

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

Autonomic effects induced by pharmacological activation and inhibition of Raphe Pallidus neurons in anaesthetized adult pigs

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Zucchelli M., Bastianini S., Ventrella D., Barone F., Elmi A., Romagnoli N., et al. (2020). Autonomic effects induced by pharmacological activation and inhibition of Raphe Pallidus neurons in anaesthetized adult pigs. *CLINICAL AND EXPERIMENTAL PHARMACOLOGY & PHYSIOLOGY.*, 47(2), 281-285 [10.1111/1440-1681.13194].

Availability:

This version is available at: <https://hdl.handle.net/11585/710227> since: 2020-01-09

Published:

DOI: <http://doi.org/10.1111/1440-1681.13194>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

Autonomic effects induced by pharmacological activation and inhibition of Raphe Pallidus neurons in anaesthetized adult pigs

Mino Zucchelli¹, Stefano Bastianini¹, Domenico Ventrella², Francesca Barone², Alberto Elmi², Noemi Romagnoli², Timna Hitrec³, Chiara Berteotti³, Alessia Di Cristoforo³, Marco Luppi³, Roberto Amici³, Maria L. Bacci², Matteo Cerri³

Laboratory or Institute

¹ Neurosurgery, IRCCS Istituto delle Scienze Neurologiche di Bologna - Bellaria Hospital, Bologna, Italy

² Department of Veterinary Medical Sciences, Alma Mater Studiorum - University of Bologna

³ Department of Biomedical and NeuroMotor Sciences, Alma Mater Studiorum - University of Bologna

Short title: Raphe Pallidus stimulation in pigs

Author for correspondence:

Matteo Cerri - Department of Biomedical and NeuroMotor Sciences - Alma Mater Studiorum - University of Bologna - Piazza di Porta S.Donato, 2 40126 Bologna Italy - email: matteo.cerri@unibo.it - Phone: +39 051 2091731 - Fax: +39 051 2091737

Current Authors Addresses

Matteo Cerri, Roberto Amici, Marco Luppi, Chiara Berteotti, Timna Hitrec, Alessia Di Cristoforo, Stefano Bastianini: Department of Biomedical and NeuroMotor Sciences - Alma Mater Studiorum - University of Bologna - Piazza di Porta S.Donato, 2 40126 Bologna - Italy

Mino Zucchelli: Neurosurgery, IRCCS Istituto delle Scienze Neurologiche di Bologna
Bellaria Hospital, Bologna, Italy

Domenico Ventrella, Maria Laura Bacci, Noemi Romagnoli, Alberto Elmi, Francesca Barone:
Department of Veterinary Medical Sciences - Alma Mater Studiorum - University of Bologna - Via Tolara di Sopra, 50 40064, Ozzano dell'Emilia - ITALY

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 CO₂, 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 CO₂ (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 organs¹. Anatomically, neurons within this region have been shown to be multi-synaptically connected with many organs, such as the thyroid², the liver³, the bones⁴, the adrenergic cells in the adrenal gland⁵, the skeletal muscles⁶, the heart^{7,8}, the smooth muscle in the cutaneous blood vessels⁹, the white adipose tissue¹⁰, 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 BAT¹², and to the heart¹³, to increase heart rate (HR), expired CO₂, BAT and core temperature^{12,1}, and to promote cutaneous vasoconstriction¹⁴. In free behaving rats similar effects were also reported¹⁵. In conscious rabbits and piglets, cutaneous vasoconstriction induced by cold exposure was prevented by the activation of GABA_A¹⁶, or 5-HT_{1A} receptors¹⁷. In humans, the brainstem region where the RPa is located was shown to be activated by cold exposure¹⁸. On the contrary, in free behaving rats, the inhibition of the RPa was reported to induce a marked vasodilation¹⁵, 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 travel²¹⁻²³.

Before being tested in humans, this procedure described by Cerri and coworkers²⁰ should be tested in a larger animal model. Many studies have recently proposed swines as an appropriate model in many field of translational medicine²⁴⁻⁻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 GABA_A-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 T_{core} after the injection of either GABA_A or muscimol (Figure 1). In the two animals where a thermocamera was available, the ear pinna temperature decreases after the injection of GABA_A but rapidly increases after the injection of muscimol (see supplementary information).

Discussion

f

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 ETCO₂ 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 rodents¹². It is possible to interpret such findings hypothesizing that GABA_A diffused to neighborhood region critically involved in the control of 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 AP²⁹. The large doses of the GABA_A 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 GABA_A 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 data 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 effects³⁰. 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 (T_{core}). 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 were 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; $30\mu\text{L}$) 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 GABA_Azine was derived by the dosage/Kg used in rats (5pmol/100 nl), but very almost no effects were evoked. Since accordingly to several authors³¹⁻³³, the dosage of drugs to be used in piglets is larger than that calculated by the simple adjustment by their weight, the dose of GABA_Azine injected was therefore increased.

After an effective GABA_Azine 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 GABA_Azine injection. Post GABA_Azine-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 GABA_Azine 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 $p < 0.05$.

Acknowledgment

The research was funded by the Ministero della Salute, grant number GR-2011- 02352269/Ricerca Finalizzata.

References

1. Morrison SF, Madden CJ, Tupone D. Central neural regulation of brown adipose tissue thermogenesis and energy expenditure. *Cell Metab.* 2014;19(5):741-756.
2. Kalsbeek A, Fliers E, Franke AN, Wortel J, Buijs RM. Functional connections between the suprachiasmatic nucleus and the thyroid gland as revealed by lesioning and viral tracing techniques in the rat. *Endocrinology.* 2000;141(10):3832-3841.
3. Kalsbeek A, La Fleur S, Van Heijningen C, Buijs RM. Suprachiasmatic GABAergic inputs to the paraventricular nucleus control plasma glucose concentrations in the rat via sympathetic innervation of the liver. *J Neurosci.* 2004;24(35):7604-7613.
4. Denes A, Boldogkoi Z, Uherezky G, et al. Central autonomic control of the bone marrow: multisynaptic tract tracing by recombinant pseudorabies virus. *Neuroscience.* 2005;134(3):947-963.
5. Morrison SF, Cao WH. Different adrenal sympathetic preganglionic neurons regulate epinephrine and norepinephrine secretion. *Am J Physiol Regul Integr Comp Physiol.* 2000;279(5):R1763-1775.
6. Billig I, Foris JM, Card JP, Yates BJ. Transneuronal tracing of neural pathways controlling an abdominal muscle, rectus abdominis, in the ferret. *Brain Res.* 1999;820(1-2):31-44.
7. Standish A, Enquist LW, Escardo JA, Schwaber JS. Central neuronal circuit innervating the rat heart defined by transneuronal transport of pseudorabies virus. *J Neurosci.* 1995;15(3 Pt 1):1998-2012.
8. Ter Horst GJ, Van den Brink A, Homminga SA, et al. Transneuronal viral labelling of rat heart left ventricle controlling pathways. *Neuroreport.* 1993;4(12):1307-1310.
9. Rathner JA, McAllen RM. Differential control of sympathetic drive to the rat tail artery and kidney by medullary premotor cell groups. *Brain Res.* 1999;834(1-2):196-199.

10. Nguyen NL, Randall J, Banfield BW, Bartness TJ. Central sympathetic innervations to visceral and subcutaneous white adipose tissue. *Am J Physiol Regul Integr Comp Physiol.* 2014;306(6):R375-386.
11. Cano G, Passerin AM, Schiltz JC, Card JP, Morrison SF, Sved AF. Anatomical substrates for the central control of sympathetic outflow to interscapular adipose tissue during cold exposure. *J Comp Neurol.* 2003;460(3):303-326.
12. Morrison SF, Sved AF, Passerin AM. GABA-mediated inhibition of raphe pallidus neurons regulates sympathetic outflow to brown adipose tissue. *Am J Physiol.* 1999;276(2 Pt 2):R290-297.
13. Cao WH, Morrison SF. Disinhibition of rostral raphe pallidus neurons increases cardiac sympathetic nerve activity and heart rate. *Brain Res.* 2003;980(1):1-10.
14. Blessing WW. Lower brainstem pathways regulating sympathetically mediated changes in cutaneous blood flow. *Cell Mol Neurobiol.* 2003;23(4-5):527-538.
15. Cerri M, Zamboni G, Tupone D, et al. Cutaneous vasodilation elicited by disinhibition of the caudal portion of the rostral ventromedial medulla of the free-behaving rat. *Neuroscience.* 2010;165(3):984-995.
16. Ootsuka Y, Blessing WW. Inhibition of medullary raphe/parapyramidal neurons prevents cutaneous vasoconstriction elicited by alerting stimuli and by cold exposure in conscious rabbits. *Brain Res.* 2005;1051(1-2):189-193.
17. Brown JW, Sirlin EA, Benoit AM, Hoffman JM, Damall RA. Activation of 5-HT1A receptors in medullary raphe disrupts sleep and decreases shivering during cooling in the conscious piglet. *Am J Physiol Regul Integr Comp Physiol.* 2008;294(3):R884-894.

18. McAllen RM, Farrell M, Johnson JM, et al. Human medullary responses to cooling and rewarming the skin: a functional MRI study. *Proc Natl Acad Sci U S A*. 2006;103(3):809-813.
19. Zaretsky DV, Zaretskaia MV, DiMicco JA. Stimulation and blockade of GABA(A) receptors in the raphe pallidus: effects on body temperature, heart rate, and blood pressure in conscious rats. *Am J Physiol Regul Integr Comp Physiol*. 2003;285(1):R110-116.
20. Cerri M, Mastrotto M, Tupone D, et al. The inhibition of neurons in the central nervous pathways for thermoregulatory cold defense induces a suspended animation state in the rat. *J Neurosci*. 2013;33(7):2984-2993.
21. Cerri M. The Central Control of Energy Expenditure: Exploiting Torpor for Medical Applications. *Annu Rev Physiol*. 2017;79:167-186.
22. Gemignani J, Gheysens T, Summerer L. Beyond astronaut's capabilities: The current state of the art. *Conf Proc IEEE Eng Med Biol Soc*. 2015;2015:3615-3618.
23. Cerri M, Tinganelli W, Negrini M, et al. Hibernation for space travel: Impact on radioprotection. *Life Sci Space Res (Amst)*. 2016;11:1-9
24. Vodicka P, Smetana K, Jr., Dvorankova B, et al. The miniature pig as an animal model in biomedical research. *Ann N Y Acad Sci*. 2005;1049:161-171.
25. Lind NM, Moustgaard A, Jelsing J, Vajta G, Cumming P, Hansen AK. The use of pigs in neuroscience: modeling brain disorders. *Neurosci Biobehav Rev*. 2007;31(5):728-751.
26. Swindle MM, Makin A, Herron AJ, Clubb FJ, Jr., Frazier KS. Swine as models in biomedical research and toxicology testing. *Vet Pathol*. 2011;49(2):344-356.

27. Verma N, Rettenmeier AW, Schmitz-Spanke S. Recent advances in the use of *Sus scrofa* (pig) as a model system for proteomic studies. *Proteomics*. 2011;11(4):776-793.
28. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerds V. The pig: a model for human infectious diseases. *Trends Microbiol*. 2012;20(1):50-57.
29. Natarajan M, Morrison SF. Adrenal epinephrine secretion is not regulated by sympathoinhibitory neurons in the caudal ventrolateral medulla. *Brain Res*. 1999;827(1-2):169-175.
30. Cao WH, Morrison SF. Brown adipose tissue thermogenesis contributes to fentanyl-evoked hyperthermia. *Am J Physiol Regul Integr Comp Physiol*. 2005;288(3):R723-732.
31. Messier ML, Li A, Nattie EE. Inhibition of medullary raphe serotonergic neurons has age-dependent effects on the CO₂ response in newborn piglets. *J Appl Physiol (1985)*. 2004;96(5):1909-1919.
32. Darnall RA, Harris MB, Gill WH, Hoffman JM, Brown JW, Niblock MM. Inhibition of serotonergic neurons in the nucleus paragigantocellularis lateralis fragments sleep and decreases rapid eye movement sleep in the piglet: implications for sudden infant death syndrome. *J Neurosci*. 2005;25(36):8322-8332.
33. Hoffman JM, Brown JW, Sirlin EA, et al. Activation of 5-HT_{1A} receptors in the paragigantocellularis lateralis decreases shivering during cooling in the conscious piglet. *Am J Physiol Regul Integr Comp Physiol*. 2007;293(1):R518-527.

Figure legends

Figure 1 shows the 10-min average values (\pm SEM) of shivering, heart rate (HR), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), end-tidal CO₂ (ETCO₂), core temperature (T_{core}), 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 CO₂ (ETCO₂), and core temperature (T_{core}) 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).