Research report

Electrophysiological responses of the ventrolateral periaqueductal gray matter neurons towards peripheral bladder stimulation

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ABSTRACT

Introduction: Many of the currently available therapies for urinary incontinence target the peripheral autonomic system, despite many etiologies residing in the central nervous system. Following previous experiments that determined the ventrolateral column of the periaqueductal gray matter (vlPAG), to be the main afferent station of bladder sensory signals, we aimed for electrophysiological characterization of vlPAG neurons using single unit recording.

Methods: 15 rats were anesthetized and underwent implantation with electrodes at the dome and the neck of the bladder, to electrically stimulate the detrusor. After craniotomy, a glass micropipette was inserted in vlPAG to record neuronal action potentials. The detrusor was stimulated by a series of 20 Hz pulses, for a total duration of 50 s at an intensity of 2 mA, for each vlPAG neuron selected. Single unit recordings were performed on a total of 26 neurons. Confirmation of electrode position was made by iontophoretic ejection of Pontamine sky blue.

Results: The firing rate of vlPAG neurons decreased significantly during the stimulation period. Peristimulus time histogram (PSTH) analysis showed 24 out of 26 neurons to be unresponsive to stimulation. All recorded vlPAG neurons showed irregular firing patterns.

Conclusions: The change in firing rate may point to an overall inhibitory influence of bladder stimulation on vlPAG neurons. These data suggest an inhibitory relay station at the vlPAG, before sensory bladder signals would affect pontine micturition center. The lack of the inhibitory effect on PSTH may be due to a longer interval between neuronal response and the stimulation.

1. Introduction

Even though there is evidence that the central nervous system (CNS) plays a critical role in the causality of urinary incontinence, most therapeutic approaches focus on peripheral (nervous) targets (i.e. the muscarinic acetylcholine receptor) (Emily et al., 2018). Several structures within the CNS have been identified to play a role in the regulation of urine storage and voiding. Examples are the pontine micturition center, parts of the limbic system such as the anterior cingulate gyrus, and parts of the spinal cord such as the sacral nucleus of Onuf. These structures form a complex network, which receive afferent sensory information and provide efferent motor signals to the bladder.

One important structure in bladder control is the periaqueductal gray matter (PAG). The PAG is a midbrain structure, which is involved in the sensori-motor regulation of bladder function. Besides, it plays a role in autonomic (Rathner and Morrison, 2006; Farkas et al., 1998) and emotional (Mouton and Holstege, 1994) control and nociception (Budai et al., 1998). The PAG is composed of separate columns (Bandler and Shipley, 1994; Parvizi et al., 2000). Various studies mentioned the vlPAG as the column associated with bladder function (Mitsui et al., 2003; Taniguchi et al., 2002; Duong et al., 1999; Matsuura et al., 1998). This has been proven by methods probing both ascending and descending tracts. Noxious stimuli at the level of the bladder increased c-Fos expression in vlPAG in rats (Mitsui et al., 2003). On the other hand,
electrical or chemical stimulation of the vlPAG in rats resulted in contraction of the bladder or increased micturition frequency (Taniguchi et al., 2002; Stone et al., 2015). Likewise, injection of inhibitory mediators into the vlPAG attenuated bladder contractions and external urethral sphincter electromyographic activity in rats (Matsura et al., 1998, 2000). Nevertheless, these experiments which stimulate the PAG and record the response in the bladder, have a major shortcoming. Stimulation of PAG has a broad spectrum of effects over autonomic and behavioral systems, which may mask true bladder-specific functionality (Nashold et al., 1969; Jenck et al., 1995). PAG activation has also been shown by fMRI studies in both storage and voiding phases of micturition (Tai et al., 2009). It can be stated, that there is a reciprocal communication pathway between the PAG and the bladder.

There is evidence for the efficacy of sacral neuromodulation for the treatment of functional pelvic disorders, like voiding dysfunction (Sukhu et al., 2016; Rosen et al., 2015; Pettit et al., 2002). In order to explore novel targets for central neuromodulatory approaches and optimize stimulation parameters to restore normal function, it is essential to unravel the functional connectivity of brain structures such as the PAG with the bladder. In our previous studies, we specified particular PAG columns which were activated after bladder stimulation, and defined specific bladder stimulation parameters (Meriaux et al., 2018). We selected stimulation parameters which would induce the micturition reflex, i.e. increase intravesical pressure, and would not arouse pain in the animal (Meriaux et al., 2018). Immunohistochemistry showed that, by using these stimulation parameters, sensory neurons were activated in the spinal cord (Meriaux et al., 2018). In the next step, we identified the cell types of the activated neurons within vlPAG (Zare et al., 2018).

After the identification of a sensory vlPAG-bladder relationship in the previous experiments we aimed at identifying the functional relationship between these two. We did so by evaluating the neurophysiological response of the vlPAG by means of extracellular single unit recordings in anesthetized rats. We hypothesized that vlPAG neurons would react to bladder stimulation, by changing their firing rate.

2. Materials and methods

2.1. Surgical preparation

Fifteen male Sprague Dawley rats (350 g) were anesthetized by intraperitoneal administration of urethane (7.5 ml/kg loading dose and 0.3 ml/repetitive dose for maintenance) from a 20% of weight urethane solution (Sigma Aldrich). Body temperature was controlled and maintained at 37 °C by means of a heating pad. A midline suprapubic incision was made and the bladder was exposed. Two unipolar ventricular pacing electrodes intended for human use (Medtronic®, Minneapolis, USA, STREAMLINE™) were fixed by means of a suture at the dome and the neck of the bladder (Fig. 1). The electrodes were then connected to a stimulator (DLS100, World Precision Instruments, Sarasota, Florida) attached to a pulse generator (DS8000, World Precision Instruments).

The rats were then mounted on a standard rat stereotaxic apparatus (Stoelting Co, Illinois, USA). A craniotomy was performed on top of the vlPAG region according to the coordinates relative to Bregma as described in a standard rat brain atlas (−6.96 mm up to −8.16 mm rostro-caudal relative to Bregma, and a maximum of 1.2 mm mediolaterally and bilateral) (Paxinos and Watson, 2007). Considerable care was taken to avoid hemorrhage from the sagittal sinus during the durotomy. To achieve this, a fine needle tip (30 G) was used to lacerate the edges of craniotomy window away from the sinus. Paw withdrawal and corneal reflexes were frequently checked throughout the experiment and additional urethane was injected if required.

2.2. Recording

We used Kwik-Fil™ filamented borosilicate glass capillaries (outer diameter: 1.5 mm, inner diameter: 0.84 mm)(World precision instruments, Sarasota, Florida) to record extracellular single cell units. The glass micropipette was pulled by a pipette puller (Narashige, Japan). Consequently, the pulled tip of the pipette was broken to a diameter of 1 to 2 μm under microscopic vision. The micropipette was backfilled with 0.5 M NaCl solution and inserted into the vlPAG to record extracellular single units. The glass pipette was descended slowly from the cortical surface as a reference point in the ventral direction by means of a hydraulic drum Microdrive (FHC, Bowdoin, ME USA) until the coordinates of the PAG in the dorsoventral direction were reached. Adequate grounding and referencing was implemented in order to reduce (environmental) noise. The micropipette was subsequently connected to an electrophysiology workstation (Alpha Omega Engineering, Nazareth, Israel). Recordings were performed on 26 neurons in total, in 15 rats, within the area of the vlPAG. A baseline recording took place after the neuron had stabilized. Subsequently recording took place until 600 action potentials had been recorded to correct for variance in firing frequency of individuals neurons. Then the detrusor was stimulated by a biphasic pulse of 20 Hz (a pulse period of 50 ms) and a pulse width of 0.5 ms (a duty cycle of 0.01) for 50 s, at an intensity of 2 mA for each neuron. These stimulation parameters were based on our previous experiments (Meriaux et al., 2018) and aimed specifically at inducing the micturition reflex. Neurons, that were lost during this period were excluded from the analysis.

2.3. Iontophoresis

After the final recording, iontophoretic injection of 4% Pontamine sky blue (SIGMA-ALDRICH) diluted in 0.5 M of NaCl was performed with the following parameters: direct current, −20 μA, with a pulse period of 14 s and a pulse width of 7 s (50% duty cycle). The current was set for 15 min using the pipette as anode (DS8000 stimulator, A360 isolator, World Precision Instruments, Sarasota, Florida) in order to verify the recording area in all animals. To give bipolar stimulation, the anode consisted of tungsten electrode inserted into the capillary glass filled with Pontamine sky blue solution, and the cathode as a stainless steel screw secured on top of the skull (1 × 2 mm, GHW Modellbau, Germany).

2.4. Histological confirmation of the electrode location

Finally, rats were decapitated, the brain was quickly removed and was frozen in −40°C 2-methyl-butane (isopentane) for further histochemical confirmation of electrode position. Brains were serially cut by
cryostat (Leica CM3050S) in 25 μm thick sections, fixed in 4% para-formaldehyde, and nuclei were visualized by means of fluorescent staining with 1:500 Hoechst solution (blue fluorescence) to facilitate neuroanatomical identification of the PAG within the brainstem. The fluorescent properties of Pontamine Sky blue, making it to appear red, were used to obtain pictures under the microscope in the blue (for background Hoechst staining) and red (for Pontamine) filter emission spectra, which were consequentially merged and the injection site was confirmed.

2.5. Analysis

For all neurons the coordinates relative to Bregma in all dimensions were checked offline again for reflecting a region within the predetermined range of the vlPAG. The analysis was performed in Spike2® (Cambridge electronic design, Cambridge, ENGLAND), SPSS® version 23 and MicroSoFt® Excel® 2016. Average neuronal firing rate was calculated during two minutes before the start of stimulation (consequently called baseline), during stimulation, and during three consecutive periods lasting one minute each, after the end of the stimulation. We have chosen to use these time periods in order to differentiate the immediate effect during the stimulation, and the immediate, intermediate and long-term result after the stimulation. For counting the firing rate during the 50 s stimulation period, the stimulation artefacts were separated and removed using Spike2® waveform analysis and differentiation tools.

Furthermore, peristimulus time histograms (PSTH) during the stimulation period were made for each individual neuron in order to evaluate the direct response of the neuron during stimulation. For this purpose, the optimal bin size was calculated for each neuron individually, as published before (Shimazaki and Shimomoto, 2007) and getting assistance from a JAVA web application (Shimazaki, 2010). This optimization resulted two bins during the 50 ms stimulation pulse period for all vlPAG recorded units. Since there were 2 bins in vlPAG recorded neurons PSTH, we considered the 2nd bin as the baseline relative to the 1st bin. Then we considered the 1st bin (which is right after the stimulation) as the reaction to the stimulation, and we compared it’s ratio to the 2nd bin. Stimulation or inhibition reaction were defined, if the 1st bin was more or less than the 2nd bin, with the associated quantification cutoff (70% difference). Tailored to our specific settings and corresponding to previous literature (Beyeler et al., 2010), we defined a responsive unit as showing at least 70% difference in the average firing rate, comparing the average frequency before and after the stimulus. Thus, excitation is defined as the average firing rate at the second bin to be less than 70% of the first bin, and inhibition as the average firing rate of the first bin to be less than 70% of the second bin. Regarding the neuron in the lateral PAG (Fig. 3b), the excitatory response was defined if the bin amplitude was more than the mean of the number of spikes recorded during the 25 ms preceding the onset of stimulation, plus twice the standard deviation of this mean (Beyeler et al., 2010).

In order to evaluate the firing properties and more specifically the regularity of the recorded neurons, interspike intervals (ISI) were calculated. ISI coefficient of variation (CoV) defined as standard deviation of ISI divided by average ISI for each neuron, was measured as an index of the regularity of firing of neuron (Holt et al., 1996).

3. Results

26 neurons recorded within the vlPAG fired with an average frequency of 4.5 ± 4.7 Hz at baseline. Their frequency ranged from 0.1 to 17.9 Hz. The relative firing rate of vlPAG neurons calculated as the fraction change of the firing rate relative to the period before stimulation, significantly decreased during the stimulation period, with a mean of 0.83 (Fig. 2). To perform a repeated measures ANOVA, we first checked for normality and then conducted a Mauchly’s test, which indicated a violation of the assumption of sphericity (p = 0.000). Hence we performed a multivariate analysis of variance (MANOVA) which resulted F(4,22) = 2.976, p = 0.042, Wilk’s Lambda = 0.649 (Fig. 2).

Follow-up test with Bonferroni correction resulted a p = 0.03 regarding the periods before and during the stimulation. Two by two follow-up comparison of other time points did not show any significance. PSTH analysis showed no reactivity in 24 out of 26 neurons within vlPAG. One neuron showed inhibitory and one excitatory response, according to the defined criteria (Section 2.5) (Fig. 3). One neuron in the lateral column of the PAG showed an excitatory response (Fig. 3B).

ISI coefficient of variation(CoV) of the recorded neurons ranged from 0.15 to 2.0 with a mean of 0.99(Fig. 4). The position of the electrodes were confirmed by microscopic observation of the Pontamine Sky Blue marking in brain sections (Fig. 5).

4. Discussion

Here we aimed for obtaining a clearer view of the role of the vlPAG in bladder function. Electrophysiological characteristics of PAG neurons were described in a number of papers considering micturition or noxious stimuli (Crook and Lovick, 2016; Sharma et al., 1999), but none of them were focusing on the vlPAG alone. Previous studies with single unit recordings in PAG showed different neuronal firing patterns during the micturition reflex within ventrolateral and lateral columns, some increasing, some decreasing and some unresponsive during micturition (Kengo and Yamamoto Gen, 2006). Since individual PAG neurons were important to us, and we had no aim of restricting this to a particular phase of the micturition cycle, for example during the filling phase or the voiding phase, we did not perform intravesical catheterization accompanied by pressure measurements. The stimulation parameters we used were based on our previous experiment which lead to bladder contraction without inducing voiding and pain (Meriaux et al., 2018). This paradigm is unique because it creates the possibility to study the reaction of the PAG without confounding factors. The diverse responses of neurons may reflect different neuronal types corresponding to different functions like detrusor contraction, sphincter relaxation or neurons with no micturition related effect. The average decrease in firing rate during the stimulation period found in our study indicates an overall inhibitory influence of the electrical bladder stimulation on vlPAG neurons.

PSTH analysis denied instant excitatory or inhibitory effect of the
stimulation on the neuronal firing in the majority of the recorded units. So at least the decrease in firing rate during the stimulation period could not be explained by connections which would involve less than 50 ms (stimulation pulse period) of signal propagation time lapse between the stimulation and the recording sites. Another explanation is that the non-responsive cells were not sensory neurons, but motor neurons or interneurons. An inhibition of vlPAG neurons via polysynaptic instead of monosynaptic connections, may also explain the decrease in average firing rate versus no overall change in PSTH. There is also the possibility that these neurons were not engaged in the physiology of bladder, since PAG has a broad spectrum of functions. To exclude nociceptive signals secondary to these stimulation parameters, behavioral tests had been done in freely moving rats (Meriaux et al., 2018). We used a different experimental paradigm in our previous experiments (Meriaux et al., 2018; Zare et al., 2018) by measuring cFos activated cells in vlPAG. Since neurochemical activation is latent, and very different from instantaneous electrophysiological responses of neurons, those cFos activated neurons (Meriaux et al., 2018; Zare et al., 2018) were most probably from different cell populations than the inhibited neurons in this work. Further experiments need to be done to combine the neurochemical and the electrophysiological properties of the cells. This includes juxtacellular labelling of the electrophysiologically responsive neurons, and identifying their neurotransmitter content. Thereafter, these identified cells may be specifically stimulated by optogenetic or chemogenetic (DREADD) techniques, and the intravesical pressure would subsequently be measured.

In parallel, we describe a simpler method of microscopic pontamine sky blue observation. Fluorescent properties of pontamine sky blue have been already used for counterstaining in immunofluorescence (Cowen et al., 1985). But to our knowledge, there was no report of using its fluorescent properties for localization of electrode position. As pontamine sky blue has a bright red fluorescence, traces of it can be easily detected by fluorescent microscopy. This would be particularly helpful when the localization needs to be in a tiny restricted area. Heavier dye injection into the region to make its detection easier by light microscopy would result in progressive dispersion of the dye to a more distant periphery of the pipette tip, decreasing the precision of the localization. Double staining with Hoechst and visualization in blue spectrum gives a scheme of the background anatomical location, which can be merged with pontamine, visible in the red spectrum. This may be a better alternative to bright-field microscopy, with a more precise localization, without the need to inject so much dye into the region (Fig. 5).

4.1. Conclusions

Reduced firing rate in the vlPAG may be due to an overall inhibitory effect of the peripheral stimulation on these neurons. One my suggest an inhibitory relaying action of the vlPAG, before sensory bladder signals would affect pontine micturition center. Diverse electrophysiological responses from the vlPAG neurons following bladder stimulation predict involvement of a heterogeneous cell population in bladder control in this region.

Conflict of interest

None.
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Fig. 5. Recording electrode position verification. Transverse sections of PAG stained with Hoechst (observed in blue spectrum) (a), to clarify the borders of PAG. The individual columns are separated according to a standard stereotactic atlas (b). Pontamine sky blue can be observed in red spectrum(c) and merged to give a composite image (d) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

b1: The vPAG area.
b2: area of the dorsal raphe and cranial nerve nuclei.
b3: Central aqueduct.
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