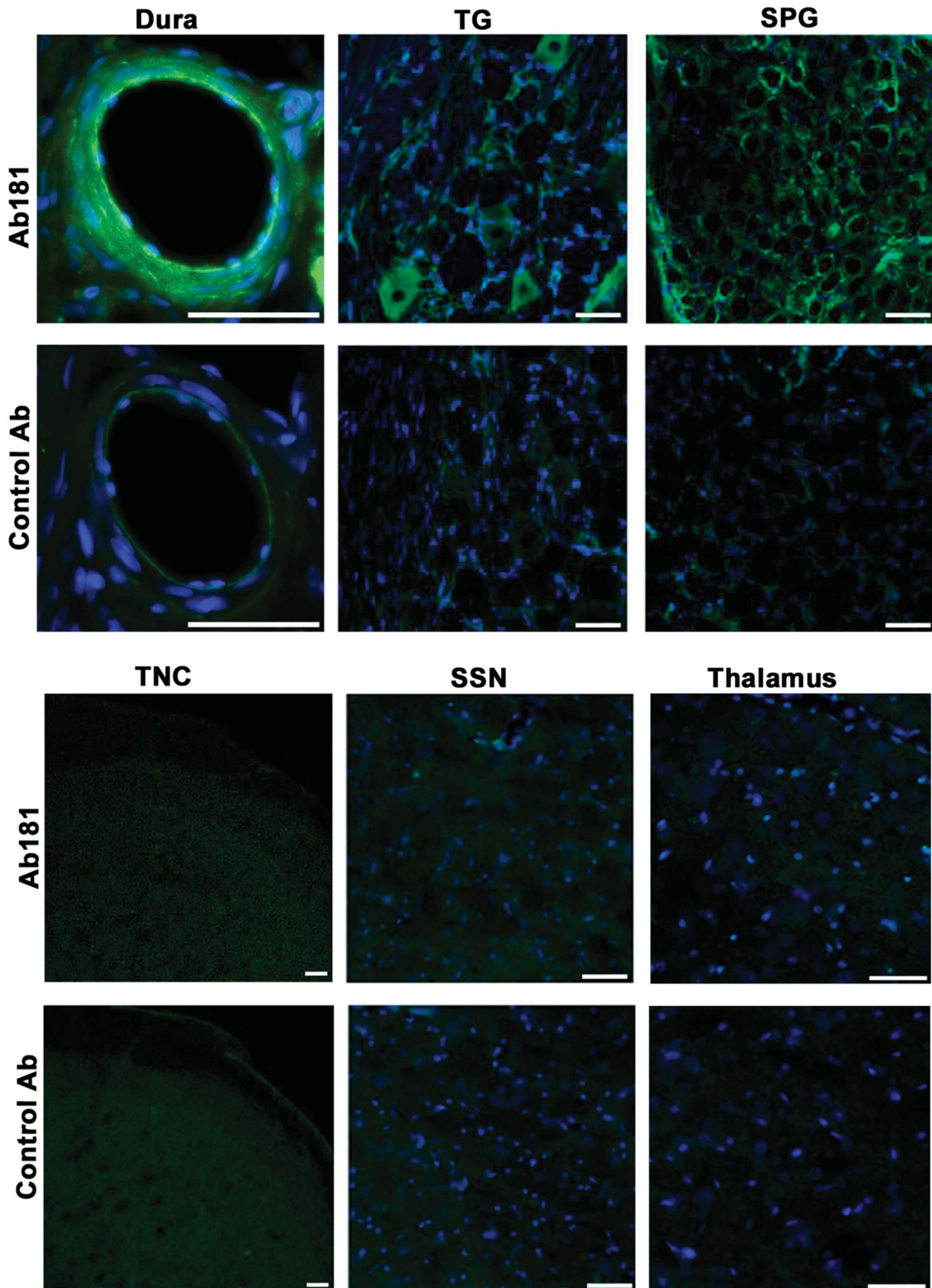
Figure 7. Distribution of Ab181 in tissues after intravenous dosing. *Ex vivo* detection of the IgG portion of Ab181 or control antibody after intravenous dosing in dural vessels, trigeminal ganglion (TG), and sphenopalatine ganglion (SPG) as well as spinal trigeminal nucleus caudalis (TNC), superior salivatory nucleus (SSN), and thalamus. Staining of neuronal and glial structures is detected in dura, TG, and SPG of Ab181-dosed rats, but not in TNC, SSN, or thalamus. Only background staining is detected after intravenous dosing with control antibody (lower panels). Scale bars 50 μm.

This would be in line with the results obtained from the electrophysiological experiments showing no effect on neuronal background activity and the immunohistochemical analysis in which Ab181 was detected in the relevant peripheral structures but not within the CNS. These findings suggest that Ab181 does not cross the blood–brain barrier in a significant concentration, a finding that is expected given the molecular size of the antibody. It cannot be excluded that a small amount of antibody reaches the CNS but remains below the immunohistochemical detection limit; however, target coverage with such small amount is unlikely sufficient for a full antagonism effect. Therefore, the current study with a selective PAC_1 antagonist antibody supports the hypothesis that peripheral PAC_1 receptor inhibition can be sufficient to abort or prevent attacks of cluster headache and migraine.

Recently, a randomized, placebo-controlled trial (phase 2) with a monoclonal PAC_1 receptor antibody (AMG 301) was reported in abstract form to be ineffective in the preventive treatment of migraine. In the context of our data, some comments are relevant. First, in our experiments, we used an antibody that was developed for rodents and differs significantly in its pharmacological properties, including its affinity to the PAC_1 receptor, for which it is more potent than the one used in the human phase 2 trial. Second, although the available evidence suggests that the PAC_1 receptor plays a relevant role in trigeminal activation, the possibility that VPAC receptors are clinically significant is an open issue. The fact that VIP may trigger migraine attacks in subset of migraineurs may support this hypothesis.[2] Finally, in the clinical trial, patients were not stratified by the presence or absence of cranial autonomic symptoms. Therefore, it may be hypothesized that the ability of PACAP38 to induce migraine attacks may require an action on PAC_1 receptors on trigeminal and parasympathetic neurons thereby increasing the activation of the trigeminoautonomic reflex. If this would be the case, one could speculate of a higher relevance of this potentially therapeutic mechanism in migraine with cranial autonomic symptoms or in cluster headache.

Taken together, the findings of our studies suggest that the PAC_1 antibody Ab181 can inhibit nociceptive neuronal traffic, so that this pharmacological approach may offer a new strategy for the preventive treatment of primary headache disorders, including cluster headache and migraine.

Conflict of interest statement

J. Hoffmann is consulting for and/or serves on advisory boards of Allergan, Autonomic Technologies, Inc (ATI), Chordate Medical AB, Eli Lilly, Hormosan Pharma, Novartis and Teva. He has received honoraria for speaking from Allergan, Chordate Medical AB, Novartis and Teva. He received personal fees for Medico-Legal Work as well as from Sage Publishing, Springer Healthcare and Quintessence Publishing. S. Miller is an employee of Amgen, Inc. S. Akerman reports an unrestricted grant, honoraria and travel reimbursements from electroCore LLC, unrelated to the submitted work. H. Sun is an employee of Amgen, Inc. L. Shi is an employee of Amgen, Inc. D. Zhu is an employee of Amgen, Inc. S. Lehto is an employee of Amgen, Inc. H. Liu is an employee of Amgen, Inc. R. Yin is an employee of Amgen, Inc. B.D. Moyer is an employee of Amgen, Inc. C. Xu is an employee of Amgen, Inc. P.J. Goadsby reports grants and personal fees from Amgen and Eli-Lilly and Company, and personal fees from Alder Biopharmaceuticals, Allergan, Autonomic Technologies, Inc, Biohaven Pharmaceuticals, Inc, Electrocore LLC, eNeura, Impel Neuropharma, MundiPharma, Novartis, Teva Pharmaceuticals, and Trigemina, Inc. WL Gore, and personal fees from MedicoLegal work, Massachusetts Medical Society, Up-to-Date, Oxford

University Press, and Wolters Kluwer; and a patent Magnetic stimulation for headache assigned to eNeura without fee. The remaining authors have no conflicts interest to declare.

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