Vessel diameter measurements at the medullary brainstem in vivo as an index of trigeminal activity

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Abstract

Activity within the CNS can be quantified by a variety of methods. Here, we present an indirect method utilizing the neuro-vascular coupling via a continuous measurement of the vessel diameter. In anaesthetized rats, induced neuronal activity in the trigeminal system could be detected via arterial diameter measurements at the back of the medullary brainstem. Building upon the previously described diameter plugin, in images aligned to compensate for ventilation and heart-rate associated movement, automated processing allowed an evaluation immediately after acquisition. Electrical stimulation of facial trigeminal areas or the cranial dura mater caused vasodilatation of arteries in the dorsal medullary brainstem, which was abolished after application of the CGRP receptor antagonist olcegepant. No change in diameter was observed in corresponding veins. Intravenous infusion of sodium sulfide, which induces nitroxyl formation, also induced vasodilatation of brainstem-supplying arteries. Both experiments show a functional role of CGRP in the trigeminal nuclear brainstem complex. The presented method allows estimating central activity at the spinal level by vascular responses.

1. Introduction

Neuronal activity of central structures can be measured by a variety of methods, directly via electrical recording or more recently by optical methods. In addition there are indirect methods of activity measurements. These include post mortem measures, e.g. measurements of immediate early gene expression, which provide information across large areas though at the expense of temporal resolution. Another method utilizes the coupling of blood flow to neuronal activity, driven by vasodilatory mediators released from activated afferent neurons; functional magnetic resonance imaging is a prominent example. The change in meningeal vessel diameter in response to electrical or chemical stimulation of peripheral trigeminal terminals at the dura mater can be monitored by video microscopy in open and closed cranial windows (Akerman et al., 2001; Gozalov et al., 2005; Williamson et al., 1997). We investigated whether activity in the first relay station of the trigeminovascular system, the trigeminal nuclear brainstem complex, can be monitored in vivo by measuring the diameter of pial blood vessels in rats. An established rat model with preparation of the dura and medullary brainstem and an offline plugin for ImageJ for vessel diameter measurement was employed for this task.
A large fraction of the trigeminal afferents release calcitonin gene-related peptide (CGRP) and the critical site of action of CGRP within the trigeminal system is debated (Edvinsson and Warfvinge, 2013; Fischer, 2010). Recent years have seen a substantial shift from a peripheral site of action towards a role of the transmission at the central end of primary afferents (Sixt et al., 2009). As such, it was of interest to quantify activity via an index in the trigeminal medullary brainstem. Total blood flow at the brainstem can also be measured via Laser Doppler flowmetry (Goadsby and Classey, 2000), but allows no differentiation between arterial and venous vessels. For video microscopy in the brainstem several issues had to be overcome, this includes a stable clear view of the target and coping with movement arising from ventilation and heartbeat. Several of these improvements allowed setting up this indirect measure of neuronal activation, which needs to prove its place among competing approaches.

2. Results

Pial blood vessels of the medullary brainstem had a similar pattern in all specimens. Two or three winding arterial vessels on each side of the medulla cross the left or right arm of the dorsal vein, from which several branches arise. Each image was focused on an area that contained at least one measurable arterial and venous vessel. Fig. 1 shows two sample images, the response of vessels to facial and dural electrical stimulation is visualized by digital subtraction. As a test to enhance contrast, a dye which resides intravascularly was infused (Evans Blue 80 mg/kg). Vessel darkening was clearly visible; this improved the precision of diameter measurement (variance $-39\%$, $p < 0.001$) and reduced the number of outliers ($\pm 2$ SD: $-63\%$, $\pm 3$ SD: $-75\%$, both $p < 0.005$, Supplemental Fig. S1).

Electrical stimulation in the infraorbital area of the facial skin caused an increase in arterial diameter in the medullary brainstem. During stimulation for one minute the dilatation reached a plateau, the diameter during the last 30 s of electrical stimulation was $105 \pm 1\%$ compared to baseline ($p = 0.028$, $n = 6$ animals, Fig. 2A, Supplemental Fig. S2A). This dilatation reversed within a few minutes and could be reproduced thereafter. Dural stimulation for one minute caused a substantial vasodilatation of posterior spinal arteries to $111 \pm 2\%$ of baseline ($p < 0.001$, $n = 10$ animals, Fig. 2B, Supplemental Fig. S2B). In experiments with two subsequent electrical dura stimulations, the flow change induced by the second stimulation was $101 \pm 41\%$ of the first response ($n = 8$, $r = 0.35$).

After infusion of olcegepant 1 mg/kg the electrically stimulated vasodilatation was abolished ($101 \pm 3\%$, $p = 0.039$ compared to control stimulation, Fig. 2B, Supplemental Fig. S2C). In the surrounding veins of various size, the electrical stimulation of the face or the dura did not cause changes in diameter ($n = 6$ and 11 animals, Fig. 2B). We observed also a dilatation at the brainstem on the contralateral side to electrical dura and facial stimulation (Supplemental Fig. S3). The infusion of saline had no effect on blood pressure and vessel diameter (Supplemental Fig. S4A). However, infusion of sodium sulfide 2.3 mg/kg into the femoral vein over the period of one minute caused an immediate reduction in blood pressure which was rapidly recovering and overshooting at the end of the infusion (Fig. 3A, Supplemental Fig. S4B). With a slight delay and a similar time course as observed...
after electrical stimulation, a lasting arterial vasodilatation occurred (108±2%, p=0.018, n=7 animals). This vasodilatation was again abolished after infusion of olcegepant 1 mg/kg (101±1%, p=0.003, n=6 animals, Fig. 3A, Supplemental Fig. S4C). Olcegepant also inhibited the previously observed changes in blood pressure. The diameter of surrounding veins did not change after NaCl or Na₂S infusion (n=6 and 8 animals, Fig. 3B).

Pial arteries of different size were observed on the surface of the spinal medulla, linked to the medullary surface by delicate pial connective tissue. Arterial vessels had a round profile and were characterized by a thick smooth muscular layer immunopositive for smooth muscle actin (SMA) and the CGRP receptor components RAMP1 (Fig. S5) and CLR (not shown) indicating the presence of CGRP receptors. The venous vessels showed no immunoreactivity for CGRP receptor components.

3. Discussion

The diameter of blood vessels supplying the dorsal medullary brainstem was measured from images. The change in arterial diameter in response to electrical stimulation within trigeminal tissues as well as intravenous infusion served as an index of activation. This site was chosen due to the relevance for the neurobiology of headache (Goadsby et al., 2009).

3.1. Technical considerations

The main issue was to establish a stable and unobstructed view of the dorsal brainstem. To this end we first attempted to fill the cavity with extracellular fluid and to confine this volume by topping it with glass or acrylic glass. However, without continuous replacement the view deteriorated due to either plasma extravasation or slow bleeding. Another unsuccessful attempt was to fill the cavity with a transparent fluid with higher density than water. Fluorohexacarbon served that purpose but did not avoid gathering of intransparencies at its surface. A roll-pump-driven fluid exchange resulted in a systematic surface variation which substantially altered the field of view. Finally, a gravity-driven continuous superfusion and drainage fulfilled all requirements, artificial cerebrospinal fluid might be preferable to saline in future experiments.

Major sources of brainstem movement are respiration and heartbeat; due to the movement also the fluid level and therefore the reflection at the fluid surface changes. Respiration can be set faster and shallower but heart-beat-associated movement appeared to be the principal source of movement.

Fig. 2 – Arterial dilatation at the medulla in response to electrical stimulation. (A) The facial skin innervated by the maxillary nerve was stimulated by 10 Hz pulses for 60 s. The arterial diameter increases in response to electrical stimulation (E-Stim). (B) Electrical stimulation at the dura mater caused arterial vasodilatation, which was abolished 5 min after infusion of olcegepant (1 mg/kg). Time course of the normalized vessel diameter in response to 60 s of electrical stimulation at the dura. After intravenous application of 1 mg/kg olcegepant this response is abolished. The venous diameter did not change upon electrical stimulation at the dura mater. (C) Bar charts summarizing the arterial and venous diameter measured within the last 30 s of electrical stimulation compared to the 30 s before. Data are mean ± sem. *p < 0.05 vs baseline. †p < 0.05 compared to before olcegepant infusion.
Muscle activity due to electrical stimulation was suppressed by gallamine paralyzing the muscles. The plugins TurboReg and StackReg helped that the investigator-drawn line for analysis stayed on the blood vessel despite image movement. Shorter exposure times with a monochrome camera could have reduced the fraction of noisy images. The blood brain barrier restricts the albumin-bound Evans Blue dye to the intravascular space. Despite the good contrast by hemoglobin, infusion of a dye can improve the measurement, and specialized approaches might benefit from this option.

3.2. Stimulation-induced vasodilatation

Due to the higher innervation density compared to the dura, we started with electrical stimulation in the face. Bipolar stimulation caused vasodilatation of pial arteries of the dorsal medullary brainstem. However, electrical stimulation across the superior sagittal sinus caused an even greater vasodilatation. Given the large area of view, only the vessels supplying activated areas are expected to show a diameter change. Therefore we focused on the sites to which the majority of meningeal afferents project and where we have previously recorded electrical activity with highest probability, i.e., the rostral and caudal medullary brainstem, containing the subnuclei interpolaris (SpI) and caudalis (SpC) of the spinal trigeminal nucleus. This might also explain the smaller responses to facial stimulation, for which stimulated afferents primarily end more orally in the subnuclei oralis (SpO) and interpolaris (SpI). Venous vessels did not show changes in diameter. Rather hypothetically, an axon reflex by a neuron with one ‘peripheral terminal’ at brainstem-supplying vessels might explain the observed dilatation in response to the stimulation of dural or facial terminals. In sample experiments vessels at the contralateral side of the electrical stimulation showed a stimulation-induced vasodilatation. This needs to be further investigated as despite known anatomical projections no such response at the contralateral side was observed at spinal level (He et al., 2015).

3.3. Limitations of neurovascular coupling

Spatial precision of neurovascular coupling is limited by the inter-vessel distance (Pawlik et al., 1981). The maximal increase of cerebral blood flow in response to a brief period of neural activation occurs with a delay of 5 s (Li and Freeman, 2007). The amplitude of the blood flow increase is correlated to other measures of the neuronal activity (Kameyama et al., 2008; Logothetis and Wandell, 2004). However, the generated neuronal input will typically not activate all neurons supplied by the measured blood vessel. A 5% vasodilatation is just above the detection limit at the noise levels in our diameter measurement. However, as 5% vasodilatation of a resistance vessel, corresponding to a 22% ($=1.05^2$) increase in flow, may indicate a much larger local blood flow increase. In turn, only a limited vasodilatation

Fig. 3 – Vessel diameter in response to intravenous infusion of Na$_2$S. (A) The arterial diameter dilates and the arterial blood pressure falls in response to 2.3 mg/kg Na$_2$S over 60 s. After intravenous application of 1 mg/kg olcegepant, a second Na$_2$S challenge induces no change in arterial diameter. The bottom panel illustrates the immediate decrease in systemic arterial pressure, the counteraction and the overshoot after the Na$_2$S infusion, which are absent after olcegepant infusion. (B) The venous diameter is not changed by the infusion of Na$_2$S. (C) Bar charts summarizing the response measured within the last 30 s of Na$_2$S infusion compared to the baseline 30 s before stimulation. Data are mean $\pm$ sem. $^*p<0.05$ vs baseline. $^#p<0.05$ compared to before olcegepant infusion.
could be expected even from a substantial rise in local activity.

3.4. Stimulation-induced increase in blood flow depends on the CGRP receptor

A considerable fraction of central terminals contain CGRP and release it upon stimulation (Kageneck et al., 2014; Wild et al., 2015). The action of CGRP released from central trigeminal terminals is not entirely clear. It has been shown that it does act as a co-transmitter for glutamate (Storer et al., 2004) and inhibition of this receptor reduces neuronal activity (Fischer et al., 2005). While CGRP in the spinal trigeminal nucleus might facilitate glutamatergic transmission, the latter is not completely CGRP-dependent. However, CGRP antagonists completely inhibited the stimulated vasodilatation, which argues for an additional and more important target of CGRP involved in neurovascular coupling, e.g. astrocytes or direct innervation of the CGRP receptor-expressing vessels by CGRP releasing fibers. There is a controversial debate about the possible site of action of CGRP antagonists, for which we refer the reader to additional literature (Effekhari and Edvinsson, 2010; Messlinger et al., 2012; Tfelt-Hansen and Olsen, 2011).

3.5. Na2S induced vasodilatation in brainstem vessels

The reaction of NO with H2S caused an increased meningeal blood flow by forming HNO (Dux et al., 2015). Injection of Na2S 0.03–0.5 mg/kg leads to a substantial reduction of the heart rate, the total resistance and therefore the systemic blood pressure (Yoo et al., 2015). In the present study, a slow infusion over one minute still caused a substantial reduction of systemic blood pressure. A robust arterial vasodilatation was also observed in the dorsal brainstem. The increase in diameter correlates well to the reduction in blood pressure, the secondary counteraction of blood pressure is also coded in a slight additional increase in diameter. Na2S dissociates immediately in the vascular system and sulfide converts endogenous nitric oxide to nitroxyl (HNO), which is an agonist at the TRPA1 receptor channel expressed by peptidergic trigeminal afferents. The inhibition of both underlines the previously described relevance of the HNO-TRPA1-CGRP pathway for vascular regulation (Dux et al., 2015; Eberhardt et al., 2014). Activation of TRPA1 leads to CGRP release upon influx of calcium, and CGRP causes vasodilatation of pial arterial vessels by activating vascular CGRP receptors. The larger average diameter of veins, in which less absolute diameter changes are required for a similar relative flow changes might explain why we were unable to detect an increased venous outflow which is likely to accompany the arterial vasodilatation.

3.6. Conclusion

Stimulated activity caused vasodilatation in arteries of the dorsal brainstem. This was used to demonstrate Na2S-evoked central trigeminal activity; the respective vasodilatation depends on the activation of CGRP receptors. The presented approach utilizes metabolic and neurovascular changes to provide a new index of trigeminal activity in vivo.

4. Experimental procedure

4.1. General preparation

All animal experiments were performed with the permission of the ‘Animal Care Authority’ of the local district government (Ansbach, Germany), in accordance with guidelines and regulations of animal care provided in Council Directive 2010/63EU of the European Parliament and in compliance with the ARRIVE guidelines. Adult Wistar rats (300–400 g) of either sex bred in-house were anesthetized with isoflurane 4% applied in a box and then maintained at isoflurane 2% throughout the experiment. This was sufficient to suppress nociceptive reflexes evoked by noxious pinch stimuli of the hind paw. The body temperature was held at 37 °C with a feedback controlled homeothermic system (Foehr Medical Instrument, Seeheim, Germany). Plastic catheters were inserted into the femoral vein for infusions and into the femoral artery for continuous monitoring of blood pressure. A tracheal tube was inserted after tracheotomy. Ointment (Bepanthen, Roche, Mannheim, Germany) was used to prevent drying of the eyes. The animal was placed in a stereotaxic frame with the head held by ear bars. The ventilation frequency was adjusted to maintain the expiratory CO2 at 2.5–3.5% in order to suppress spontaneous breathing, an increase in ventilation frequency at lower volume reduced breathing-induced movement of the brainstem. The muscle relaxant gallamine (Relaxan, Sigma-Aldrich) was applied before the experiment to avoid movement during electrical stimulation. Three minutes after gallamine infusion, the mean arterial blood pressure rose by 5.5±2.2% (p=0.026, t-test dependent samples).

4.2. Specific preparation, stimulation and recording

An incision was made along the midline of the scalp, and the skull was exposed. For electrical stimulation at the dura mater a small area of the parietal bone left and right of the superior sagittal sinus were removed (Fischer et al., 2005) or thinned with a dental drill. No differences were observed between the earlier experiments removing all layers of the bone and the later experiments with electrical stimulation through a thin layer of bone. The neck muscles were divided in the midline and retracted to get access to the atlanto-occipital ligament. The ligament together with the adhering spinal dura mater was incised paramedially and along the occipital bone; the resulting flap was pinned aside. The opened cisterna magna was continuously rinsed by a gravity-driven flow of isotonic saline to remove turbid particles and hold the liquid level constant for an optimal camera view.

For electrical stimulation two needle electrodes were pinned into the right facial skin innervated by the maxillary nerve, both were positioned on a line connecting the corner of the mouth and the inner corner of the eye. The facial stimulation site was primarily chosen due to the innervation density of the whisker pad, less due to innervation pattern of the dural stimulation site which has maxillary fractions. For the dural stimulation gold wire electrodes were placed between bregma and lambda at the exposed dura on both
sides of the superior sagittal sinus. Electrical stimulation was performed at intervals of at least 5 min for periods of 60 s with rectangular pulses of 0.5 ms duration, 2–10 V at 10 Hz. The stimulation strength was set as high as possible, but ensuring that no relevant effects on the systemic blood pressure occurred during stimulation. It should be considered that variability in stimulated tissue is likely due to the inter-individual anatomy underlying the resistance distribution. In addition, the chosen parameters might also activate some cortical neurons below the electrodes. The brainstem was illuminated by a 150 W halogen cold light source with flexible light guides (Leica KL1500 LCD) and an area of the right side of the brainstem containing pial vessels was recorded with a 3 megapixel color digital camera (Leica DFC220) attached to a stereo microscope. The camera was directed to an area covering the trigeminal subnuclei interpolaris and caudalis, the characteristic posterior spinal artery was found caudal of the obex. The recorded vessels were located 0.42 ± 0.03 mm caudal and 0.25 ± 0.02 mm lateral of the obex. Scheduled image acquisition was controlled with open-source Manager software add-on for ImageJ. Within Manager, a standard twain driver was used for the described camera, exposure time was adjusted to about 20 ms and the image was cropped to the region of interest within the focal plane.

4.3. Experimental protocols and substances

Based on previous experience of the kinetics of vessel diameter changes, images were stored at intervals of 3 s. Episodes of image stacks covered 30 s of reference before stimulation, the 60 s of electrical stimulation or intravenous infusion and 90–210 s thereafter. Olegepeant (BIBN4096BS) was a generous gift from Boehringer-Ingelheim (Siberach, Germany), sodium sulfide (NaS) was purchased as anhydrous, 100 mM stock solutions were prepared in a glove box using argon-bubbled nanopure water and stored in glass vials with PTFE septa at +4 °C. In some experiments, blood flow at the dura was monitored by a laser Doppler system (DRT4, Moor Instruments, Axminster, UK) in parallel as an independent means of evaluating the electrical stimulus. Evans Blue 80 mg/kg was diluted in saline and infused intravenously.

4.4. Image analysis

In the open source package ImageJ, images of one stimulation episode were loaded as a stack and reduced to the 8-bit green component. Subsequent images were subjected to an affine transformation to match the first image (co-register) using two plugins; TurboReg aligns two images and was driven by StackReg for the whole stack http://bigwww.epfl.ch/thevenaz/stackreg (Thevenaz et al., 1998). Image processing was automated by macros for minor tasks like the extraction of green substacks from RGB images, saving coregistered stacks, and calculating a subtraction image between two times, typically the maximum diameter change minus the condition immediately before. These macros were combined and modified to present vessel diameter changes in a series of images within less than one minute at reduced resolution (typically after 2 × 2 binning of the images). In addition, another macro performed these tasks at full resolution and for all subfolders of a user-selected path. This was used to process all images at the end of an experimental day. All macros are available on request.

The previously described DIAMETER plugin was employed to measure selected vessels. Briefly, the inner vessel diameter defined by the red blood cell column is quantified by a full width at half-maximum algorithm. The robustness of this algorithm was improved by a change in calculation of the reference intensities outside the blood vessel; now the median intensity (previously the brightest three consecutive points) outside the blood vessel is used. Five parallel shifted measurements are made by the plugin based on a line selection across a vessel by the operator, the results are pasted from the clipboard into an excel template spreadsheet (available on request) which contained further data preprocessing, including outlier rejection, averaging, visualization in absolute and normalized form and quantification of the diameter change at the end of the stimulation period.

4.5. Immunohistochemistry

Immunohistochemistry was performed as previously described (Seiler et al., 2013). Briefly, rats were deeply anaesthetized and perfused through the left ventricle with isotonic saline followed by 4% PFA in 10 mM phosphate-buffered saline (PBS, pH 7.4). After laminectomy the medulla together with C1–3 were carefully dissected including spinal dura mater and spinal roots, stored in PBS and then in a solution of 30% sucrose in PBS both for one day. The specimen was rapidly frozen in butyl butane and cut into 14 μm horizontal sections using a cryostat (Leica, Bensheim, Germany). The sections were mounted on poly-L-lysine-coated slides (Sigma-Aldrich, Steinheim, Germany), and preincubated for one hour at room temperature with a solution of 5% donkey serum (Dianova, Hamburg, Germany) and 1% bovine serum albumin in PBS supplemented by 0.5% Triton X-100. Sections were incubated with primary antibodies directed against human receptor activity modifying protein (RAMP1) raised in goat (Merck and Co., Inc. Rahway, NJ) 1:100 and against smooth muscle actin (SMA) raised in rabbit (Abcam ab 5694, Cambridge, UK) 1:250 at room temperature overnight. Sections were incubated with fluorescently labeled secondary antibodies for one hour, using donkey anti-goat IgG coupled to Alexa Fluor 555 and donkey anti-rabbit IgG coupled to Alexa 488 (1:1000, Molecular Probes, Eugene, OR, USA). Sections were coverslipped in Fluoromount G (SouthernBiotech, Birmingham, AL). Images were obtained using a LSM 780 confocal-laser scanning system mounted on an inverted Axio Observer Z1 equipped with a 63 × oil-immersion objective with a numerical aperture of 1.4 (Carl Zeiss MicroImaging GmbH, Jena, Germany). Samples were sequentially scanned by an Argon laser, for Alexa Fluor 488 using 488 nm excitation and 493–630 nm emission, for Alexa Fluor 555 using 514 nm excitation and 545–697 nm emission.

4.6. Statistical analysis

Statistical comparisons were performed with the Statistica 8 (Statsoft, Tulsa, OK). Two groups were analyzed with matched or independent t-tests, for n = 10 non-parametric Wilcoxon or U-tests were applied. p < 0.05 was considered to be significant. Data are presented as mean ± SEM if not noted otherwise.
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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2015.12.013.

References