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The rostral ventro-medial medulla (RVM) control of cutaneous vasomotion of paws and tail in the rat. Implication for pain studies.

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This paper is dedicated to our colleague and friend Bernard Pollin, who passed away while our work was under redaction and whose contributions to this work were invaluable.

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ABSTRACT

Thermal neutrality in rodents is achieved by large cyclic variations of the sympathetic drive of the vasomotion of the tail and paws, the most widely used target organs in current acute or chronic animal models of pain. Given the pivotal functional role of rostral ventro-medial medulla (RVM) in nociception and rostral Medullary Raphe (rMR) in thermoregulation, two largely overlapping brain regions, we aimed at circumscribing the brainstem regions that are the source of premotor afferents to sympathetic preganglionic neurons that control the vasomotor tone of the tail and hind-paws. A thermometric infrared camera recorded indirectly the vasomotor tone of the tail and hind-paws. During the control period, the rat was maintained in vasoconstriction by preserving a stable, homogeneous and constant surrounding temperature, slightly below the core temperature. The functional blockade of the RVM/rMR by the $GABA_A$ receptor agonist muscimol (0.5 nmol, 50 nl) elicited an extensive increase of the temperature of the paws and tail, associated to slight decrease of blood pressure and heart rate. Both the increased heat loss through vasodilatation and the decrease heart-induced heat production elicited a remarkable reduction of the central temperature. The effective zones were circumscribed to the parts of the RVM/rMR facing the facial nucleus. They match very exactly the brain regions often described as specifically devoted to the control of nociception. Our data support and urge on the highest cautiousness regarding the interpretation of results aimed at studying the effects of any pharmacological manipulations of RVM/rMR with the usual tests of pain.

INTRODUCTION

Numerous studies have explored the descending systems that control the spinal transmission of nociceptive messages, notably through the Rostral Ventromedial Medulla (RVM) in the brainstem. The RVM includes the nucleus raphe magnus and the neighboring part of the reticular formation, which extends under the gigantocellular reticular nucleus (Fields et al. 2006). Two main categories of neurons, so-called "on-" and "off-" cells, were highlighted that: (1) exhibit irregular spontaneous activities in opposition of phase (Barbaro et al. 1989) and (2) are activated and inhibited by nociceptive stimuli, respectively (Fields et al. 1983; Vanegas et al. 1984). These two neuronal types were proposed to belong to a double spino-bulbo-spinal positive feedback loop that facilitates the spinal transmission of nociceptive messages, either directly or through a mechanism of disinhibition, the "on-" and "off-" cells being supposed to produce an excitatory and an inhibitory drive on the spinal neurons, respectively.

However, RVM neurons could play a much less specific role than that of a descending pathway modulating nociception at spinal level (Le Bars et al. 2001; Lefler et al. 2008; Lovick 1997; Mason 2001; 2005a; 2005b; 2006; 2011; 2012; Thurston and Helton 1996; Thurston and Randich 1992; 1995). For example, we described recently the probable involvement of "on-" and "off-" cells in autonomic regulation, notably thermoregulation (El Bitar et al. 2014b).

On the basis of three animal models used in thermoregulation studies, namely the tail of the rat, the ear of the rabbit and the interscapular brown adipose tissue (BAT) of the rat, it became apparent that neurons in the midline region of the ventral medulla play a critical role in responding to cold (Blessing et al. 1999; McAllen et al. 2010, Morrison 1999, 2011; Morrison et al. 1999, 2008, 2012; Nakamura 2011; Nakamura and Morrison 2008, 2011; Rathner et al. 2001; Rathner and McAllen 1999; Tanaka et al. 2002). Broadly, cooling the animal results in (1) reduced heat loss through increased skin vasoconstriction of the ear in the rabbit or the tail in the rat and (2) increased heat production by BAT in the rat. These phenomena are triggered by activation of sympathetic preganglionic neurons in the spinal intermediolateral cell column, themselves under the control of premotor neurons located in the rostral Medullary Raphe (rMR), a region immediately rostral to the rostral pole of the inferior olivary complex, that includes the raphe pallidus, the raphe magnus and the laterally extending parapyramidal nucleus (Blessing 2003; McAllen et al. 2010; Morrison 2011; Nakamura 2011; Nakamura and Morrison 2008, 2011), where the expression of Fos immunoreactivity is increased following cold exposure (Bonaz and Taché 1994; Cano et al. 2003; Morrison et al. 1999; Nakamura et al., 2004).

Yet a possible discrepancy was noted. Studies focusing on the descending systems postulated to control nociception emphasize a region which includes the nucleus raphe magnus and the gigantocellular reticular nucleus pars alpha, mainly at the level of the facial nucleus (e.g. Brink et al. 2006; Carlson et al. 2007; Fields et al. 1995; Heinricher and Kaplan 1991; Heinricher and Tortorici 1994; Kaplan and Fields 1991; Morgan and Fields, 1994; Neubert et al. 2004; Vanegas et al. 1984; Thurston and Helton, 1996; Thurston and Randich, 1995; Xu et al. 2007), while studies focusing on thermoregulation emphasize a slightly more caudal region including the raphe pallidus (e.g. Blessing and Nalivaiko, 2001; Cao and Morrison 2003; Cao et al. 2004, 2010; Cerri et al. 2010; Fan et al. 2007; Madden and Morrison 2003, 2005;

Morrison 1999, 2003, 2004; Morrison et al. 1999, 2000; Nakamura and Morrison 2007, 2011; Nakamura et al. 2004; Ootsuka and McAllen 2005; Rathner et al. 2001, 2008; Salo et al. 2009; Tanaka et al. 2002, 2007; Yoshida et al. 2003).

The aim of the present study was therefore to map exactly the brainstem regions that contain the premotor sympathetic neurons controling the vasomotor tone of the tail and hind-paws of the rat, and to compare these locations with that of nociception-related brainstem areas. This choice was made specifically because many behavioral models of pain/nociception are based on the assessment of the response of a rodent to a thermal stimulus applied to the tail or a hind-paw (reviewed in Le Bars et al. 2001, 2009).

Specifically, we measured in 160 adult male Sprague-Dawley rats, the dynamic changes in vasomotor tone induced by microinjections of muscimol within these regions. During the injection, the rats were maintained in a stable state of tail and hind-paw vasoconstriction. Microinjection of muscimol within the RVM/rMR was expected to elicit vasodilation of the tail and hind-paw. When a vasomotor response was present, the latency at which vasodilation occurred was used as an index of the distance between the injection site and the structure generating the response.

GLOSSARY

7	*Facial nucleus*
α	*Slope of the squared temperature variation elicited by a radiant heat source ($°C^2/s$)*
BAT	*Brown adipose tissue*
bpm	*Beats per minute*
ΔT_{skin}	*Amplitude of the skin temperature variation (°C)*
Δt	*Duration of the ascending phases of vasodilatation ($= t_{max} - t_{min}$)*
$ETCO_2$	*End-tidal CO_2*
GiA	*Gigantocellular reticular nucleus pars alpha*
HLI	*Heat Loss Index*
HR	*Mean heart rate (bpm)*
LPGi	*lateral paragigantocellular nucleus.*
MAP	*Mean arterial blood pressure (mmHg)*
ml	*Medial lemniscus*
paw-contra	*Mid-plantar area on the hind-paw, contralateral to the injection site*
paw-ipsi	*Mid-plantar area on the hind-paw, ipsilateral to the injection site*
PPy	*Parapyramidal nucleus*
py	*Pyramidal tract*
RMg	*Raphe magnus nucleus*
rMR	*Rostral Medullary Raphe*
RPa	*Raphe pallidus nucleus*

ROI	*Region of interest*
RVM	*Rostral Ventro-medial Medulla*
RVM/rMR	*Combination of RVM and rMR*
s	*spread of the sigmoid curve*
t_{min}	*Beginning time of the ascending phase of vasodilatation (min)*
t_{max}	*End time of the ascending phase of vasodilatation (min)*
t_x	*Abscissa of the inflection point of Boltzmann sigmoid (min)*
T_{adj}	*Sigmoid Boltzmann curve adjusted to the recorded temperature*
T_{amb}	*Ambient temperature (°C)*
T_{core}	*Core body temperature (°C)*
T_{max}	*Adjusted maximal skin temperature at the end of the process of vasodilatation (°C)*
T_{min}	*Adjusted skin temperature during the control period (°C)*
$T_{paw\text{-}contra}$	*Temperature of the plantar aspect of the hind-paw, contralateral to the injection site (°C)*
$T_{paw\text{-}ipsi}$	*Temperature of the plantar aspect of the hind-paw, ipsilateral to the injection site (°C)*
T_{skin}	*Temperature of the skin (°C)*
$T_{tail\text{-}dist}$	*Temperature of the distal part of the tail (°C)*
$T_{tail\text{-}mid}$	*Temperature of the mid part of the tail (°C)*
$T_{tail\text{-}prox}$	*Temperature of the proximal part of the tail (°C)*
T_x	*Ordinate of the inflection point of Boltzmann sigmoid (°C)*
tail-dist	*Distal area of the tail, located at 3 cm from the tip*
tail-mid	*Intermediate area of the tail, located at mid-tail*
tail-prox	*Proximal area of the tail, located at 3 cm from the root of the tail*
TFL	*Tail-flick latency (s)*
$T_{tail\text{-}mid}$	*Temperature of the mid part of the tail (°C)*
$T_{tail\text{-}prox}$	*Temperature of the proximal part of the tail (°C)*
T_{warm}	*Warming temperature (°C)*
x	*latero-lateral coordinate with reference to the interaural line*
y	*ventro-dorsal coordinate with reference to the interaural line*
z	*rostro-caudal coordinate with reference to the interaural line*

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METHODS

Ethic statement

Animal experiments were performed with permission of the Board of the Veterinarian Services of the French Ministry of Agriculture (permit number 75-151) in accordance with the National Institute of Health's "Guide for the care and use of Laboratory animals", the European Communities Council Directive 86/609/EEC regulating animal research, and the ethics committee of the International Association for the Study of Pain (Covino et al. 1980; Zimmermann 1983). The Committee of Ethics for the Animal Experiment of our Institution approved the procedures.

Animals

Experiments were performed on 160 adult male Sprague-Dawley rats (Janvier Labs, Saint-Berthevin, France) weighing 320-370 g. They were housed in groups of 3-4 per cage, allowed free access to food and water with a 12 h alternating light-dark cycle, and acclimatized to the laboratory for at least one week before the experiment. The experiments were conducted between 9 AM and 5 PM.

Experimental procedure

The animals were deeply anesthetized with 2.5% halothane in 100% oxygen. A tracheal cannula was inserted and the ventilation was controlled mechanically with an open circuit respirator equipped with a scavenging system, at a rate of 50 breaths/min. The tidal volume was adjusted to maintain a normal end-tidal CO_2 ($ETCO_2$). The expiratory halothane level and $ETCO_2$ were assessed with a capnometer (Capnomac II, Datex Instruments, Helsinki, Finland), recorded each ten seconds and under control of alarms throughout the experiment. The mean arterial blood pressure (MAP) and heart rate (HR) were monitored continuously via a catheter inserted into the common carotid artery and connected to a computer via a transducer. MAP and HR were calculated and recorded using the NOTOCORD® blood pressure analyzer system.

The body of the animal was wrapped up in a water-warming pad connected to an extra-capacity water circulator (TP 220-Kent, scientific corporation) sparing the head, the paws and the tail. The heating blanket was covered with an isothermic metalized polyester film ("survival blanket") to stabilize the space temperature around the body. A two channel OMEGA® HH506RA digital thermometer and two VIP-T-CT25515 Probes (0.1°C resolution) were used to measure the core temperature T_{core} (throughout a rectal probe inserted 10 cm) and the heating temperature T_{warm} (probe placed between the warming pad and the trunk of the rat). We adjusted T_{warm} in order to maintain (1) T_{core} stable within normal physiological values and (2) the tail and hind-paws in vasoconstriction.

The rat was mounted on a Horsley-Clarke stereotaxic frame. 0.5 ml of xylocaine 2% was injected subcutaneously in the scalp, followed by a 2-cm midline incision. After trepanation, a small incision of the dura-mater was made to introduce the tip of a microinjection glass needle.

After surgery, halothane was decreased to 0.8-0.9% with oxygen being kept at 100%, the tidal volume was adjusted to keep the $ETCO_2$ around 3.5% and at least 30-min were waited before starting the

148 experimental procedure. After 15-min of control period in steady vasoconstriction, 57 ng (50 nl) muscimol
149 were injected in the RVM/rMR (target zone between -0.8 and -3.3 rostral to inter-aural line) over 60-
150 seconds. Since RVM and rMR largely overlap, we will refer to "RVM/rMR" for brain regions that include
151 the raphe pallidus, raphe magnus, parapyramidal nucleus and the reticular formation that extends under the
152 gigantocellular reticular nucleus (Mason 2001, 2005a, 2005b, 2006, 2011). Control experiments were
153 conducted with the same procedure, except that muscimol was injected outside the RVM/rMR.
154
155 ***Experimental conditions***
156 The experiments were made in anesthetized rat with unremitting halothane level [0.85 (0.83-
157 0.86)%], while ventilation was controlled in order to achieve a stable and normal acid-base equilibrium
158 during the control period $ETCO_2$ = 3.65 (3.57-3.73)%. The mean room temperature T_{amb} was stable at 24.2
159 (24.1-24.4)°C. The paws and tail of the rat were maintained in vasoconstriction during the control period by
160 preserving a stable, homogeneous and constant surrounding temperature [T_{warm} = 37.4 (37.2-37.5)°C], ≈
161 0.3°C below T_{core} [37.7 (37.6-37.8)°C], and kept constant till the end of the experiment. In the control period,
162 MAP and HR were 83.9 (77.4-90.4) mmHg and 321 (309-332) bpm, respectively.
163
164 ***Muscimol preparation***
165 We prepared muscimol solution in a concentration of 0.01 nmol/nl, added pontamine sky blue to
166 identify the site of injection, fractioned the solution in 2 µl aliquots for single use and preserved them at -
167 20°C. Muscimol produces a rapid and persistent hyperpolarization of neurons (Hikosaka and Wurtz 1985;
168 Martin and Ghez 1999) based on its high affinity and selectivity for the $GABA_A$ receptor (Beaumont et al.
169 1978; Enna and Snyder 1975; Gallagher et al. 1983; Krogsgaard-Larsen et al. 1977; Nicholson et al. 1979).
170 The day of the experiment, we filled the circuit of the glass needle with paraffin to wash out air bubbles,
171 aspirated 0.5 µl of muscimol solution at the tip of the needle using a 1-µl Hamilton syringe and introduced
172 the needle in the brain at the end of the surgery..
173
174 ***Thermographic recordings***
175 Under stable environmental temperature, skin temperature is a reliable indicator of skin blood flow
176 variations (El Bitar et al. 2014a, Hertzman 1953). Heat transmission from deeper tissue to the skin occurs by
177 conduction and vascular convection, and then heat dissipation to the environment through conduction,
178 convection, evaporation and radiation processes. The spatial and temporal evolution of the skin temperature
179 at the level of the tail and paws was monitored using a JADE MWIR (3-5 µm optical bandpass) camera
180 (CEDIP Infrared Systems, Croissy-Beaubourg, France) with a 500 µs integration time, which supplied
181 images of 320x240 pixels at 1 Hz with a sensitivity of 0.02°C at 25°C. The camera was placed 1.5 m upright
182 to the scene and was controlled by the software Cirrus© (CEDIP Infrared Systems, Croissy-Beaubourg,
183 France). It was calibrated by means of a black body as previously described (Benoist et al. 2008).
184 Recently, we verified the highly significant positive linear correlation between skin temperature and
185 skin blood flow measured by a laser-Doppler probe. The mean time lag between vasodilatation and skin

temperature increase was estimated at around half a minute (El Bitar et al. 2014a). Such an approach based on thermal imaging has the advantage of giving accurate measures of temperature at both temporal and spatial levels.

Analysis of the thermographic films was made using the software Altair© (CEDIP Infrared Systems, Croissy-Beaubourg, France). Ten regions of interest (ROI) were defined in the recorded scene, each comprising 10 pixels. Five ROIs were located on the dorsal aspect of the tail. A proximal ROI (tail-prox) was placed 3 cm from the root of the tail, an intermediate ROI (tail-mid) was placed at the middle of the tail, and a distal ROI (tail-distal) was placed 3 cm from the tip of the tail. The remaining two zones were distributed equidistantly between tail-prox and tail-mid and tail-mid and tail-dist. Two additional ROIs were located on the plantar aspect of each hind paw; their final designation was defined respective to the side of muscimol microinjection, ipsilateral (paw-ipsi) or contralateral (paw-contra), as determined following histological controls. Finally, a ROI was located over a piece of wood placed in the scene to monitor the ambient temperature ($T_{ambient}$). For each time point, the mean of the ten pixels defining each ROI was computed to obtain one single temperature time course for each ROI ($T_{tail\text{-}prox}$, $T_{tail\text{-}mid}$, $T_{tail\text{-}dist}$, $T_{paw\text{-}left}$, $T_{paw\text{-}right}$ and $T_{ambient}$).

Histological identification of microinjection sites

At the conclusion of the experiment, the rat was deeply anesthetized with 3% halothane and the brain was perfused through the heart with 0.9% NaCl, followed by 10% formaldehyde and removed. The brain was frozen, cut in serial 100-µm thick sections and Nissl-stained with cresyl violet or carmin. Sites of microinjections were determined from microscopic visualization of the serial sections and reported on schemas of frontal sections of the brain (Paxinos and Watson 2005). Coordinates were then expressed relative to the interaural line: x-axis = latero-lateral; y-axis = ventro-dorsal; z-axis = rostro-caudal. The center of injection was easy to delimit. On average ~1 hour post injection, the overall rostro-caudal diffusion of pontamine blue was 1.1 mm (1.0-1.2 mm), and two times larger than diffusion in the coronal plane.

Assessing the dynamic effects of muscimol on skin temperature

The skin temperature evolution following muscimol microinjection matched a sigmoid curve. The curves were adjusted to a Boltzmann sigmoid according to the equation $T_{adj} = T_{min} + \Delta T_{skin}/\{1 + \exp[-(t - t_x)/s]\}$, using the SigmaPlot© software. Time t = 0 corresponded to the end of the microinjection (Fig. 1A). The following parameters were fitted to the data: T_{min} (adjusted skin temperature during the control period), T_{max} (adjusted maximal skin temperature at the end of the process), $\Delta T_{skin} = T_{max} - T_{min}$ (amplitude of the skin temperature variation), t_x (abscissa of the inflection point of the sigmoid), T_x (ordinate of the inflection point of the sigmoid), s = spread of the sigmoid curve. These parameters were calculated for the proximal and distal parts of the tail, and for each hind-paw. The regression coefficients R^2 of the adjusted curves were always highly significant (> 0.98).

The regulation of the laboratory air-conditioning system was a source of change of ambient temperature in the ~ 0.5°C range. This represented ~10% of the magnitude of the ascending phase of

muscimol-induced increases in skin temperature at the hind-paws and distal part of the tail and ~15% of the increases in skin temperature at the proximal tail. Therefore, the beginning (t_{min}) of the ascending phase of vasodilatation at the hind-paws and distal part of the tail was calculated by considering the 10% percentile of ΔT_{skin}: $t_{min} = t_x - S*Ln(1/0.1-1)$ (Fig. 1B). A slightly different cutoff (15%) was chosen to estimate the onset of vasodilation at the proximal part of the tail, because of the slower evolution of the process at that location. The end (t_{max}) of the ascending phase of vasodilatation was calculated by considering the 90% percentiles of ΔT_{skin}: $t_{max} = t_x - S*Ln(1/0.9-1)$.

Then, the latency of the vasomotor reactions were divided into three groups according to the distribution of the beginning time t_{min} of the ascending phase of vasodilatation: $t_{min} < 7.5$ min, $7.5 < t_{min} < 15$ min and $t_{min} > 15$ min. This categorization was justified by the clear bimodal distribution of t_{min}, centered on 3-5 and 10-12 min, respectively (Fig. 1C). Beyond 15 min, the distribution of t_{min} was flat, and the observed effects if any were interpreted as resulting from uncontrolled diffusion of muscimol (Edeline et al. 2002). Together with a small volume of injection (50 nl) and a large number of negative sites of injection, this approach provided an acute delineation of the structures controling sympathetic cutaneous vasoconstrictor activity.

Three-dimensional mapping of response latencies

The large number of microinjection sites provided the opportunity of building a three-dimensional mapping of response latencies as a function of injection site, using interpolation. The interpolation was performed by computing an average of the responses obtained at all injection sites, weighted by the distance between the interpolated voxel and each injection site. This ensured that the interpolated values were most dependent on the responses of the nearest injection sites. In addition, because of the non-homogeneous sampling of the interpolated volume, a mask was used to exclude voxels that were not close to at least one injection point.

For each measurement location (left paw, right paw, proximal tail, distal tail), a 3D volume representing response latencies as a function of injection site was obtained using interpolation. The responses obtained from the left and right paw were merged, by expressing the location of the injection site as ipsilateral (positive x-axis) vs. contralateral (negative x-axis) relative to the paw. The interpolated 3D volume extended from -2 mm to +2 mm along the x-axis, -1.5 mm to +1 mm along the y axis, and -1 mm to +3.5 mm along the z-axis (200 points along each dimension). For each voxel of the volume, an interpolated latency was obtained by computing a weighted average of all latency values:

$$\text{latency}(x, y, z) = \frac{\sum w_i \text{latency}_i}{N}$$

The weight assigned for each latency value (w_i) was determined by the distance between the voxel (x, y, z) and the injection site (x_i, y_i, z_i):

$$w_i = \frac{1}{\left(\sqrt{(x_i - x)^2 + (y_i - y)^2 + (z_i - z)^2}\right)^6}$$

This weight is inversely proportional to the sixth power of the distance. This ensured that at any given location of the interpolated volume, interpolated latencies were more dependent on the latencies of the nearest injection site. Because the 3D volume was not homogeneously sampled (i.e. injection sites were clustered in some regions of the volume), a mask was used to exclude voxels, which were not located at a distance < 0.5 mm of at least one injection point. Finally, because injection at a large amount of sites did not elicit any response, a second mask was computed to distinguish voxels located in regions where injection elicited a response from voxels located in regions where injection did not elicit a response after 55 min of recording. After assigning a latency of 55 min to these trials, the second mask was computed by taking all voxels of the interpolated volume having interpolated latencies above an arbitrary interpolated latency value of 53.5 min.

Heat loss Index

The skin is an interface between core temperature (T_{core}) and ambient temperature, (T_{amb}) acting as a radiator. In addition to active changes secondary to vasomotor tone of skin vessels, the temperature T of the skin can be affected passively by either T_{amb} or T_{core}). To cancel out these passive changes, we computed the Heat Loss Index (HLI) = $(T_{skin} - T_{amb})/(T_{core} - T_{amb})$ as initially proposed by Székely (1986). The value of HLI can vary between 0 and 1, representing complete vasoconstriction ($T = T_{amb}$) and complete vasodilatation ($T = T_{core}$), respectively (Gordon et al. 2002; Romanovsky et al. 2002).

Analysis of vital signs

To study the systemic effects of muscimol on MAP, HR, $ETCO_2$ and T_{core} with respect to the microinjection sites, we divided the experiments in three groups on the basis of the beginning time t_{min} of the ascending phase of vasodilatation. Group 1: (1) $t_{min} < 15$ min for all considered skin areas either on the tail or the paws. Group 2: $t_{min} < 15$ min for at least one but not all the areas considered on the tail or the hind-paws. Group 3: $t_{min} > 15$ min for all considered skin areas either on the tail or the paws. The mean of each variable during the control period was considered as the reference level, and the temporal evolution was calculated in terms of variation of this level, namely T_{core} (Δ°C), MAP (ΔmmHg), HR (Δ°C) and $ETCO_2$ (Δ%).

Data processing and statistical analyses

The temporal evolution of the mean skin temperature, MAP and HR were synchronized and measured with a one-second-time resolution. The corresponding time courses were downsampled to a one-minute time resolution by averaging. Data were expressed as means (± confident interval 95%). The comparisons were done using the Mann Whitney U test and the Friedman repeated measures ANOVA on ranks test.

RESULTS

The paws and tail of the rat were successfully maintained in vasoconstriction during the control period by preserving a stable, homogeneous and constant surrounding temperature T_{warm}, adjusted to a few tenths degrees below T_{cor}, and by keeping this temperature constant throughout the experiment. Following a 15-min period of control in steady vasoconstriction, muscimol (0.5 nmol, 50 nl) was injected over 60-seconds.

We will first describe in details a typical recording. Then the effects of muscimol will be detailed with reference to the injection sites determined post-mortem. Emphasis will be made on the comparison between hind-paws (ipsilateral vs. contralateral to the injection site). We seized the opportunity of the large number of microinjection sites to build three-dimensional mappings of response latencies as a function of injection site, using interpolation. Results will also be synthesized by taking into account concomitant variations of MAP, HR and T_{core}. Finally, the potential effect of muscimol on a widely used nociceptive test, the tail-flick test, will be assessed by using the present results to compute expected variations in tail-flick latency elicited by the effects of muscimol on both tail temperature and T_{core}.

A typical example of the effects of muscimol microinjection within the RVM/rMR

An example of a thermographic film is provided as supporting information (Supporting video), from which a series of seven images recorded during 30-min are shown in figure 2A. These images correspond to a typical example of a microinjection centered to the RMg, as determined following histological examination (Fig. 2H). Figure 2B shows the temporal evolution of the temperature of (1) the hind-paws, ipsilateral ($T_{paw-ipsi}$) and contralateral ($T_{paw-contra}$) to the microinjection site, (2) five sites on the tail and (3) a small piece of wood, indicator of the ambient temperature (T_{amb}). Figure 2C shows the temporal evolution of the core temperature (T_{core}). During the control period, T_{amb} and T_{core} were stable while the hind-paws and tail were in vasoconstriction with skin temperatures slightly above T_{amb}. Following the injection of muscimol, a progressive increase of skin temperature is observed, starting at the ipsilateral paw ($T_{paw-ipsi}$: 4.9 min post-injection), followed by the proximal tail ($T_{tail-prox}$: 6.6 min), the distal tail ($T_{tail-dist}$: 11.6 min), and, finally, the contralateral paw ($T_{paw-contra}$: 12.6 min). The time courses of $T_{paw-ipsi}$ and $T_{paw-contra}$ were similar, increasing by 8.2 and 7.4°C, within 7.7 and 8.0 min respectively, and remaining stable afterwards. The temporal evolution of the temperature of the tail was quite different, taking 26.6 min to achieve a 6.4°C increase for $T_{tail-prox}$ vs. 21.2 min to achieve a 7.8°C increase for $T_{tail-dist}$. The intermediate parts of the tail showed a similar evolution whose parameters spread out between those of the extreme parts.

Shortly after vasodilatation of the ipsilateral hind-paw, one sees a decrease in core temperature (T_{core}) (Fig. 2C) that was accentuated by the later vasodilation of the tail and contralateral hind-paw. Overall, T_{core} was reduced by 0.8°C within the 30 min post-injection period despite the active warming that remained stable all over the experiment. The Heat Loss Indexes are shown in figure 2D for each site of T_{skin} skin recording.

MAP (Fig. 2E) and HR (Fig. 2F) were also affected by muscimol. Both parameters dropped and stabilized within 8 min, that is a few minutes earlier than the vasodilatation. At 30 min, the final MAP and HR were 36% and 20% less than during the control period, respectively. These variations were associated with a decrease of $ETCO_2$ (Fig. 2G).

From such an example, one can infer that microinjection of muscimol centered on the RMg is able to block the drive of the sympathetic control of vasomotion of the hind-paws and the tail, leading to an increase in their skin temperature and a subsequent decrease in T_{core}. The effects were dominant on the part of the body ipsilateral to the microinjection site. These variations were associated with a decrease in MAP, HR and $ETCO_2$.

Overall effect of muscimol microinjection within the RVM/rMR on the vasomotion of the hind-paws

During the control period, both hind-paws were vasoconstricted at a stable skin temperature, close to the ambient temperature. The microinjection of muscimol in the RVM/rMR elicited an increase of the plantar skin temperature, indicating increasing blood flow. This reaction always started first in the ipsilateral hind-paw. Following adjustment of each individual curve to a sigmoid by a Boltzmann regression (see Fig. 1AB), the experiments were divided in three groups on the basis of the distribution of the beginning (t_{min}) of the ascending phase of vasodilatation at the hind-paw ipsilateral to the microinjection site (Fig. 1C): group 1 (acute onset group: $t_{min} < 7.5$ min); group 2 (intermediate onset group: $7.5 < t_{min} < 15$ min); group 3 (late onset or no response group: $t_{min} > 15$ min). The corresponding temporal evolutions of adjusted skin temperatures are shown in Figure 3 for the 3 groups. Regarding the two groups showing responses with acute or intermediate onset, the ipsilateral and contralateral curves were essentially parallel, but the response elicited in the latter was postponed by 11-12 minutes. The ascending phase of vasodilatation Δt was short lasting (in the 6-8 min range) and large (in the 7-8°C/min range), therefore steep (in the 1°C/min range). The corresponding numerical data are provided in table 1A.

Figure 4 shows the corresponding localization of the muscimol microinjection sites reported on the atlas of the rat brain by Paxinos and Watson (2005). For clarity of presentation, the data related to the hind-paws, ipsilateral and contralateral to the microinjection sites, are shown on the right and left parts of the figure, respectively. The black, grey and white circles represent microinjection sites related to experiments belonging to the first, second and third groups, respectively. Regarding the ipsilateral hind-paw, the most effective sites are located between planes -1.3 and -2.8 mm with reference to the inter-aural line, mainly in raphe pallidus, inner layer of raphe magnus and parapyramidal nucleus (Fig. 4B). These points are surrounded laterally and rostro-caudally by the second group (grey symbols), often localized in the outer layer of the raphe magnus. The non-reactive regions are outside these zones. The effects of microinjection on the contralateral hind-paw (Fig. 4A) were very much dependent on its proximity to the midline. Microinjections distant from the midline by less than 0.1 mm exhibited an acute onset effect, and those by less than 0.3 mm an intermediate onset effect.

We seized the opportunity of the large number of microinjection sites to build a three-dimensional mapping of response latencies as a function of injection site, using interpolation (Fig. 5). Since the effects were clearly lateralized, the responses obtained from the left and right paw were merged, by expressing the location of the injection site as ipsilateral (positive x-axis) vs. contralateral (negative x-axis) relative to the paw. The earliest responses latencies were observed between planes z = -1.3 and z = -2.8 mm both from the midline (raphe pallidus and raphe magnus) and a more lateral part centered on the lateral paragigantocellular nucleus (LPGi) and the Parapyramidal nucleus (PPy).

Overall effect of muscimol microinjection within the RVM/rMR on the vasomotion of the tail

During the control period, the tail was vasoconstricted, with a decreasing thermal gradient from $T_{tail\text{-}prox}$ to $T_{tail\text{-}dist}$ [27.1 (26.9-27.3) vs. 24.9 (24.7-25.1)°C; $P < 0.001$]. The microinjection of muscimol within the RVM/rMR elicited an increase of the tail skin temperature. However, a proximal-distal gradient was also seen regarding the reactivity of the tail. Knowing these gradients, we analyzed the proximal and distal parts of the tail, separately, using the type of grouping already made but based on the starting point of the acute ascending phase of vasodilatation on the distal part of the tail (1^{st} group (acute onset group): $0 < t_{min} < 7.5$ min; 2^{nd} group (intermediate onset group): $7.5 < t_{min} < 15$ min; 3^{rd} group (late or none reactive): $t_{min} > 15$ min), following sigmoidal adjustment of each individual. The corresponding temporal evolutions of adjusted skin temperatures are shown in Figure 6 for the 3 groups. Regarding the first two groups, the corresponding numerical data are provided in table 1B. By comparison with the paws the process was slower (in the 0.3-0.5°C/min range) and less pronounced (in the 4-6°C range), possibly because we recorded the dorsal facet of the tail (see discussion).

Figure 7 shows the corresponding localization of the muscimol microinjection sites for the proximal (Fig. 7A) and the distal (Fig. 7B) parts of the tail. Again, the black grey and white symbols represent the acute, intermediate and late or none reactive group respectively. The black symbols are located mainly between planes -1.3 and -2.8 mm, close to the midline, in the raphe pallidus and raphe magnus. The grey symbols surround the black ones and are located in the raphe magnus and parapyramidal region. In these two groups, the proximal and distal parts of the tail reacted concomitantly. Overall, the proximal part of the tail was more responsive to the microinjection; in particular, one can see several points from which a vasodilatation was elicited on the proximal but not on the distal part of the tail, at least within the 15-min early period. Most of these points were however located in the raphe magnus and parapyramidal nucleus.

Figure 8 represents the three-dimensional mapping of response latencies t_{min} as a function of injection site. For both the proximal (Fig. 8A) and the distal (Fig. 8B) parts of the tail, the earliest response latencies were observed between planes z = -1.3 and z = -2.8 mm from the midline (raphe pallidus and raphe magnus) and a more lateral part. Interestingly, the lateral part centered on the Parapyramidal nucleus was restricted to planes z = -2.3 to -2.8 mm without any obvious participation of the lateral paragigantocellular nucleus.

406 ***Comparison of Heat Loss Indexes in various vasomotor states***

407

408 The potential extent of skin temperature variations was physically restricted to the T_{amb}-T_{core} range.
409 In terms of thermoregulation, these changes are best described using the Heat Loss Index as it eliminates the
410 passive changes and range limitation due to any T_{amb} and T_{core} variations (Gordon et al. 2002; Romanovsky
411 et al. 2002; Székely 1986): HLI = $(T_{skin}-T_{amb})/(T_{core}-T_{amb})$. Table 2 compares results of skin temperature,
412 converted in terms of HLI, obtained in the present study (acute onset group 1, black symbols) to previous
413 experiments performed on anesthetized rats maintained in thermo-neutral conditions (El Bitar et al. 2014a).
414 The HLI values in the control period of the present study were close to those recorded during
415 vasoconstrictions in our previous study and identical in terms of ranking ($T_{tail\text{-}dist} < T_{paw\text{-}ipsi} < T_{tail\text{-}prox}$).
416 However, the HLI values following muscimol microinjection were 20-25% lower than the HLI seen during
417 the maximal physiological vasodilatation achieved during thermo-neutrality. This observation suggests that
418 the ongoing hind-paws and tail fiber sympathetic activity was not completely silenced following the
419 muscimol microinjections, in spite of an apparent ceiling effect.

420

421 ***Overall results, including the effects on vital signs***

422

423 In order to homogenize the results for further analyzes, the experiments were reorganized in three
424 new groups, again on the basis of the onset time t_{min} of the ascending phase of vasodilatation (Fig. 9). The
425 first includes experiments where t_{min} was < 15 min for <u>all</u> considered skin areas either on the tail or the paws
426 (black circles in figure 9A). They were located between planes -0.8 and -2.8 mm, not more lateral than 0.3
427 mm from the midline, mainly in the inner part of raphe magnus and raphe pallidus. The second includes
428 experiments where t_{min} was < 15 min for <u>at least one</u> but not all the areas considered on the tail or the hind-
429 paws (grey circles in figure 9A). They were located in the raphe magnus and parapyramidal area,
430 surrounding the preceding group. The third group includes experiments where t_{min} was > 15 min for <u>all</u>
431 considered skin areas either on the tail or the paws (white circles in figure 9A). The corresponding effects of
432 microinjection of muscimol on $T_{paw\text{-}ipsi}$, $T_{paw\text{-}contra}$, $T_{tail\text{-}prox}$ and $T_{tail\text{-}dist}$ are summarized in figures 9B, C, D and
433 E, respectively.

434 Figure 9F shows the concomitant effects on T_{core}. There was no statistical difference among the three
435 groups concerning T_{core} during the control period (P = 0.94). After the muscimol injection, the mean
436 temperatures diverged strongly with progressive drops for the 1st and 2nd group. The effects of muscimol
437 microinjections on MAP, HR and $ETCO_2$ are presented in Figures 9G, H and I, respectively. For all three
438 groups, we report a transitory increase of MAP and HR in the first minute following the microinjection of
439 muscimol. Thereafter, the curves declined slowly and slightly till the end of the experiments. Regarding the
440 first two groups, the corresponding numerical data are provided in table 3. These results are in line with
441 previous reports in anesthetized rats following microinjection of muscimol in the RVM/rMR, describing
442 slight or non-significant decreases in MAP, HR and $ETCO_2$ (Bernard et al. 2008; Blessing and Nalivaiko
443 2001; Nakamura and Morrison 2007; Tanaka et al. 2007; Zaretsky et al. 2003a; 2003b). Note that our

recordings were made under an anesthetic regime that preserves withdrawal reflexes while most earlier reports were obtained with deeper regimes that could have masked the effects reported here.

Modeling the effect of muscimol injection within the RVM/rMR on the tail-flick test

In a previous study, we proposed and verified experimentally a simple model to compute the expected tail-flick latency (TFL) of a rat exposed to a source of radiant heat applied onto the tail (Benoist et al. 2008). The model takes into account the power of the radiant heat source, the initial skin temperature, the core temperature and the site of stimulation on the tail, and has been applied successfully to reconstruct TFLs following a conditioned stress response (Carrive et al. 2011). Here, the model was used to compute the predictable variations of TFL introduced by muscimol. Decisional and motor latencies were estimated to be 134 and 4 ms, respectively (Benoist et al. 2008). Considering a site of stimulation on the mid-tail, the distance to the dorsal horn entry zone is approximately 200 mm. The model provides the following equation for the expected tail-flick latency: $TFL\ (s) = [(36.8 - 0.73*T_{mid\text{-}tail})^2/\alpha + 90/(0.041*T_{core} - 0.47) + 110/(0.041*T_{mid\text{-}tail} - 0.47) + 138]/1000$ where α is the slope of the squared temperature variation (in $°C^2/s$) generated by the power of the radiant heat source.

Let us recall at this point that, when the skin is exposed to a constant power source of infrared radiation, the temperature increases with the square root of time, according to the law of radiant heat transfer $T = T_{mid\text{-}tail} + a.t^{0.5}$ or, expressed in terms of temperature variation $T - T_{mid\text{-}tail} = a.t^{0.5}$. This quadratic relationship becomes linear in t by squaring the two terms of the equation: $(T - T_{mid\text{-}tail})^2 = a^2.t = \alpha.t$ where α is the slope of the squared temperature variation generated by the power of the radiant heat source [see Fig. 2A in (Benoist et al. 2008)]. The predictive model of TFL was fully verified following variations of the radiant heat source (i.e. α) or the basal temperature of the skin (i.e. $T_{mid\text{-}tail}$ here) [see Fig. 8 in (Benoist et al. 2008)]. In the classical tail-flick test, the principal source of variation introduced by experimenters is the power of the electrical bulb used for heating the skin of the animal. The investigator adjusts the radiant heat emission with a rheostat to achieve a predetermined TFL in the control situation, most commonly in the 2-4 seconds range (Le Bars et al. 1999, 2009). In the model, such latencies are achieved for α in the 0.08-0.2$°C^2/s$.

The model was applied to the data of animals in which the beginning of the ascending phase of vasodilatation was < 15 min either on the tail or the paws (black group in figure 9). The model foretells a 30-35% decrease of the TFL, thirty minutes following muscimol administration (Fig. 10). It appears therefore that, regardless of any other additional possible causes, the vasodilatation of the tail is a major source of variation of the TFL following muscimol administration in RVM/rMR.

DISCUSSION

Muscimol was injected in the lower brainstem with the aim of assessing the role of the RVM/rMR on the control of sympathetic drive to the tail and hind-paws. Microinjections within this brainstem region increased the skin temperature of the hind-paws and tail as a result of reduced vasomotor tone. The effects were dominant on the hind-paw ipsilateral to the microinjection site. Increased heat loss through vasodilatation of the tail and paws were associated with a drop of T_{core} and slight decreases of MAP, HR and $ETCO_2$.

Following some technical considerations regarding the microinjection procedure, we will discuss the following points: (1) general findings; (2) lateral localization of effective sites; (3) rostro-caudal localization of effective sites; (4) pharmacological manipulations of RVM/rMR in pain studies.

Technical considerations regarding the microinjection procedure

The kinetic and volume of efficient microinjection is determined by numerous factors: the concentration, rate of delivery and properties of both the drug - coefficient of diffusion, binding, catabolism - and the tissue - volume fraction, tortuosity - (Syková and Nicholson 2008). In nuclear and cortical homogenous regions, drug distributions are typically spherical or in the shape of drop (Bondareff et al. 1970; Martin 1991; Myers 1966).

In a "mapping" study aiming at demarcating a functional region in the brain, both positive and negative results are significant. A negative result obtained with a large dose is a very convincing result in this respect. Considering the large volume of the explored brain structure (roughly 3 x 4 x 2 = 24 mm^3), a compromise was necessary to both find significant positive results with minimal doses and to avoid useless negative results. Studies on the involvement of these regions in pain/nociception and thermoregulation have used muscimol injections in the 10-1000 pmol / 60-1000 nl ranges (Bernard et al. 2008; Blessing and Nalivaiko 2001; Brink et al. 2006; Cao et al. 2004, 2010; Cerri et al. 2010; Fan et al. 2007. Gilbert and Franklin 2001; Heinricher and Kaplan, 1991; Martenson et al. 2009. Meng et al. 1998; Morrison 1999, 2003; Nakamura and Morrison 2007, 2011; Ootsuka and McAllen 2005; Rathner et al. 2008; Vianna et al. 2008; Zaretsky et al. 2003a, 2003b). Knowing that muscimol does not spread appreciably because it is a potent ligand for neuronal and glial $GABA_A$ receptors, we decided to use a small volume of injection (50 nl = 0.05 mm^3, that is a ~0.5 mm diameter for an ideal sphere) but a relatively high concentration. This was supposed to provide an effective radius of blockade in the ~ 1 mm range (Martin 1991; Martin and Ghez 1999; Malpeli 1999; Edeline et al. 2002). Following similar microinjections using [^{3}H]muscimol, autoradiographic analyses showed a spread ~1.5 mm from the injection site at 15 min (Edeline et al. 2002; Martin 1991). Martin and Ghez (1999) used glucose metabolism to assess the extent of inactivation and observed a central core of blockade (~ 1 mm radius) surrounded by an extensive region of reduced metabolism, possibly due to reduced synaptic activity of neurons receiving projections from the core region. In summary, muscimol binds

515 strongly and diffuses slowly outward at effective concentrations from the region immediately inundated by
516 the bulk flow (Malpeli 1999).

517 Since myelinated fiber bundles impede muscimol diffusion (Allen et al. 2008), it is likely that the
518 particular architecture of the RVM/rMR with a predominance of rostro-caudal fibers and a low neuronal
519 density favors a rostro-caudal diffusion of the product. In addition, the two paired bundles of myelinated
520 fibers that cover the brainstem floor, namely the pyramidal tract and the medial lemniscus, constitute a
521 strong diffusion barrier. Accordingly, the post-mortem examination of pontamine spread revealed an
522 elongated pattern of diffusion two times longer in the rostro-caudal direction as compared to the coronal
523 plane.

524 Using these microinjections, we were able to build response maps on the basis of 160 experiments.
525 Together with the small volume of injection and the large number of negative sites of injection (60%), this
526 approach provided (1) an acute delineation of the structures able to block sympathetic cutaneous
527 vasoconstrictor activity; (2) the possibility of a temporal analysis of these effects; and (3) the resultant
528 capacity of defining a brain region as the core of effects, surrounded by intermediate and then ineffective
529 zones.

530 Because of such a large number of injection sites, we introduced a new procedure to compute three-
531 dimensional interpolated maps of response latencies and overall sympathetic blockade as a function of
532 injection site. The interpolation was performed by computing an average of the responses obtained at all
533 injection sites, weighted by the distance between the interpolated voxel and each injection site. This ensured
534 that the interpolated values were most dependent on the responses of the nearest injection sites. In addition,
535 because of the non-homogeneous sampling of the interpolated volume, a mask was used to exclude voxels
536 that were not close to at least one injection point. The obtained volumes provide an interesting mean to
537 assess the relationship between the effects of the injection and the anatomical location of the injection.

538

539 ***General findings***

540

541 Our results are largely in agreement with earlier reports regarding the sympathetic drive of the tail.
542 Microinjection of glycine, GABA or muscimol in the RVM/rMR blocks the activation by cold or fever of
543 sympathetic fibers that innervate the tail (Blessing and Nalivaiko 2001; Cerri et al. 2010; Korsak and Gilbey
544 2004; Ootsuka and McAllen 2005; Ootsuka et al. 2004; Rathner et al. 2008; Vianna et al. 2008). On the other
545 hand, microinjection of glutamate or bicuculline increases the activity of vasomotor sympathetic nerves of
546 the tail, thus decreasing the blood flow, without affecting the mesenteric vascular bed (Blessing and
547 Nalivaiko 2001; Morrison 2001; Rathner and McAllen 1999). Several aspects of our contribution should be
548 highlighted.

549 The experimental conditions were close to those of thermoneutrality (El Bitar et al. 2014a). T_{warm} was
550 adjusted to ~ 0.3°C below T_{core}. Demonstrating their vasoconstricted state, the temperature of the distal part
551 of the tail was very close to the ambient temperature (~ 0.7°C above $T_{ambient}$). It then increased by ~ 6°C
552 following the injection of muscimol in responsive brainstem sites. In comparable experimental conditions,

553 microinjection of GABA or muscimol in RVM/rMR blocks the activity of the sympathetic fibers innervating
554 the rat tail (Korsak and Gilbey 2004; Ootsuka and McAllen 2005).
555 We have previously discussed (El Bitar et al. 2014a) that the vasomotor tone of the dorsal facet of
556 the tail is less reactive than the ventral facet, notably because the ventral artery is larger than the lateral
557 arteries, while the ventral vein is thinner than the lateral veins (Knoppers 1942; Thorington 1966; Wu et al.
558 1995; Young and Dawson 1982). It follows that the temperatures of the dorsal and ventral facets of the tail
559 are identical during vasoconstriction but shift by ~ 3°C during full vasodilatation. The recorded effects from
560 the tail were thus underestimated, as compared to those seen on the hind paws.
561 A major novelty of our study was to include the paws to these basic phenomenological observations.
562 Such as for the tail, the hind-paws were in a stable vasoconstricted state, as demonstrated by the fact that the
563 temperature of the hind-paws was close to the ambient temperature (2-3°C above $T_{ambient}$). This increased by
564 ~ 7°C following the injection of muscimol in the active sites. We have already discussed the involvement of
565 the paws in rat thermoregulation (El Bitar et al. 2014a). The feet make up approximately 10% of the total
566 surface area of the body, slightly more than the tail (Lin et al. 1979). It follows that the substantial muscimol-
567 induced vasodilatation of the paws contributed significantly to the core temperature drop (~ 1°C within half
568 an hour).
569 Our purpose was not to confirm well-documented notions. We specifically aimed at mapping the
570 brainstem regions that contain the premotor sympathetic neurons that control the vasomotor tone of the tail
571 and hind-paws because most models of pain/nociception are based on behavioral responses elicited by
572 *thermal* stimulation (reviewed in Le Bars et al. 2001, 2009). We undertook this large study because studies
573 on pain/nociception have emphasized the Rostral Ventromedial Medulla (RVM), which includes the nucleus
574 raphe magnus and the gigantocellular reticular nucleus pars alpha (mainly at the level of the facial nucleus),
575 while studies on thermoregulation have emphasized a slightly shifted more caudal region centered on the
576 raphe pallidus. In addition, we described in a previous work that neurons involved in pain/nociception
577 located in the brainstem are probably also implicated in autonomic regulation, notably cutaneous vasomotion
578 (El Bitar et al. 2014b).
579 The results of the present study suggest that the regions described as being involved in
580 pain/nociception modulation and the regions involved in thermoregulation are spatially matched, at least
581 functionally regarding the vasomotor tone of the tail and hind-paws.
582
583 ***Localization of effective sites in coronal planes***
584
585 The most effective sites were restricted to well-circumscribed regions. Numerous negative
586 microinjections sharpened the delineation. Our observations regarding the tail are in keeping with the
587 mapping by Korsak and Gilbey (2004) who reported that GABA microinjections in the region of raphe
588 pallidus and magnus markedly decreased the sympathetic cutaneous vasoconstrictor activity in the dorsal
589 collector nerves of the tail, while injections more dorsal or lateral tended to produce either a smaller decrease

or have no effect. Blessing and Nalivaiko (2001) also emphasized the raphe magnus, raphe pallidus and parapyramidal nucleus in this respect, although they did not provide a graphical mapping of the effects.

Only microinjections in RMg, RPa and PPy elicited a vasodilatation in the ipsilateral hind-paw within less than 7.5 min and those distant from the midline by less than 0.3 mm induced a vasodilatation in the contralateral hind-paw within the same period. These observations deserve two comments. First, within the coronal plane, the lateral diffusion of muscimol was limited, as expected. Second, projections of RVM/rMR cells are mainly lateralized, predominantly controlling vasomotor tone of the ipsilateral body side. Our results are in keeping with studies showing that RVM/rMR neurons send axons towards the spinal cord mainly through the ipsilateral dorso-lateral funiculus (Basbaum and Fields 1979; Fields et al. 1995; Lefler et al. 2008; Light 1985). Microinjections close to the midline also elicited early vasodilatation of the tail, the most effective sites being restricted to the same well-circumscribed regions. Neurons in the PPy present morphological, histo-chemical (Helke et al. 1989; Lynn et al. 1991) and functional (Blessing and Nalivaiko 2000) properties similar to those of the RPa. Some authors consider therefore PPy as part of RPa (e.g. Cano et al. 2003).

Interestingly, brainstem neurons activated both antidromically from the lumbar dorso-lateral funiculus and by mild cooling of the animal core temperature, were found in both raphe pallidus and magnus (Rathner et al. 2001). Such neurons could be involved in the results presented here. However, there are other control loops that regulate the central temperature through the vessels in the hairy skin, the interscapular brown adipose tissue (BAT) and the fusimotor fibers to limb muscles, all being silenced by neuronal inhibition of the raphe pallidus and/or magnus (McAllen et al. 2010). Although they all should have a sympathetic premotor relay in these nuclei, these four sympathetic thermo-effector outflows differ in terms of thermal thresholds, relative responsiveness to core temperature, patterns and driving by neural pathways, and thus probably do not "crosstalk" at this level (McAllen et al. 2010; Nagashima et al. 2000; Ootsuka and McAllen, 2006 ; Romanovsky 2007 ; Tanaka et al. 2007). The relative contribution of single individual neurons to these functions remains an open question.

Rostro-caudal localization of effective sites

Overall, the injection sites which elicited the most widespread and early latency vasodilation were located in the coronal planes posterior to the interaural line by 1.3-2.6 mm. 85% of experiments where the vasodilatation of all areas either on the tail or the paws occurred in less than 15 min were found in these regions. Again our observations agree with the report by Korsak and Gilbey (2004) showing a similar rostro-caudal extension of their effective sites for blocking by GABA the sympathetic vasoconstrictor drive in the tail. Similarly, brainstem neurons both antidromically activated from the lumbar dorso-lateral funiculus and activated by mild cold were found in the raphe nuclei between the coronal planes posterior to the interaural line by 1.8-2.3 mm (Rathner et al. 2001).

Overall, the literature related to thermoregulation points to coronal planes immediately rostral to the rostral pole of the inferior olivary complex at the level of the caudal half of the facial nucleus. This brainstem

region is often referred as to the rostral raphe pallidus, although it generally does include the nucleus raphe magnus (e.g. Madden and Morrison 2003, 2005; Morrison 1999, 2003; Morrison et al. 1999, 2000; Rathner et al. 2008). In fact, very few studies were devoted to a systematic mapping.

As pointed out by Nason and Mason (2004), it is unlikely that a microinjection into any single nucleus in RVM/rMR will affect neurons confined to that nucleus because these neurons have large dendritic arbors that cross cytoarchitectonic boundaries (Gao and Mason 1997; Mason et al. 1990; Newman 1985; Potrebic and Mason 1993). Blessing (2003) discussed the conventional anatomical demarcation into RMg and RPa by raising the following points. The delimitation of boundaries is based particularly on the work of Taber et al. (1960) that follows the atlas of Meessen and Olszewski (1949) for rabbits and the atlas of Olszewski and Baxter (1954) for humans. However, the rabbit atlas combines all the ventral midline neurons in the rostral medulla and caudal pons as "RMg" and does not use the term "RPa." In contrast, the human atlas uses "RPa" and not "RMg". However, by convention, RMg in the rat is more rostral and dorsal, and RPa is more caudal and ventral (Paxinos and Watson 2005).

We noted that the Heat loss Indexes following muscimol microinjection were 20-25% lower than the HLI seen during the maximal physiological vasodilatation achieved in rats maintained in thermo-neutral conditions (El Bitar et al. 2014a), suggesting that the ongoing hind-paws and tail fiber sympathetic activities were not completly silenced following the microinjections, in spite of a clear ceiling effect when microinjections were effective. The most parsimonious explanation for this observation is the oblong geometry of the involved brainstem structures, ~12 times longer than the diameter of the injected volume, preventing the drug to reach all structures potentially involved in controling vasomotor tone.

In summary, there are morphological and functional reasons to consider the RVM and rMR nuclei as a single RVM/rMR entity, as already proposed by Mason et al. (2001, 2005a, 2005b, 2006, 2011).

Is the RVM really specific for pain/nociception?

Our study shows, in the rat, a mutual covering of the regions involved in thermoregulation (at least, regarding the vasomotor tone of the tail and hind-paws) with those reported as being specifically involved in pain/nociception.

In spite of several warnings initiated by Thelma Lovick and Peggy Mason (Le Bars et al. 2001; Lefler et al. 2008; Lovick 1997; Mason 2001; 2005a; 2005b; 2006; 2011; 2012), the specificity of the RVM regarding the control of pain/nociception mechanisms is repeatedly put forward or assumed (e.g. Fields et al. 2006; Heinricher and Ingram, 2008; Basbaum et al. 2009; Heinricher et al. 2009). RVM neurons do not only project towards the dorsal horn of the spinal cord (Fields and Basbaum 1978; 1999), but also to the intermediolateral cell column, with a high degree of collateralization in both the rostro-caudal and dorso-ventral axes (Bacon et al. 1990; Basbaum et al. 1978; Hossaini et al. 2012; Lefler et al. 2008; Loewy 1981; Morrison and Gebber 1985).

Numerous neurons in RVM/rMR were identified as sympathetic premotor neurons by early retrograde trans-synaptic labeling with the pseudo-rabies virus (Smith et al. 1998; Strack et al. 1989).

Quantitative data, obtained from 6 days survival experiments, revealed ~5 times more labeled neurons in raphe magnus and gigantocellular reticular pars alpha nuclei than within the raphe palidus nucleus (Smith et al. 1998). Smith et al. (1998) found the highest density at the level of the facial nucleus but the coronal planes facing the rostral part of the facial nucleus were not represented. Therefore, the neurons labeled in that study could have been mainly located in the RVM/rMR at the level of facial nucleus, although we cannot infer their rostral extent. However, it was stated more recently that transneuronally labeled neurons are distributed through the parapyramidal region, including all RVM/rMR elements (Tóth et al. 2006), at least within the coronal planes from 1.3 to 3.3 mm posterior to the interaural line (Nakamura et al., 2004). Finally, cold exposure increases the expression of Fos immunoreactivity not only in RPa and PPy (Bonaz and Taché 1994; Cano et al. 2003; Martinez et al. 2001; Morrison et al. 1999) but also in RMg, between the coronal planes posterior to the interaural line by 1.3-3.3 mm (Nakamura et al., 2004).

If one moves back to the literature related to nociception, namely the so-called "on-" and "off-" cells recorded within the medial brainstem, one is struck by the similar rostro-caudal distribution of the sites, all pointing out the coronal planes including the facial nucleus. Interestingly, Vanegas et al. (1984) attempted to map "on-" and "off-" neurons that project to the spinal cord, and found that these were located in brain regions 1.9-2.7 mm posterior to the interaural line. In summary, the most effective sites for blocking by muscimol the cutaneous vasoconstrictor fibers matches exactly the brainstem sites where "on-" and "off-" cells were recorded over the last years, notably those projecting to the spinal cord.

Pharmacological manipulations of RVM/rMR in pain studies

Administration of muscimol exclusively within RVM/rMR results in a significant decrease in TFL while administration of the competitive $GABA_A$ receptor antagonist bicuculline (but not the glycine receptor antagonist strychnine) induces an increase of the TFL (Drower and Hammond 1988; Gilbert and Franklin 2001; Heinricher and Kaplan 1991; Heinricher and Tortorici 1994; Nason and Mason 2004). Similar results have been reported with paw withdrawal and hot-plate tests (Gilbert and Franklin 2001; Martenson et al. 2009).

These increases or decreases of TFL are generally interpreted in terms of hypo- or hyper-algesia, respectively. However, other interpretations are conceivable, available and advisable. The tail-flick test does not achieve the criterion of construct validity because it does not effectively measure the targeted construct, i.e. a quantitative nociceptive response, presumed to reflect an animal's perception of pain (Le Bars et al. 2001, 2009). Indeed, the reaction time measured in the conventional tail-flick test (and all other tests using conventional progressive heating) is the sum of (1) the time to achieve the threshold for the behavioral reaction and (2) the behavioral latency (Benoist et al. 2008; Pincedé et al. 2012). If the reaction time is the only measured end-point, there is no way of knowing whether the variation was produced by changes of either the basal skin temperature or the threshold temperature, or both. Using a simple model for computing the TFL in the rat, taking into account the power of the radiant heat source, the initial skin temperature, the core temperature and the site of stimulation on the tail (Benoist et al. 2008), we predicted that the change in

tail temperature 15 minutes after effective muscimol injection would lead to a 30-35% reduction of the TFL. Interestingly, Heinricher and Kaplan (1991) described a decrease of the TFL of approximately 30% at 12 min post-injection of muscimol (50 ng; 0.5 µl), maintained throughout the half an hour observation period, a finding reproduced by Meng et al. (1998). Both observations match remarkably the predicted variations of TFL related to changes in baseline skin temperature. Interestingly, Tjølsen and Hole (1997) attributed the reduction of TFL following lesions of the raphe-spinal serotoninergic system to the increase in skin temperature.

It should be noted that such considerations are not restricted to the tests based on the utilization of thermal stimuli. For example, the vasodilatation of the paws induced by high ambient temperatures exacerbates the second phase of the formalin test (Rosland 1991; Tjølsen et al. 1992). It follows that the increased responsiveness to formalin injection following muscimol administration in RVM/rMR (Gilbert and Franklin 2001) could also be largely due to the unavoidable vasodilatation.

Conclusion

The functional blockade of the RVM/rMR by muscimol elicits an important increase of skin temperature of the paws and tail, eliciting a reduction of the core temperature. The effective zones match very exactly the brain regions defined as specifically devoted to the control of nociception. Because changes in skin temperature can have a strong effect on the usual tests of pain using radiant heat such as the TFL, our results indicate that the evidence that pharmacological manipulations of RVM/rMT modulate pain-specific descending modulatory pathways could, actually, be explained by unaccounted changes in vasomotor tone.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: NEB, BP, DLB. Performed the experiments: NEB, BP, DLB. Analyzed the data: NEB, BP, DLB, AM, GH. Wrote the paper: NEB, BP, AM, DLB.

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1032

FIGURE LEGENDS

Figure 1. Procedure to analyze the muscimol-induced variations of skin temperature. **A & B.** Abscissa: time in min with reference to the end of the microinjection. Ordinate: temperature in °C. The grey area corresponds to the time intervals < 7.5 min and 7.5-15 min post-injection, delimited by vertical dotted lines. **A.** Analysis of temperature variations observed from an ipsilateral hind-paw ($T_{paw\text{-}ipsi}$). The thin yellow line is the temporal evolution of the temperature recorded during the 5-min control period and the following 30-min post-injection. The thick yellow line is the corresponding adjusted sigmoid Boltzmann curve $T_{adj} = T_{min} + \Delta T_{skin}/\{1 + \exp[-(t - t_x)]/s\}$, with $R^2 = 0.99$ (equation in the upper left corner of the graph). The adjusted parameters of the function are shown in red, namely the adjusted skin temperature during the control period (T_{min} = 24.2°C), the adjusted maximal skin temperature at the end of the process (T_{max} = 32.4°C), the amplitude of skin temperature variation ($\Delta T_{skin} = T_{max} - T_{min}$ = 8.2°C), the inflection point (t_X, $T_x = T_{min} + \Delta T_{skin}/2$) [(8.8 min, 28.3°C)] of the sigmoid represented by a red point. The beginning (t_{min}) and end (t_{max}) of the ascending phase of vasodilatation were then calculated by considering the 10 and 90% percentiles of ΔT_{skin}: $t_{min} = t_x - \text{Ln}(1/0.1\text{-}1)*s^{-1}$ = 4.9 min and $t_{max} = t_x - \text{Ln}(1/0.9\text{-}1)*s^{-1}$ = 13.9 min, represented by white points. **B.** Calculation of the beginning of the ascending phase of vasodilatation observed from the corresponding three other recorded zones ($T_{paw\text{-}contra}$, $T_{tail\text{-}prox}$, $T_{tail\text{-}dist}$). The thin and thick curves represent the genuine and adjusted curves, respectively. The green, dark blue and light blue curves correspond to the contralateral hind-paw, proximal and distal parts of the tail, respectively (corresponding equations provided in the upper left hand corner of the graph). Calculations of t_{min} were made as in A, except for the proximal part of the tail for which a 15% percentile was used in place of the 10% (see text). **C.** Histogram showing the distribution of the beginning of the ascending phase of vasodilatation (t_{min}) for the ipsilateral hind-paws, allowing the categorization into three groups: group 1: $\mathbf{t_{min} < 7.5}$ **min**; group 2: $\mathbf{7.5 < t_{min} < 15}$ **min**; group 3: $\mathbf{t_{min} > 15}$ **min.**

Figure 2. Example of effects elicited by muscimol microinjection in the RVM. **A.** Example of pictures taken at 0, 5, 10, 15, 20, 25, 30 min post-injection of a thermographic movie recorded with a 320*240 pixels resolution (See the corresponding Supporting video S1). The false colors scale of temperature is shown in the right hand part of the picture. **B.** Temporal evolution of the temperatures recorded during the 5-min control and 30-min post-injection periods. Abscissa: time in min. Ordinate: temperature in °C. The eight analyzed zones (10 pixels each) are indicated on the drawing in the right hand part of the figure. $T_{paw\text{-}ipsi}$ (yellow) and $T_{paw\text{-}contra}$ (green) corresponds to the skin temperature of the ipsi- and contralateral hind-paw, respectively. $T_{tail\text{-}prox}$ (dark blue) and $T_{tail\text{-}dist}$ (light blue) corresponds to the skin temperatures of the proximal and distal part of the tail, respectively; there are three additional intermediate blue sites between these two sites. T_{amb} (brown) corresponds to the ambient temperature measured from a small piece of wood. Note the stability of the control period in vasoconstriction with skin temperatures near T_{amb}. Following the microinjection, the ipsilateral hind-paw skin temperature raised first, followed by the proximal and distal parts of the tail and at last the contralateral hind-paw. **C.** Temporal evolution of core temperature (T_{core}). Abscissa: time in min. Ordinate: temperature in °C. Note the decrease in core temperature, starting shortly after the rise of $T_{paw\text{-}ipsi}$. **D.** Corresponding Heat loss Index [$HLI = (T_{skin} - T_{amb})/(T_{core} - T_{amb})$ (Romanovsky et al. 2002)] for the seven skin areas considered. **E.** Temporal evolution of MAP. Abscissa: time in min. Ordinate: MAP in mmHg. Note the slight transitory increase in MAP shortly after the microinjection, followed by a sustained drop and then a progressive stabilization after 8-min post-injection. **F.** Temporal evolution of HR. Abscissa: time in min. Ordinate: HR in beat per minute (bpm). Note the slight transitory increase in HR shortly after the microinjection, followed by a sustained drop and then a progressive stabilization after 9-min post-injection. **G.** Temporal evolution of $ETCO_2$. Abscissa: time in min. Ordinate: $ETCO_2$ in %. Note the parallel changes in E, F and G. **H.** Localization of the site of injection drawn on a frontal section of the brain in plane -2.3 mm caudal to the inter-aural line. The center of the injection site is located 0.3 mm lateral to the midline and 0.4 mm below the inter-aural line. Abbreviations: 7: facial nucleus, GiA: gigantocellular reticular nucleus pars alpha; ml: medial lemniscus; PPy: parapyramidal nucleus; py: pyramidal tract; RMg: raphe magnus nucleus; RPa: raphe pallidus nucleus.

Figure 3. Temporal evolution of the skin temperature recorded from the hind-paws, following muscimol microinjections. Abscissa: time in min, Ordinate: temperature variation in °C (mean ± 95% confidence interval). As indicated in the left hand lower corner, yellow and green curves are data adjusted to a Boltzmann sigmoid (see Fig. 1) from the ipsi- ($T_{paw\text{-}ipsi}$) and contralateral ($T_{paw\text{-}contra}$) hind-paw skin temperature, respectively. The experiments were divided in three groups on the basis of the distribution of the beginning (t_{min}) of the ascending phase of vasodilatation from the ipsilateral hind-paw. **A.** Group 1: $t_{min} <$ 7.5 min). **B.** Group 2: $7.5 < t_{min} < 15$ min. **C.** Group 3: $t_{min} > 15$ min. See text and table 1A.

Figure 4. Overall effects elicited by muscimol microinjection on the skin temperature recorded from the hind-paws. **A.** contralateral hind-paw; **B.** ipsilateral hind-paw. Schema of frontal sections of the brain from inter-aural z = -0.8 (top) to z = -3.3 mm (bottom), adapted from Paxinos and Watson (2005). The circles indicate the center of the corresponding injection sites, with a diameter scaled to a 50 nl sphere. The black symbols represent the early latency group with an onset of vasodilatation in less than 7.5 min. The grey symbols correspond to the intermediate latency group with an onset of vasodilatation between 7.5 and 15 min. The white symbols stand for the group that included the experiments with an onset of vasodilatation in more than 15-min (or did not react at all). Abbreviations: 7: facial nucleus; GiA: gigantocellular reticular nucleus pars alpha; ml: medial lemniscus; PPy: parapyramidal nucleus; py: pyramidal tract; RMg: raphe magnus nucleus; RPa: raphe pallidus nucleus.

Figure 5. Three-dimensional mapping of the hind-paw response latencies t_{min} as a function of injection sites. The responses obtained from the left and right paw were merged, by expressing the location of the injection site as ipsilateral (positive x-axis) vs. contralateral (negative x-axis) relative to the paw. On the left graphs, the interpolated response latencies are indicated by the false colors (scale shown in the lower right hand part of the figure). They are adjusted on frontal sections of the brain from inter-aural z = -0.8 (top) to z = -3.3 mm (bottom) with y being the ventro-dorsal coordinate, again with reference to the interaural line. The right drawings are adapted from Paxinos and Watson (2005). Abbreviations: 7: facial nucleus; GiA: gigantocellular reticular nucleus pars alpha; LPGi: lateral paragigantocellular nucleus; ml: medial lemniscus; PPy: parapyramidal nucleus; py: pyramidal tract; RMg: raphe magnus nucleus; RPa: raphe pallidus nucleus.

1117 **Figure 6.** Temporal evolution of the skin temperature recorded from the tail, following muscimol
1118 microinjections. Abscissa: time in min, Ordinate: temperature variation in °C (mean ± 95% confidence
1119 interval). As indicated in the left hand lower corner, dark blue and blue colors are data adjusted to a
1120 Boltzmann sigmoid (see Fig. 1) from the skin temperature of the proximal ($T_{tail\text{-}prox}$) and distal ($T_{tail\text{-}dist}$) part
1121 of the tail, respectively. The experiments were divided in three groups on the basis of the distribution of the
1122 beginning (t_{min}) of the ascending phase of vasodilatation on the distal part of the tail. **A.** Group 1: $t_{min} < 7.5$
1123 min). **B.** Group 2: $7.5 < t_{min} < 15$ min. **C.** Group 3: $t_{min} > 15$ min. See text and table 1B.
1124

Figure 7. Overall effects elicited by muscimol microinjection on the skin temperature recorded from the tail. **A.** proximal part of the tail; **B.** distal part of the tail. Symbols and abbreviations as in figure 4.

Figure 8. Three-dimensional mapping of the tail response latencies t_{min} as a function of injection sites. **A.** proximal part of the tail. **B.** distal part of the tail. The interpolated response latencies are indicated by the false colors (scale shown in the bottom of the figure). They are adjusted on frontal sections of the brain from inter-aural z = -0.8 (top) to z = -3.3 mm (bottom) with x and y being the lateral and ventro-dorsal coordinate, respectively, again with reference to the interaural line. Symbols and abbreviations as in figure 5.

Figure 9. A. Schema of frontal sections of the brain from inter-aural -0.8 (top) to -3.3 mm (bottom), adapted from Paxinos and Watson (2005). Symbols and abbreviations as in figure 4. The data were synthesized by regrouping the experiments in three new groups, again on the basis of the beginning time t_{min} of the ascending phase of vasodilatation, considering all skin areas either on the tail or the paws. The black symbols represent the early latency group with **t_{min} < 15 min for all** considered skin areas either on the tail or the paws. The grey symbols correspond to the intermediate latency group with **t_{min} < 15 min for at least one** of these areas. The white symbols correspond to the late latency group with **t_{min} > 15 min for all** considered skin areas. **B.** Variation of skin temperature (Δ°C) recorded from the ipsilateral hind-paw. **C.** Variation of skin temperature (Δ°C) recorded from the contralateral hind-paw. **D.** Variation of skin temperature (Δ°C) recorded from the distal part of the tail. **E.** Variation of skin temperature (Δ°C) recorded from the proximal part of the tail. **F.** Variation of core temperature (Δ°C). In the 1st group, T_{core} declined linearly as early as the first minute post-injection); for the 2nd group, T_{core} decline is parallel to the 1st's but with 15-min delay and the 3rd group's T_{core} remained stable. **G.** Variation of mean arterial blood pressure (ΔmmHg). Note a slight transitory increase of MAP in all groups, shortly after the microinjection followed by a sustained drop. This was followed by a slow decline in the first group, stabilization in the 3rd group blood, while the 2nd group spread out in-between. **H.** Corresponding variations of heart rate (Δbpm). **I.** Variation of end-tidal CO_2 (Δ%). The corresponding numerical data are provided in table 3.

Figure 10. We proposed and verified experimentally a simple model for computing the TFL in the rat, taking into account the power of the radiant heat source, the initial temperature of the skin, the core temperature or the site of stimulation on the tail (Benoist et al. 2008). This model is used here to compute the predictable variations of the TFL introduced by muscimol in the positive cases of the present experiments. Since the decisional and motor latencies were estimated 134 and 4 ms, respectively (Benoist et al. 2008) and considering a site of stimulation on the mid-tail far-off the dorsal horn entry zone by 200 mm, the model provides the following equation giving the tail-flick latency: TFL (s) = $[(36.8 - 0.73*T_{mid\text{-}tail})^2/\alpha + 90/(0.041*T_{core} - 0.47) + 110/(0.041*T_{mid\text{-}tail} - 0.47) + 138]/1000$ where α is the slope of the squared temperature variation, witness of the power of the radiant heat source. Experimental data are from the experiments where the beginning of the ascending phase of vasodilatation was < 15 min either on the tail or the paws (Black group in figure 9); they include both the mean temperatures of the core (T_{core}, brown line) and the middle of the tail ($T_{mid\text{-}tail}$, blue line). In the control situation, the model provides tail-flick latencies (red lines) in the 3-4 seconds range for α in the 0.08-0.2°C^2/s range: the model foretells a 30-35% decrease of the TFL following muscimol microinjections in the sites indicated by a black circle in Fig. 9A.

Supporting Video. Thermographic recording at 1 Hz with a 320x240 pixels resolution, compressed and presented in 14 seconds, made during the 5-min control and 30-min post-injection periods. The thermography is presented in false colors (scale in the left). The graph is the corresponding temporal evolution of the skin temperature recorded on 5 points distributed from the base to the tip of the tail (blue points) and the right (yellow point) and left (green point) hind-paws as indicated on the right hand picture. The ambient temperature was measured on an inert piece of wood (brown point). The localization of the site of injection of muscimol (50 nmol, 50 nl) is drawn on a frontal section of the brain in plane -2.3 mm caudal to the inter-aural line. Abbreviations: Gi: gigantocellular reticular nucleus; GiA: gigantocellular reticular nucleus pars alpha; ml: medial lemniscus; RMg: raphe magnus nucleus; RPa: raphe pallidus nucleus; py: pyramidal tract.

A: paws (see Fig. 3)

		T_{min} (°C)	ΔT_{skin} (°C)	t_{min} (min)	t_X (min)	Δt (min)	$\Delta T_{skin}/\Delta t$ (°C/min)
ipsilateral	group 1	25.4 (25.2-25.7)	7.4 (7.2-7.7)	3.6 (3.4-3.8)	6.6 (7.4-5.8)	6.6 (7.4-5.8)	1.2 (1.0-1.6)
	group 2	25.4 (25.2-25.7)	7.7 (7.4-8.0)	11.3 (11.2-11.5)	15.3 (16.0-14.6)	7.9 (6.7-9.0)	7.9 (6.7-9.0)
contralateral	group 1	25.6 (25.5-25.8)	7.2 (7.0-7.4)	14.6* (12.8-16.3)	18.0* (15.8-20.2)	6.8 (5.9-7.7)	1.1 (0.9-1.3)
	group 2	25.4 (25.3-25.6)	7.9 (7.6-8.2)	23.5* (22.0-25.0)	27.8* (29.6-25.9)	8.5 (7.8-9.2)	0.9 (0.8-1.0)

* $P < 0.01$ with respect to the corresponding value of the ipsilateral paw.

B: tail (see Fig. 6)

		T_{min} (°C)	ΔT_{skin} (°C)	t_{min} (min)	t_X (min)	Δt (min)	$\Delta T_{skin}/\Delta t$ (°C/min)
proximal	group 1	27.7 (27.4-28.0)	4.4* (4.1-4.7)	2.4* (2.4-2.5)	8.1 (8.9-7.2)	14.2* (11.9-16.5)	0.3* (0.2-0.4)
	group 2	27.7 (27.3-28.2)	4.4* (4.0-4.9)	8.2* (8.1-8.4)	16.1 (16.9-15.3)	15.7* (13.7-17.7)	0.3* (0.2-0.4)
distal	group 1	25.2 (25.0-25.5)	5.6* (5.2-6.0)	2.4* (2.0-2.8)	8.6* (7.1-10.2)	14.7* (11.2-18.2)	0.4* (0.3-0.5)
	group 2	25.4 (25.0-25.8)	6.2* (5.7-6.7)	11.2 (11.0-11.4)	17.5* (18.6-16.4)	12.5* (10.7-14.4)	0.5* (0.4-0.6)

* $P < 0.05$ with respect to the corresponding value of the ipsilateral paw.

Table 1 (see Fig. 3 and 6). Results (expressed as means ± confident interval 95 %) obtained following Boltzmann sigmoid regression of individual curves of skin temperature following microinjections of muscimol (see Fig. 1). For both the paws (A) and the tail (B), the experiments were divided in three groups on the basis of the beginning time t_{min} of the ascending phase of vasodilatation. Group 1: **t_{min} < 15 min for all** skin areas; Group 2: **t_{min} < 15 min for at least one** skin area; Group 3 (not shown): **t_{min} > 15 min for all** skin areas (see Fig. 3 and 6). Keys: t_X = abscissa of the inflection point of the sigmoid, Δt = duration of the ascending phase of vasodilatation, T_{min} = adjusted skin temperature during the control period, ΔT_{skin} = amplitude of skin temperature variation. $\Delta T_{skin}/\Delta t$ = speed of warming. The regression coefficients R^2 of the adjusted curves were always highly significant (> 0.98).

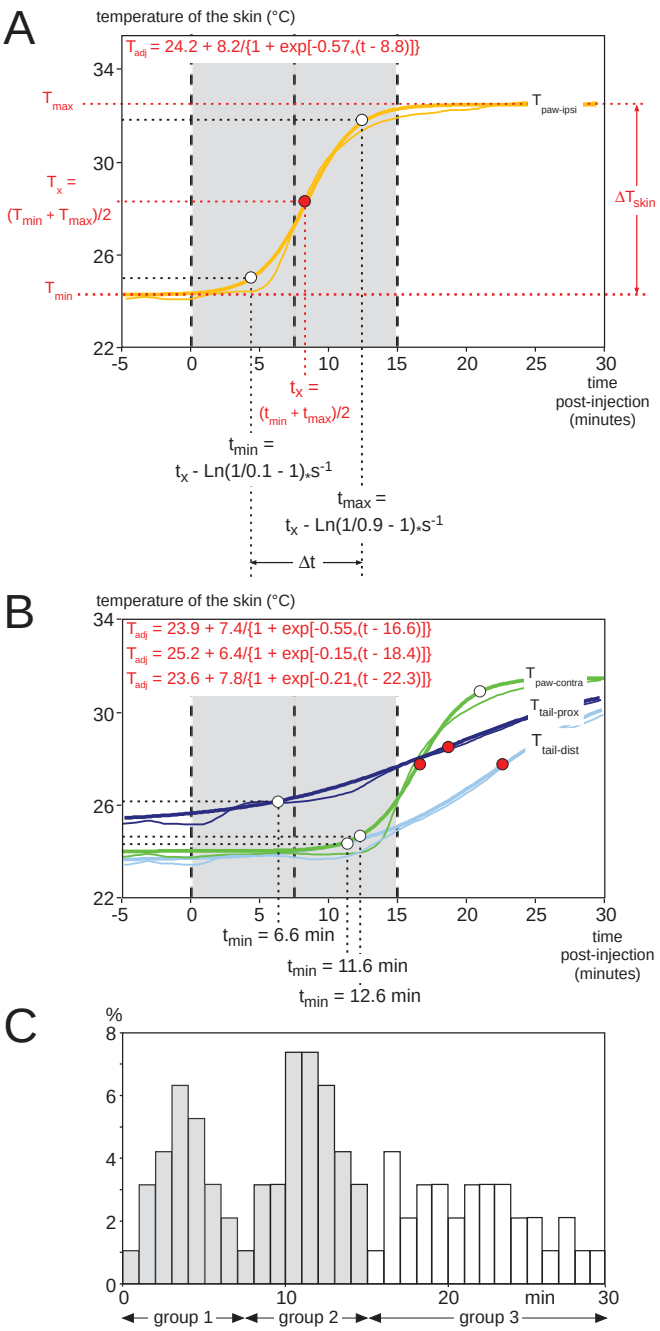
	(A) HLI during vasoconstriction			(B) HLI during vasodilation		
	(a) control period (present study)	(b) vasoconstriction periods (El Bitar et al. 2014a)	P	(a) 30 min following muscimol	(b) vasodilation periods (El Bitar et al. 2014a)	P
$T_{paw\text{-}ipsi}$	0.10 (0.10-0.11)	0.13 (0.09-0.16)	0.12	0.69 0.65-0.74)	0.92 (0.85-0.98)	< 0.00001
$T_{tail\text{-}prox}$	0.24 (0.22-0.25)	0.23 (0.21-0.26)	0.64	0.62 (0.57-0.67)	0.77 (0.72-0.83)	< 0.0001
$T_{tail\text{-}dist}$	0.04 (0.04-0.04)	0.03 (0.01-0.05)	0.06	0.55 (0.46-0.63)	0.74 (0.69-0.80)	< 0.0001

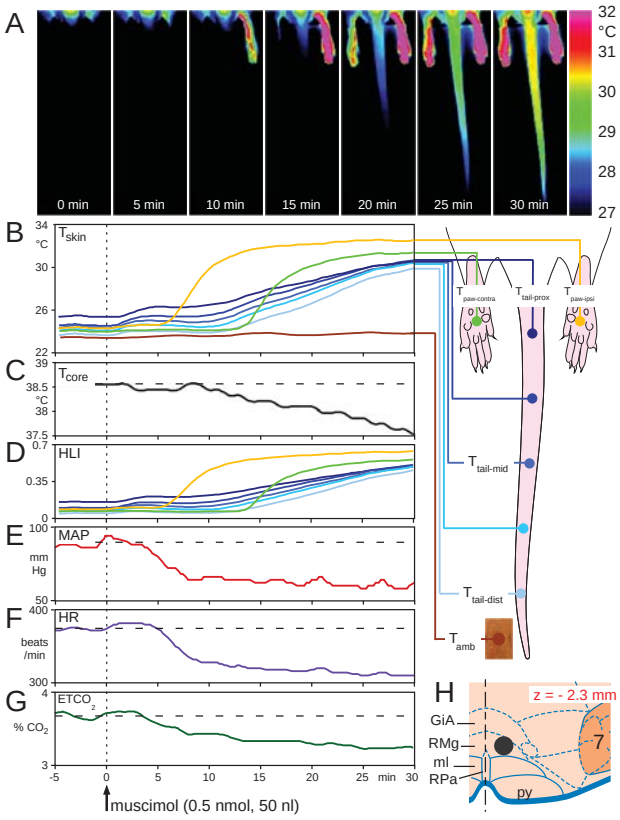
Table 2. Summary of skin vasomotor tone expressed in terms of Heat Loss Index [HLI = $(T_{skin}-T_{amb})/(T_{core}-T_{amb})$] (Gordon et al. 2002; Romanovsky et al. 2002; Székely 1986), represented as means and their 95% confidence interval. Results obtained from current study (a) are compared to a previous study (b) in the extreme vasomotor tone status, namely vasoconstriction (A) and vasodilation (B). During vasoconstriction, there were no significant statistical differences between HLI observed in the two studies. By contrast, the HLI for both hind-paws and following muscimol microinjections were significantly lower than the HLI seen during the physiological vasodilation elicited during thermo-neutrality (El Bitar et al. 2014a).

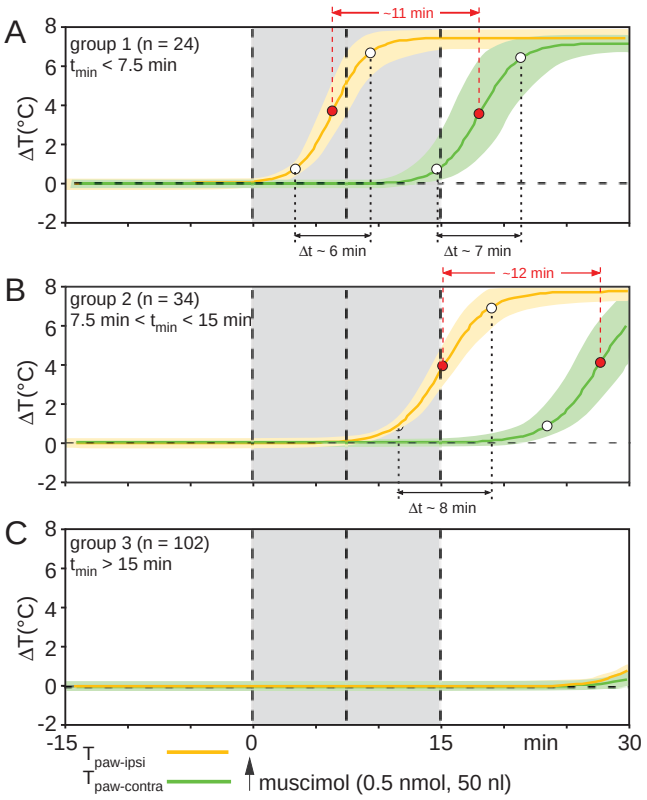
	Skin area	T_{min} (°C)	ΔT_{skin} (°C)	t_{min} (min)	t_x (min)	Δt (min)	$\Delta T_{skin}/\Delta t$ (°C/min)	ΔT_{core} (°C)	ΔMAP (mmHg)	ΔHR (bpm)	$\Delta ETCO_2$ (%)
Group 1	ipsilateral paw	26.1 (25.7-26.4)	7.3 (6.9-7.6)	5.8 (5.4-6.2)	8.8 (7.8-9.8)	5.9 (4.6-7.1)	1.2 (1.0-1.7))	0.96 (0.85-1.06)	23.9 (20.1-27.7	39 (31-48)	0.5 (0.4-0.5)
	contralateral paw	25.9 (25.7-26.1)	6.9 (6.2-6.6)	9.6 (8.7-10.4)	13.0 (11.6-14.2)	6.7 (5.7-7.6)	1.0 (0.8-1.2)				
	proximal tail	27.8 (27.4-28.1)	4.2 (3.9-4.5)	5.3 (5.0-5.6)	10.9 (9.8-11.9)	14.2 (12.4-16.0)	0.3 (0.2-0.4)				
	distal tail	25.3 (25.0-25.5)	5.6 (5.2-5.9)	5.1 (2.4-7.7)	11.7 (10.3-13.1)	13.2 (10.8-15.6)	0.4 (0.3-0.5)				
Group 2	ipsilateral paw	25.2 (25.0-25.3)	7.7 (7.5-8.0)	10.9** (10.4-11.3)	14.8** (13.9-15.8)	7.8 (6.9-8.8)	1.0 (0.9-1.1)	0.52** (0.37-0.67)	19.4* (12.5-26.3)	40 (34-45)	0.4 (0.4-0.5)
	contralateral paw	25.4 (25.2-25.5)	7.2 (7.1-7.4)	27.6** (26.5-28.6)	31.5** (32.8-30.)	7.2 (7.1-7.4)	1.0 (1.0-1.0)				
	proximal tail	25.6 (25.2-25.9)	6,0 (5.7-6.4)	10.2** (10.1-10.3)	24.8** (23.7-26.9)	29.2 (27.2-31.2)	0.2 (0.2-0.2)				
	distal tail	24,6 (24.4-24.8)	6.4 (6.2-6.6)	21,4** (22.0-20.8)	29,1** (28.0-30.3)	15.5 (14.4 -16.5)	0.4 (0.4-0.5)				

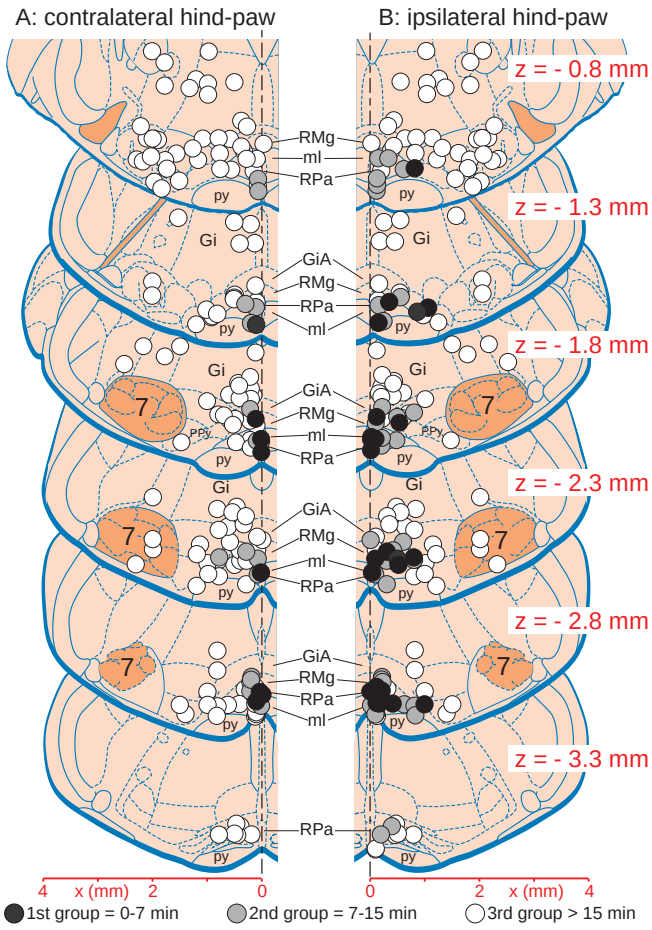
* $P < 0.05$ **, $P < 0.01$ with respect to the corresponding value in group 1.

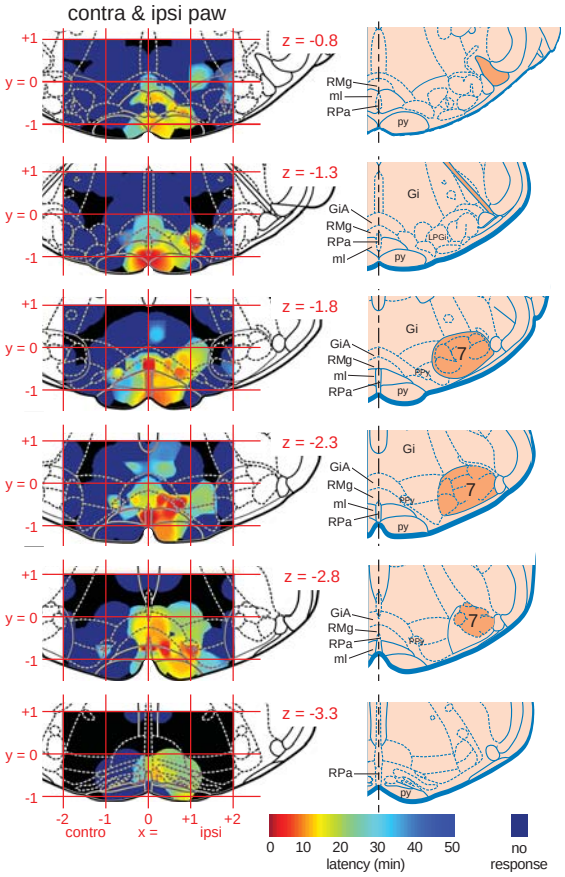
Table 3 (see Fig. 9). Results (means ± confident interval 95%) expressed in terms of difference to mean value recorded during the control period. (1) The data represented in the five left hand columns were obtained following Boltzmann sigmoid regression of individual curves of skin temperature following microinjections of muscimol (see Fig. 1). The experiments were divided in three groups on the basis of the beginning time t_{min} of the ascending phase of vasodilatation. Group 1: **t_{min} < 15 min for all** skin areas; Group 2: **t_{min} < 15 min for at least one** skin area; Group 3 (not shown): **t_{min} > 15 min for all** skin areas (see Fig. 9B-9E). Keys: T_{min} = adjusted skin temperature during the control period, ΔT_{skin} = amplitude of skin temperature variation, t_X = abscissa of the inflection point of the sigmoid, Δt = duration of the ascending phase of vasodilatation. The regression coefficients R^2 of the adjusted curves were always highly significant (> 0.98). (2) The data represented in the four right hand columns are the corresponding data related to the vital signs (T_{core}, MAP, HR and $ETCO_2$) recorded 30 minutes following the microinjection of muscimol (see Fig. 9F-9I). The mean values for T_{core}, MAP, HR and $ETCO_2$ during the control period were 37.7 (37.6 - 37.8)°C, 83 (77-90) mmHg, 320 (309-332) bpm, 3.65 (3.57-3.73)%, respectively. There was no statistical difference among the three groups concerning T_{core}, MAP, HR and $ETCO_2$ during the control period (P = 0.78, 0.25, 0.77 and 0.63, respectively).

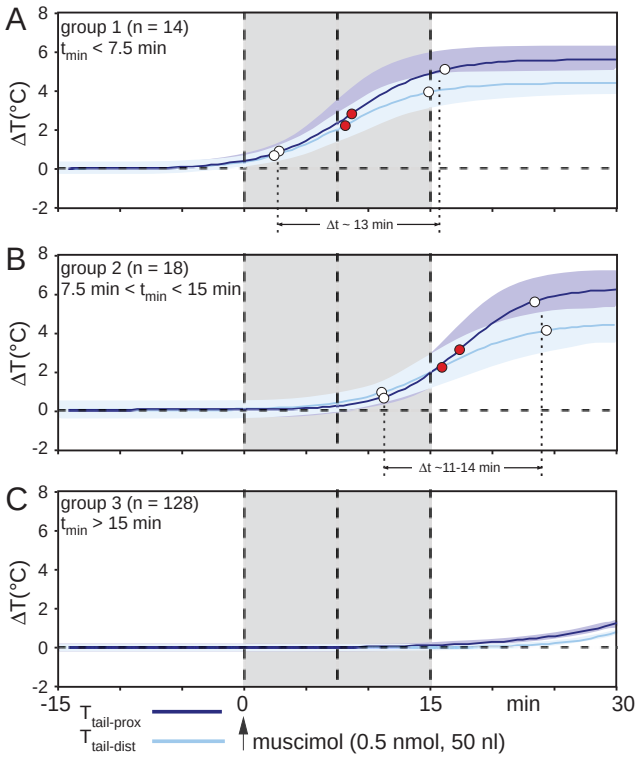

A
temperature of the skin (°C)
T_{adj} = 24.2 + 8.2/{1 + exp[-0.57*(t - 8.8)]}
T_{max}
T_{min}
T_x = (T_{min} + T_{max})/2
$T_{paw-ipsi}$
ΔT_{skin}
34
30
26
22
-5
0
5
10
15
20
25
30
time post-injection (minutes)
t_x = (t_{min} + t_{max})/2
t_{min} = t_x - Ln(1/0.1 - 1)*s^{-1}
t_{max} = t_x - Ln(1/0.9 - 1)*s^{-1}
Δt
B
temperature of the skin (°C)
T_{adj} = 23.9 + 7.4/{1 + exp[-0.55*(t - 16.6)]}
T_{adj} = 25.2 + 6.4/{1 + exp[-0.15*(t - 18.4)]}
T_{adj} = 23.6 + 7.8/{1 + exp[-0.21*(t - 22.3)]}
$T_{paw-contra}$
$T_{tail-prox}$
$T_{tail-dist}$
34
30
26
22
-5
0
5
10
15
20
25
30
time post-injection (minutes)
t_{min} = 6.6 min
t_{min} = 11.6 min
t_{min} = 12.6 min
C
%
8
6
4
2
0
0
10
20
min
30
group 1
group 2
group 3

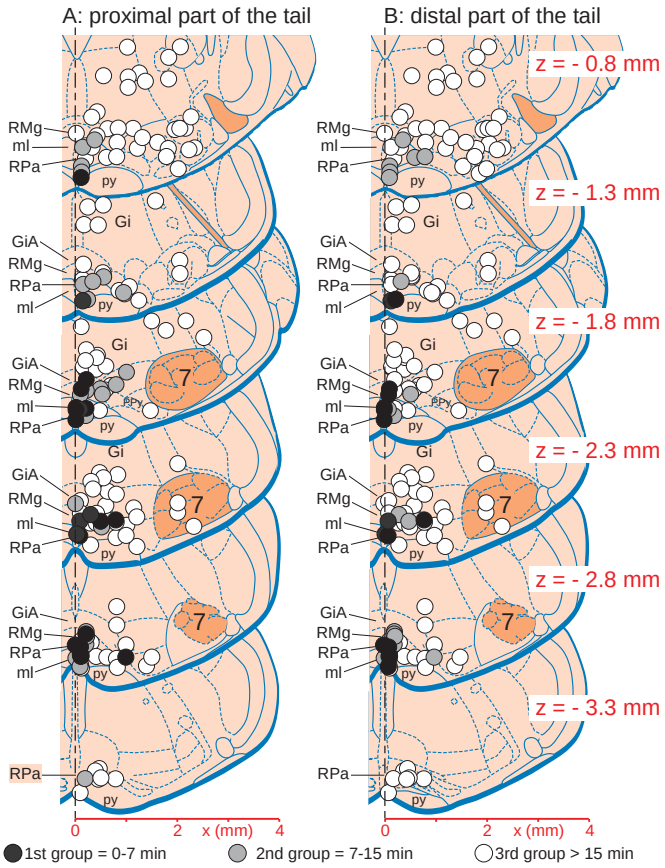

A
0 min
5 min
10 min
15 min
20 min
25 min
30 min
32
°C
31
30
29
28
27
B
T skin
34
°C
30
26
22
C
T core
39
38.5
°C
38
37.5
D
HLI
0.7
0.35
0
E
MAP
100
mm
Hg
50
F
HR
400
beats
/min
300
G
ETCO2
4
% CO2
3
-5
0
5
10
15
20
25
min
30
muscimol (0.5 nmol, 50 nl)
T paw-contra
T tail-prox
T paw-ipsi
T tail-mid
T tail-dist
T amb
H
z = - 2.3 mm
GiA
RMg
ml
RPa
py
7

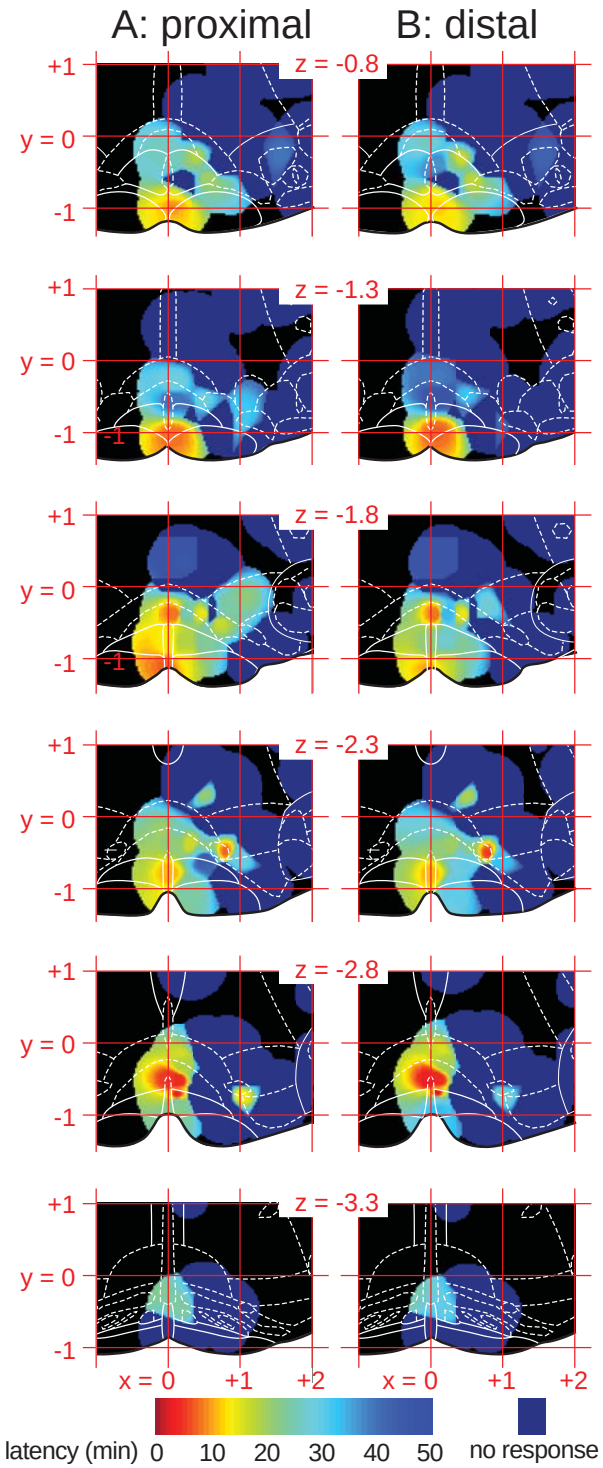

A
group 1 (n = 24)
t_min < 7.5 min
~11 min
ΔT(°C)
8
6
4
2
0
-2
Δt ~ 6 min
Δt ~ 7 min
B
group 2 (n = 34)
7.5 min < t_min < 15 min
~12 min
Δt ~ 8 min
C
group 3 (n = 102)
t_min > 15 min
-15
0
15
min
30
T paw-ipsi
T paw-contra
muscimol (0.5 nmol, 50 nl)

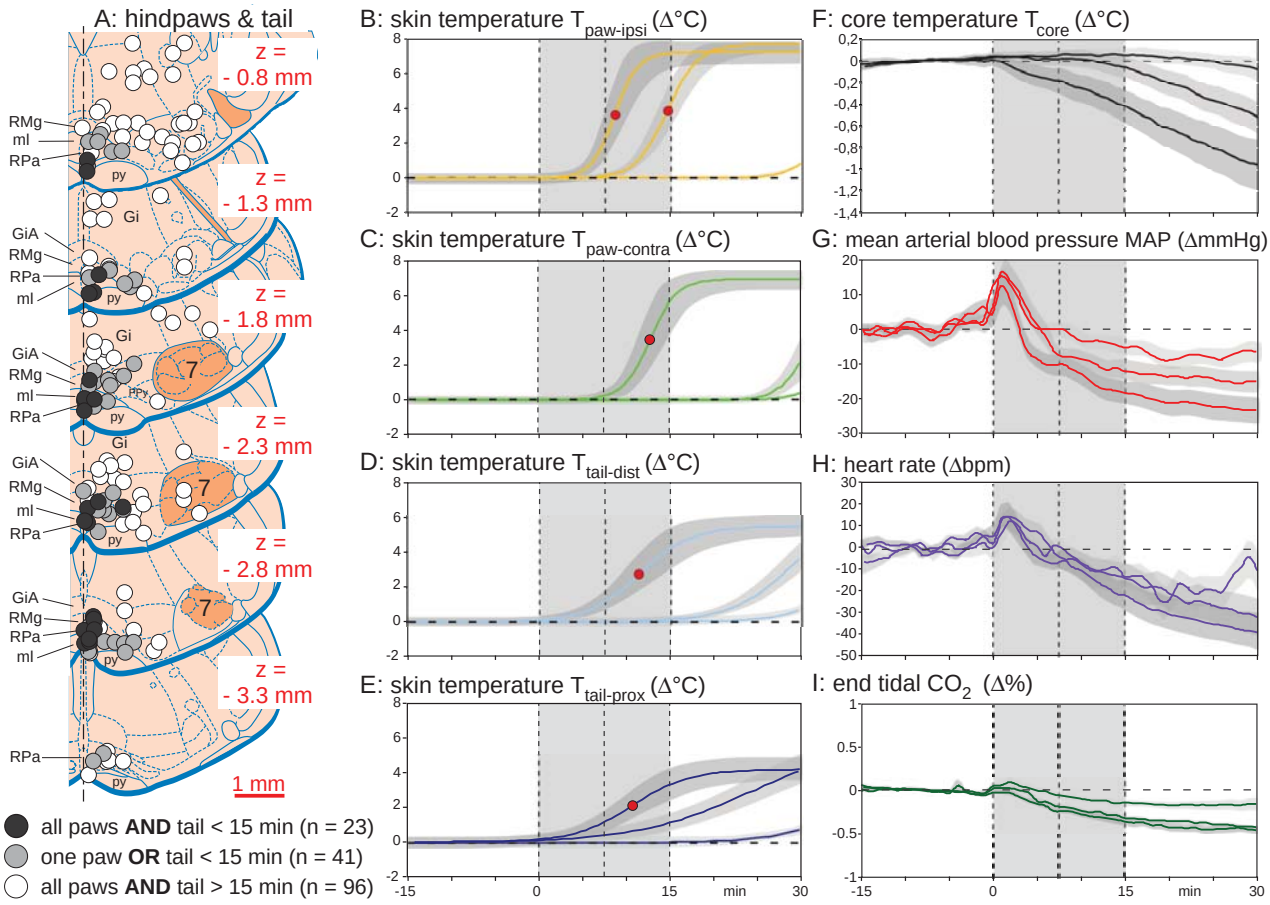

A: contralateral hind-paw
B: ipsilateral hind-paw
z = - 0.8 mm
z = - 1.3 mm
z = - 1.8 mm
z = - 2.3 mm
z = - 2.8 mm
z = - 3.3 mm
RMg
ml
RPa
GiA
py
Gi
PPy
7
4 x (mm) 2 0
0 2 x (mm) 4
1st group = 0-7 min
2nd group = 7-15 min
3rd group > 15 min

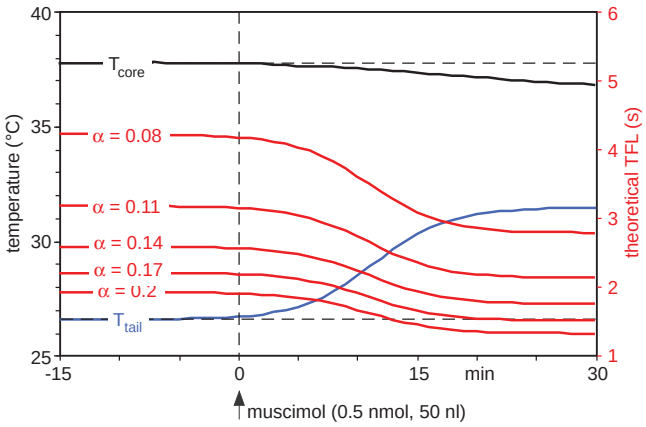

contra & ipsi paw
+1
y = 0
-1
z = -0.8
RMg
ml
RPa
py
z = -1.3
Gi
GiA
RMg
RPa
LPGi
ml
py
z = -1.8
Gi
GiA
RMg
ml
RPa
7
py
z = -2.3
Gi
GiA
RMg
ml
RPa
7
py
z = -2.8
GiA
RMg
RPa
ml
7
z = -3.3
RPa
py
-2
-1
0
+1
+2
contro
x =
ipsi
0
10
20
30
40
50
latency (min)
no
response


A
group 1 (n = 14)
t_min < 7.5 min
ΔT(°C)
8
6
4
2
0
-2
Δt ~ 13 min
B
group 2 (n = 18)
7.5 min < t_min < 15 min
ΔT(°C)
Δt ~11-14 min
C
group 3 (n = 128)
t_min > 15 min
ΔT(°C)
-15
0
15
min
30
T tail-prox
T tail-dist
muscimol (0.5 nmol, 50 nl)


A: proximal part of the tail
B: distal part of the tail
z = - 0.8 mm
z = - 1.3 mm
z = - 1.8 mm
z = - 2.3 mm
z = - 2.8 mm
z = - 3.3 mm
RMg
ml
RPa
py
Gi
GiA
7
bPy
0
2
x (mm)
4
1st group = 0-7 min
2nd group = 7-15 min
3rd group > 15 min


A: proximal
B: distal
z = -0.8
z = -1.3
z = -1.8
z = -2.3
z = -2.8
z = -3.3
+1
y = 0
-1
x = 0
+1
+2
latency (min)
0
10
20
30
40
50
no response


A: hindpaws & tail
z = - 0.8 mm
z = - 1.3 mm
z = - 1.8 mm
z = - 2.3 mm
z = - 2.8 mm
z = - 3.3 mm
RMg
ml
RPa
py
Gi
GiA
7
1 mm
all paws AND tail < 15 min (n = 23)
one paw OR tail < 15 min (n = 41)
all paws AND tail > 15 min (n = 96)
B: skin temperature T paw-ipsi (Δ°C)
C: skin temperature T paw-contra (Δ°C)
D: skin temperature T tail-dist (Δ°C)
E: skin temperature T tail-prox (Δ°C)
F: core temperature T core (Δ°C)
G: mean arterial blood pressure MAP (ΔmmHg)
H: heart rate (Δbpm)
I: end tidal CO2 (Δ%)
-15
0
15
min
30


40
35
30
25
6
5
4
3
2
1
temperature (°C)
theoretical TFL (s)
T core
α = 0.08
α = 0.11
α = 0.14
α = 0.17
α = 0.2
T tail
-15
0
15
min
30
muscimol (0.5 nmol, 50 nl)