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Abstract

The Raphe Pallidus (RPa) is a region of the brainstem that was shown to modulate the sympathetic outflow to many tissues and organs involved in thermoregulation and energy expenditure. In rodents, the pharmacological activation of RPa neurons was shown to increase the activity of the brown adipose tissue, heart rate, and expired CO_2, whereas their inhibition was shown to induce cutaneous vasodilation and a state of hypothermia that, when prolonged, leads to a state resembling torpor referred to as synthetic torpor. If translatable to humans, this synthetic torpor-inducing procedure would be advantageous in many clinical settings. A first step to explore such translatability, has been to verify whether the neurons within the RPa play the same role described for rodents in a larger mammal such as the pig. In the present study, we show that the physiological responses inducible by the pharmacological stimulation of RPa neurons are very similar to those observed in rodents. Injection of the $GABA_A$ agonist GABAzine in the RPa induced an increase in heart rate (from 99 to 174 bpm), systolic (from 87 to 170 mmHg) and diastolic (from 51 to 98 mmHg arterial pressure, and end tidal CO_2 (from 49 to 62 mmhg). All these changes were reversed by the injection in the same area of the $GABA_A$ agonist muscimol. These results support the possibility for RPa neurons to be a key target in the research for a safe and effective procedure for the induction of synthetic torpor in humans.

Keywords

Hypothermia, Raphe Pallidus, Autonomic Nervous System, Gabazine, Synthetic Torpor, Pig

Introduction

The Raphe pallidus (RPa) is a region on the ventral surface of the lower brainstem involved in the control of the autonomic outflow to many organs[1]. Anatomically, neurons within this region have been shown to be multi-synaptically connected with many organs, such as the thyroid [2], the liver [3], the bones [4], the adrenergic cells in the adrenal gland [5], the skeletal muscles [6], the heart [7,8], the smooth muscle in the cutaneous blood vessels [9], the white adipose tissue [10], and the brown adipose tissue (BAT) [11].

The activation, or the inhibition, of the RPa neurons was shown to have relevant metabolic effects in many species. In anaesthetized rats, the activation or the disinhibition of the RPa neurons was shown to activate the sympathetic outflow to the BAT [12], and to the heart [13], to increase heart rate (HR), expired CO_2, BAT and core temperature [12,1], and to promote cutaneous vasoconstriction [14]. In free behaving rats similar effects were also reported [15]. In conscious rabbits and piglets, cutaneous vasoconstriction induced by cold exposure was prevented by the activation of $GABA_A$ [16], or 5-HT_{1A} receptors [17]. In humans, the brainstem region where the RPa is located was shown to be activated by cold exposure [18]. On the contrary, in free behaving rats, the inhibition of the RPa was reported to induce a marked vasodilation [15], hypothermia [19,15] and, when prolonged, a state of synthetic torpor [20,21]. The possibility to induce a state of synthetic torpor, characterized by the development of a deep, reversible and undefended hypothermia, can be very useful in many medical conditions, such as stroke or cardiac arrest, and it's also being considered as a mean to improve the ability of humans for prolonged space travel [21-23].

Before being tested in humans, this procedure described by Cerri and coworkers [20] should be tested in a larger animal model. Many studies have recently proposed swines as an appropriate model in many field of translational medicine [24--28.]

Here we report the results from a preparatory experiment aimed at evaluating if the effects induced by the activation of the RPa neurons in the pig are comparable with what reported from other species.

Results

Compared to baseline values, the microinjection of the $GABA_A$ antagonist GABAzine induced a significant increase in HR (from 99 ± 7 to 174 ± 26 bpm; p = 0.0005), SAP (from 87 ± 7 to 170 ± 20 mmHg; p =

0.016), DAP (from 51 ± 3 to 98 ± 14 mmHg; p = 0.0310), and ETCO2 (from 49 ± 1 to 62 ± 3 mmHg; p = 0.0042) (Figure 1). Shivering became evident shortly after the injection, affecting rapidly the entire body (Figure 1 and Figure 2). The microinjection of muscimol promptly arrested shivering, and reversed the GABAzine-induced increases in HR, SAP, and DAP, restoring values similar to those of the baseline, whereas ETCO2 levels (41 ± 1 mmHg) became significantly lower (p = 0.0136) than those of the baseline (Figure 1). No significant changes were observed in Tcore after the injection of either GABAzine or muscimol (Figure 1). In the two animals where a thermocamera was available, the ear pinna temperature decreases after the injection of GABAzine but rapidly increases after the injection of muscimol (see supplementary information).

Discussion

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The main result of this study is the evidence that the activation of neurons in the medullary Raphe region in the pig induces an increase in HR, AP, ETCO2 and shivering, very similar to those previously observed in rodents [12,15].

In particular, the increase in $ETCO_2$ is suggestive of an increase in metabolic rate driven by the activated neurons within the RPa. The increase in both SAP and DAP appears to be larger than that commonly observed in anaesthetized rodents [12]. It is possible to interpret such findings hypothesizing that GABAzine diffused to neighborhood region critically involved in the control or arterial pressure, such as the rostral ventromedial medulla (RVLM). In rodents, the activation of RVLM neurons was in fact shown to evoke a drastic increase in AP [29]. The large doses of the GABAzine used in the present study may sustain such interpretation, but, if this was the case, a drastic drop in AP below the baseline level should have been expected after the muscimol injection. Interspecies variability or interaction with some of the drug used during the procedure (fentanyl) are other possible explanations of these data. Shivering was also very promptly triggered by the injection of GABAzine and equally promptly stopped by the injection of muscimol. In every animal, shivering was clearly visible and generalized to the entire body.

The increase in HR, SAP and DAP, ETCO2 and shivering which resulted from the disinhibition of RPa neurons was promptly reversed by the injection of the $GABA_A$ agonist muscimol. ETCO2 levels drop to a value significantly lower than that of the baseline. This effect suggests that the inhibition of RPa neurons may cause a reduction in metabolic rate. If this was the case, this observation would strengthen the possibility to induce synthetic torpor in the pig by inhibiting RPa neurons.

In conclusion, the presented date show that, in anaesthetized pigs, the effects induced by the disinhibition of neurons within the RPa region are very similar to what has been reported for rodents.

Methods

Ethical Approval

All procedures performed in studies involving animal were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The experiments were performed at the Physiology facility of the Department of Veterinary Medical Sciences of the University of Bologna. The study was conducted according to relevant national and international guidelines on Animal Experimentations. The procedure was reviewed and approved in advance by the Scientific Ethics Committee for Animal Experimentation of the University of Bologna and by the Italian Ministry of Public Health. All the experiments were carried out under the supervision of the Veterinary Services of the University of Bologna.

Animal care

In this study, 4 female pigs (35-70 Kg) were used. The sedative, anesthesiological and analgesic protocol was implemented according to the requests from the Local Ethical Committee for animal Care to reduce as much as possible animal suffering. Some of the requested drugs that were used, such as the fentanyl , are known to induce autonomic effects [30]. To reduce the confounding effects of such drugs, that were infused continuously during the experiment, the experimental plan was structured so to use each animals as control of itself, by comparing pre-injection with post-injection parameters.

On the day of the experiment, animals were administered with an intramuscular (IM) injection of tiletamine-zolazepam (5 mg/kg; Zoletil, Virbac, FR). Approximately 15 minutes later, general anaesthesia was induced by an injection of thiopental sodium (5 mg/kg; Pentothal Sodium, MSD, IT) through a catheter in the auricular vein. After oro-tracheal intubation, anaesthesia was maintained with isoflurane (2.5%, Isoflo, Esteve, IT) in a 1:1 mixture of oxygen and air. Throughout the entire procedure, animals were administered with a Constant Rate Infusion of fentanyl (10 mcg/kg/h, Fentanest, Pfizer, US) to ensure analgesia, alongside with regular fluid therapy (Lactated Ringer, 10 ml/kg/h). An intra-arterial catheter was implanted in the femoral artery and

connected to the anesthesia delivery unit (ADU) in order to monitor invasive blood pressure. A rectal thermocouple probe, connected to the same ADU, was used to monitor the core temperature (Tcore). Other standard parameters including: oxygen saturation, $ETCO_2$, HR, ECG, respiratory rate, and non-invasive blood pressure were constantly monitored. Body temperature was maintained around 37°C by the use of a forced air-warming device.

Surgery

Animals were positioned in ventral position; the head was fixed with tapes and slightly flexed. After a linear occipito-cervical incision, the fascia was identified and opened. The underlying muscular structures were dissected, and the periosteum of the occipital bone was exposed from the lambda to the foramen magnum. The periosteum was then opened and the posterior arch of the first cervical vertebra (C1) exposed. A complete C1 laminectomy was performed and the dura exposed. A small occipital craniectomy was obtained by drilling and by use of Kerrison rongeur. Particular attention was directed in preserving the integrity of the dura (especially near the cranio-cervical passage and far from the midline) to avoid the damage of large venous dural sinuses. The dura was opened with a small vertical incision at the level of the foramen magnum; the dural edges was suspended with retraction sutures to the muscle, the arachnoid of the cisterna magna was opened and a progressive cerebrospinal fluid withdrawal was achieved. The cerebellar tonsils were gently dissected and retracted cranially with the aid of cottonoids, to visualize the foramen of Magendie and the fourth ventricle floor.

Microinjection

Since the size of the skull in the pig can vary in a relevant way with body weight, the use of the available stereotaxic atlas of the pig brain was not reliable. To therefore identify a responsive area, an injection of the $GABA_A$ antagonist GABAzine (10 mmol; 30µL) was performed, starting with the following set of coordinates from the calamus scriptorius used as reference point and with the head tilted of 45° from the plane of the dorsal surface of the fourth ventricle : +0,5 mm on the anteroposterior axis, 0 mm on the latero-lateral axis, -17 mm on the dorsoventral axis. If no effects were induced, the anteroposterior coordinate was increased by 0.5 mm. A response was always obtained within the 4th injection. A period of at least 30 minutes was waited between each injections.

Initially, the dose of microinjected GABAzine was derived by the dosage/Kg used in rats (5pmol/100 nl), but very almost no effects were evoked. Since accordingly to several authors [31-33], the dosage of drugs to be used in piglets is larger than that calculated by the simple adjustment by their weight, the dose of GABAzine injected was therefore increased.

After an effective GABAzine injection, and at the peak of such response (approx 15 to 30 minutes after the injection), the $GABA_A$ agonist muscimol (10µL, 10mmol) was injected in the same location using a 100-µL syringe (Hamilton).

Shivering was visually assessed during the experiment by an operator according to the following arbitrary unit (AU): 0 = no shivering; 10 = modest shivering in the region of the nuchal muscle; 20 = shivering of the nuchal muscle and of the anterior limbs; 30 = extensive shivering of the entire body.

In two animals, a thermocamera was used to assess the cutaneous vasodilatory response to the injections. Representative images are available in the supplementary information section (Supplementary Figure 1)..

At the conclusion of the experiment, the injection site was marked with fluorescent microbeads. Unfortunately, most the fluorescence was washed away during the fixation process, but a small microsphere residue was still visible in the targeted area (Figure 3).

At the end of the experiment, all animals were euthanized with an IV injection of Tanax (0.3 ml/kg, Intervet Italia, IT). The brainstem was removed and fixed for 10 days with 4% paraformaldehyde and then cryoprotected (30% 200 g/L sucrose) until sinking. The medulla was cut (60 µm) coronally on a cryostat and the location of the injection sites was visually verified..

All results are shown as mean ± SEM. Baseline values were calculated as the average of the 10-min period preceding GABAzine injection. Post GABAzine-injection values were calculated as the average of the 10-min period preceding muscimol injection. Post muscimol-injection values were calculated as the average of the 10-min period which started 20 minutes after muscimol injection. The effects induced by the microinjection of either GABAzine or muscimol were statistically analyzed by comparing the post-injection levels of each variable with those of the baseline by means of a paired Student's t-test with significance at $p < 0.05$.

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Figure legends

Figure 1 shows the 10-min average values (± SEM) of shivering, heart rate (HR), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), end-tidal CO_2 (ETCO2), core temperature (Tcore), and shivering at the peak of the effects induced by the injection within the Raphe pallidus of the $GABA_A$ antagonist GABAzine (black bar), and 20 minutes after the subsequent injection in the same region of the $GABA_A$ agonist muscimol (gray bar). Results are compared with those observed immediately before the first injection (Baseline, white bar). AU = arbitrary unit

* = $p < 0.05$ GABAzine vs baseline, ** = $p < 0.01$ GABAzine vs baseline, # = $p < 0.05$ muscimol vs baseline..

Figure 2 shows an example of the effects induced on shivering, heart rate (HR), systolic (thick line) and diastolic (thin line) arterial pressure (AP), end-tidal CO_2 (ETCO2), and core temperature (Tcore) by the injection of the $GABA_A$ antagonist GABAzine in two non-responsive sites (first two arrows) and in a third responsive site (third dashed line) followed by the $GABA_A$ agonist muscimol (fourth dashed line) within the Raphe pallidus. .

Figure 3 shows an example of the location of an injection site. On the left is visible a brainstem section. The area delimited by the red rectangle is magnified on the right side. A residue of fluorescent microsphere is visible on the region where the Raphe Pallidus is located.

Supporting Informations:

Supplementary Figure 1

Supplementary figure 1 shows representative images from a thermal camera recording showing ear pinna (arrow) cutaneous temperature before the injection of GABAzine within the Raphe Pallidus (panel A), after the injection of the GABA-A antagonist GABAzine (panel B), and after the injection in the same location of the GABA-A agonist muscimol (panel C).