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CARDIOVASCULAR AND BEHAVIOURAL RESPONSES TO CONDITIONED FEAR AFTER MEDULLARY RAPHE NEURONAL BLOCKADE.

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LIST OF ABBREVIATIONS

HR – heart rate

MAP – mean arterial pressure

PAG – periaqueductal gray

RMg – raphe magnus

RPa – raphe pallidus

RVM – rostral ventromedial medulla

TBody – deep body temperature

TTail – tail surface temperature

USV – ultrasonic vocalizations

VLPAG – ventrolateral periaqueductal gray

ABSTRACT

Conditioned fear to context in the rat leads to a host of sympathetically mediated physiological changes, including a marked rise in mean arterial pressure, a delayed rise in heart rate and a marked cutaneous vasoconstriction, along with the behavioral responses of freezing and ultrasonic vocalisation. In this study we examine the role of the rostral ventromedial medulla (RVM), which includes raphe nuclei pallidus and magnus, in the expression of these changes. RVM is a major premotor sympathetic and somatic center and an important integrating center in the descending emotional motor system. To evaluate its role, conditioned fear was tested after temporary blockade with microinjections (0.4 µl) of the GABA-A receptor agonist muscimol (0.2 mM) or the glutamate receptor antagonist kynurenic acid (0.1 M). Changes in mean arterial pressure, heart rate and activity were recorded by radio-telemetry. Cutaneous vasoconstriction in the tail was recorded indirectly by infrared thermography. Muscimol and kynurenic acid had different, almost complementary effects. Muscimol abolished the skin vasoconstrictor response and significantly reduced the tachycardic response, but did not reduce the pressor response significantly and had little effect on the somatic motor components, freezing and ultrasonic vocalization. In contrast, kynurenic acid abolished ultrasonic vocalization and significantly reduced freezing but had no effect on the cardiovascular components. The results show that neurons in the rostral ventromedial medulla are implicated in the expression of some of the cardiac, vascular and somatic motor components of conditioned fear. Most importantly, these cardiovascular components are not under local glutamatergic control whereas the somatic motor components are.

Keywords: RVM, Raphe Pallidus, Raphe Magnus, Heart rate, Cutaneous blood flow, Freezing.

Fear triggers a series of autonomic changes that prepare an animal to deal with a survival challenge. This can be observed for example when a rat is re-exposed to an environment (or context) which has previously been paired with an aversive stimulus. This response, known as conditioned fear to context or contextual fear, is associated with a marked increase in mean arterial pressure, a delayed increase in heart rate, a powerful skin vasoconstriction in the tail and an increase in body temperature, while on the behavioral side the animal adopts a tense immobile freezing posture and emits ultrasonic vocalizations (Carrive, 2002, Vianna and Carrive, 2005, Carrive, 2006).

This integrated response is mediated through the activation of a descending system that spans the entire length of the neuraxis, from the limbic forebrain where contextual cues reactivate the memory of the unconditioned stimulus, to autonomic preganglionic neurons and somatic motoneurons that project out to the periphery. Holstege (1995) refers to this system as the emotional motor system. There are 3 main integrating centers in this descending motor system: the hypothalamus in the forebrain, the periaqueductal gray (PAG) in the midbrain, and the rostral ventromedial medulla (RVM) which includes the raphe nuclei raphe pallidus (RPa) and raphe magnus (RMg) (Holstege, 1995). Previous work from Ledoux et al (1988) and from our laboratory (Walker and Carrive, 2003, Furlong and Carrive, 2007) has shown that the hypothalamus and the ventrolateral PAG (VLPAG) mediate some of the autonomic and somatic motor components of the conditioned fear response. However, the role of RVM in the expression of the components of this emotional response has not yet been investigated.

Anatomical and functional attributes of RVM strongly suggest that it could play a role in fear. First, RVM receives inputs from the hypothalamus and VLPAG (Hermann et al., 1997). Second, RVM projects massively to the spinal cord (Holstege and Kuypers, 1982) where it targets sympathetic preganglionic and somatic motoneurons (Allen and Cechetto, 1994, Jansen et al.,

1995a, Billig et al., 2000, Cano et al., 2003, Kerman et al., 2003, Toth et al., 2006). With respect to sympathetic control, recent work has revealed that RPa plays an important role in thermoregulation by controlling the sympathetic outflow to skin vasculature and thermogenic brown fat (Blessing, 2003, Zaretsky et al., 2003a, Morrison, 2004, Nakamura et al., 2005). It also appears to be involved in the control of heart rate during stress because its inhibition reduces the tachycardia evoked by air-jet stress (Zaretsky et al., 2003b). With respect to somatic motor control, it has been shown that disinhibition of RVM increases muscle tone (Nason and Mason, 2004) while inhibition prevents fusimotor activity (Tanaka et al., 2006), and that direct activation produces immobility in awake rats (Morgan and Whitney, 2000). Thus RVM is an important sympathetic and somatic premotor center which could relay outputs of hypothalamus and VLPAG during fear.

In this study we test the role of RVM in the expression of the autonomic (heart rate, mean arterial pressure, skin vasoconstriction, body temperature) and somatic motor (freezing, ultrasonic vocalisation, activity) components of conditioned fear to context. This was achieved by means of chemical inhibition by local microinjections of muscimol, a GABA-A receptor agonist, or kynurenic acid, a glutamate receptor antagonist, immediately before testing the animal for conditioned fear to context.

EXPERIMENTAL PROCEDURES

The subjects were experimentally naïve male Wistar rats (350-500g) obtained from the colony of specific pathogen-free rats maintained by the University of New South Wales. The rats were kept in individual plastic home boxes (65 X 40 X 22 cm) with *ad libitum* food and water throughout the experiment. The animals were housed in the same room during the entire duration of the experiment. It was maintained on a 12-h light/dark cycle and the experiments were conducted during the light phase of the cycle. The room was air conditioned and

maintained at a constant temperature of 24-25 °C. All experiments were approved by the Animal Ethics Committee of the University of New South Wales and conformed to the rules and guidelines on animal experimentation in Australia.

Radio-telemetric probe implantation

Rats were first implanted with Data Sciences International radio-telemetric probes for recording of mean arterial pressure, heart rate and activity (PA-C40, *n*=13) or mean arterial pressure, heart rate, activity and deep body temperature (C50-PXT, *n*=3). The surgery was done in aseptic conditions and under anesthesia with a mixture of Ketamine (Ketamil, 120 mg/kg, i.p.) and Xylazine (Ilium Xylazil-20, 6.5 mg/kg, i.p.). The rats were also pretreated with the analgesic Carprofen (Rimadyl, 5 mg/kg, s.c.) and received antibiotics (Benicillin, 0.3 ml, i.p.) at the end of surgery. The probes were implanted in the peritoneal cavity, with the catheter sitting in the descending aorta at the level of the iliac bifurcation, as previously described (Carrive, 2000). During the recovery period (1 week) the animals were handled every day to habituate to the experimenter.

Guide cannula implantation

The guide cannulae were implanted 1 week after the telemetric probes. The surgery was done under the same anaesthetic, analgesic and aseptic regimen as the radio-telemetric probe implantations. Once anaesthetised, the animal's head was secured in a stereotaxic frame in the flat skull position. The scalp was removed and the skull was exposed. Four holes were drilled: three for the screws and one for the guide cannula (26 G, Plastics One), which was implanted vertically and aimed 1.5 mm above the target region, RPa. The coordinates of the targeted region were: 2.5 mm posterior to the interaural line, 0.0 mm medial, and 10.5 mm ventral to the skull surface, according to the stereotaxic atlas of Paxinos and Watson (2005). After adjustments, our final implantation coordinates were: 2.4 mm posterior to the interaural line, 0.0 mm medial, and 10 mm below the skull surface. The guide cannula was finally anchored to the

screws with dental cement. Animals were allowed to recover for at least one week before fear conditioning began.

Fear conditioning and testing

The apparatus and procedure were the same as in Vianna and Carrive (2005). Because infrared thermography requires an unobstructed view of the animal, the lids of the home boxes and conditioning footshock chambers were removed and replaced by 60 cm tall plexiglass walls high enough to prevent escape. The home box lids were substituted with the plexiglass walls at least one day before each conditioning or testing session. Conditioning and testing was done in footshock chambers (23 cm long x 21 cm wide x 60 cm tall) made of clear Perspex walls on two sides with a grid floor composed of 18 stainless steel rods (2 mm in diameter), spaced 1.5 cm apart, and wired to a shock generator. The chambers were cleaned before and after use with 0.5% acetic acid. Conditioning started at least 1 week after guide cannulae implantation and consisted of three conditioning shock sessions done on separate days over a period of 4 days. Each shock session consisted of a 40-min long exposure to the footshock chamber with four unsignaled electric footshocks (1mA, 1s) delivered at approximately t = 5, 15, 25 and 35 min.

The conditioned fear response was tested by re-exposing the rats to the aversive context of the footshock chamber for 30 min, with no shock delivery. The re-exposure was done immediately after the end of the microinjection. To control for the effect of handling, the rats were also tested in the safe context of their home box, that is, they were returned to their home box immediately after injection. These responses will be referred to as handling controls. There was never more than one test per day.

Drugs

The microinjected drugs were the GABA-A receptor agonist muscimol, (0.2 mM, Sigma), and the glutamate receptor antagonist kynurenic acid (0.1M, Sigma). The drug control was physiological saline. There were three successive experiments. In the first experiment we

compared the effects of muscimol and saline on contextual fear. Because some of the autonomic responses were affected, we then compared the effects of muscimol and saline on handling (second experiment). Finally, in the third experiment, we compared the effects of kynurenic acid and saline on contextual fear. The effects of kynurenic acid and saline on handling were not tested because kynurenic acid had no effect on the autonomic responses of fear. In all experiments the order of injection (drug/saline) was counterbalanced and fear conditioning was reinforced with an additional shock session before the kynurenic experiment.

Microinjection procedure

Each rat was gently taken out of its home box and the stylet of the guide cannula was removed. An injection cannula (33 G, Plastics One) connected to an oil-filled 10-µl Hamilton syringe via polyethylene tubing was inserted into the guide cannula and screwed to its top. The injection was monitored by observing the displacement of the junction between the oil and the injectate. A volume of 0.4 µl was injected in 20 s. One minute later, the cannula was removed, the stylet put back, and the animal introduced in the shock box or returned to its home box. The whole procedure took about 5-7 min, during which the animal was held in one hand, and gently restrained against the chest of the experimenter.

Data collection

Up to 7 parameters were recorded simultaneously: heart rate (HR), mean arterial pressure (MAP), deep body temperature (TBody, in 2 rats only), tail surface temperature (TTail), freezing, ultrasonic vocalizations (USV) and movement of the animal (Activity). HR and MAP were extracted automatically from the pulsatile blood pressure signal recorded by the radio-telemetric probes, using the ART gold software (Data Sciences International) running on a PC computer. Activity was a continuous measure of body movements, which was extracted automatically from changes in orientation of the probes. In rats implanted with C50-PXT probes, the software also extracted TBody from the peritoneal cavity. The probe signals were

sampled every 30 s from 3-s time windows and later averaged over 1-min periods. The surface temperature of the tail was recorded with an infrared digital thermographic camera (ThermaCAM P45, Flir, Sweden) placed 1 m above the animal. This camera has a thermal sensitivity of approximately 0.1 °C and a spatial resolution of 320X240 pixels. The emissivity factor was set at 0.98 which corresponds to the emissivity of the skin (see Vianna and Carrive, 2005). Infrared images were captured automatically every 2 min, starting 30 min before the test and ending 1 h after its end. Re-exposures of the rats to the shock box or to the home box (handling controls) after injection were timed so that they started immediately after an image capture. The images were analyzed with the ThermaCAM Quickview 1.1 software. TTail was extracted from single pixels in the middle section on the midline axis of the tail.

In addition, when testing fear, an experimenter sitting in the experimental room manually recorded the time spent freezing and each time an USV was emitted during the re-exposures. Freezing was sampled every 2 sec and defined as a complete absence of movement while the animal assumed a characteristic tense posture. USVs were detected with a bat detector (Mini-3, Ultrasound Device, UK) tuned to the 20-25kHz frequency range. Both parameters were entered manually using hand held buttons connected to the ART system which automatically cumulated them every 30 sec in synchrony with the telemetric data. They were later averaged over 1-min periods.

Statistical Analysis

The data were analyzed with StatView 5 (SAS Institute Inc.) using repeated measures ANOVAs. The repeated measure was time, and the independent factor was drug or saline. Statistical significance was set at $p< 0.05$. All comparisons were done between minute 1 and minute 29 of the 30 min period following re-exposure to the shock box or return to the home box.

Verification of cannula placement

At the end of the experiment, the animals were given an overdose of pentobarbitone (120 mg/kg, i.p.), intracranially microinjected with a dye (pontamine sky blue, 0.4 µl), and transcardially perfused with saline followed by paraformaldehyde (4% in Phosphate buffer 0.1M). The brains were removed and transferred in a 20 % sucrose solution for 2 days before being sectioned in a cryostat. Serial 50 µm sections were mounted and examined under a light microscope while wet. The center of the sites of injections were finally plotted on standard plates from the atlas of Paxinos and Watson (2005).

RESULTS

A total of 16 rats were implanted and tested. The intended target was RPa on the midline at AP level -2.5 mm interaural. Histology revealed that 10 injection sites had their center within 0.5 mm from the midline, 2 had their center outside this boundary and 4 were in the subarachnoid space. The 10 animals were selected for analysis. Of these 10 animals, 9 were tested with muscimol for fear, 8 of these 9 were tested with muscimol for handling and 8 were tested with kynurenic acid for fear, in this sequence (Fig 1). All saline controls were done except one (kynurenic acid for fear) due to an error in the recording of the telemetric data. The three experiments have been analyzed separately as shown on Figures 2, 3 and 4.

--------- Figure 1 here --------

Prior to the tests, the animals were at rest, usually asleep. Baseline values for HR, MAP and TTail were on average 323 bpm, 91 mmHg and 27.8 °C respectively, and there was no statistically significant difference between the different conditions (Figs 2, 3 and 4). TBody was recorded in only 2 of the 10 rats and had an average baseline value of 37.4 °C (data not shown).

Responses associated with fear after muscimol or saline.

The contextual fear response after injection of muscimol or saline is shown on Fig 2. The response after saline will be described first.

Fear after saline. Re-exposure to the shock box after RVM injection of saline evoked the typical conditioned fear response as previously described (Carrive, 2002, Walker and Carrive, 2003, Vianna and Carrive, 2005, Carrive, 2006) (Fig 2, open circles). Behaviorally, the response was characterised by an immobile freezing posture which lasted for nearly the entire duration of the re-exposure (see Freezing and Activity traces). Freezing immobility was also associated with a characteristic respiratory pattern of short inspirations followed by long expirations, and with each expiration, the emission of 22 kHz USV. The cardiovascular response was characterised by a marked increase in HR and MAP, which remained stable throughout the re-exposure (on average +27 mmHg and +115 bpm above baseline). TTail dropped down to room temperature (-4°C from baseline), which was reached halfway through the re-exposure (Fig 5). This drop reflects a reduction in blood flow due to cutaneous vasoconstriction. In the two animals in which TBody was recorded, a steady increase was observed to a maximum of + 1.1°C (both animals) by the end of the re-exposure, as previously described (Vianna and Carrive, 2005). When returned to their home box after the test, saline-injected rats first displayed a burst of activity for 10 min and then gradually returned to rest (Fig 2). Within 40-60 min, HR, MAP and Activity were back to baseline. TTail displayed a rebound increase of +8°C from room temperature to 32 ºC 10 min after the re-exposure, then returned to baseline, which was reached 70-75 min later (data not shown). TBody was also back to baseline at approximately the same time.

Fear after muscimol. RVM injections of 0.2 mM muscimol had a clear effect on the autonomic components of the fear response (Fig 2). The average HR response was reduced by 46% and the average MAP response by 40%. A repeated measure ANOVA confirmed that the reduction in HR was a significant effect ($F_{1,28}$=14.46, p=0.0016), however the apparent reduction of the

average MAP response was not ($F_{1,28}$=2.89, p=0.11). The clearest effect was on the TTail response which was completely abolished ($F_{1,13}$= 24.41, p= 0.0001) and in fact reversed to an average of 2.0 ºC above baseline (Fig 2 and Fig 4). TTail was back to baseline by the end of the re-exposure. In the two rats in which TBody was recorded, no increase was observed but instead a steady drop that reached a maximum of -1.1 and -2.0°C below baseline by the end of the re-exposure. In contrast, there was little or no change in the somatic motor components. There was on average less freezing in the second half of the re-exposure but this reduction was small and not significant ($F_{1,28}$=2.47, p=0.14). Activity was slightly higher on average but this difference was also not significant ($F_{1,28}$=2.32, p=0.15) and the USV response was the same as with saline ($F_{1,28}$=0.142, p=0.71). Within 5 mins of returning to the home box, HR and MAP were the same as saline and TTail was close to baseline. All variables returned to baseline within 40-70 min (up to 90 min for TBody in one rat).

--------- Figure 2 here --------

Responses associated with handling after muscimol or saline.

Eight of the 9 rats tested above were reinjected with muscimol and saline and returned instead to the safe context of their home box (Fig 3). These handling controls were done to test the specificity of the effect of muscimol on the fear response and the aversive context.

Handling after saline. Return to the home box after RVM saline evoked the same 10 min long burst of activity (Fig 3) observed in animals returned to their home box after the fear test (Fig 2). This was associated with a marked increase in HR and MAP similar to that observed with fear, but within 30 min, both variables were back to baseline. The average TTail response also dropped initially (-2.6°C) but not as low as during fear and was back to baseline within 20 mins. It peaked 10 min later, +3.0°C above baseline and remained elevated until the end of the recording session. In one animal in which TBody was recorded, a small increase in temperature

(+0.38°C) was observed which peaked 10 min after returning to the home box.

Handling after muscimol. RVM injections of 0.2 mM muscimol had no major effect on the handling response, compared to saline (Fig 3). Small reductions of the average HR, MAP and TTail responses were observed, but these were not statistically significant (HR, $F_{1,28}$=1.65, p=0.22; MAP, $F_{1,28}$=1.87, p=0.20; TTail, $F_{1,13}$=0.02, p=0.90). There was no difference in the Activity response either (Activity, $F_{1,28}$=2.32, p=0.15). However, in the only animal in which TBody response was recorded, the increase in temperature was not seen. We observed instead a gradual drop which peaked -1.5 °C below baseline 45 min after return to the home box.

--------- Figure 3 here --------

Responses associated with fear after kynurenic acid or saline.

The contextual fear response after injection of kynurenic acid or saline is shown on Fig 4. This experiment was conducted to find out if glutamatergic transmission in RVM plays a role in the expression of the fear response. The fear response after saline was the same as in the first experiment and will not be described again.

Fear after kynurenic acid. In contrast to muscimol, the RVM injection of 0.1M kynurenic acid had no effect on the autonomic components of the fear response (Fig 4). The HR, MAP and TTail responses were not different from those evoked after saline ($F_{1,28}$<0.64, p>0,44; Figs 4 and 5). In the two rats in which TBody was recorded we observed the same steady rise as in saline which peaked +1.3 and +1.9°C above baseline by the end of the re-exposure. In contrast to this lack of effect on autonomic variables, there were marked changes in the somatic motor components. Freezing was significantly reduced to 56% of saline values ($F_{1,28}$=17.26, p=0.001), USV was almost abolished ($F_{1,28}$=10.68, p=0.006) and Activity was increased almost four-fold (392% of saline; $F_{1,28}$=10.48, p=0.006). Finally, there was no difference between saline and

kynurenic acid injected animals in the recovery period.

The effect of kynurenic acid on handling was not tested since none of the autonomic variables were affected and since freezing and vocalizations are specific to fear.

--------- Figure 4 here --------

--------- Figure 5 here --------

Other controls.

As mentioned earlier, two animals tested with muscimol were not included in the analysis because their injection sites were more than 0.5 mm from the midline (Fig 1, open circles). Muscimol had no effect on the fear response of these animals. In both cases HR increased (+143 and +159 bpm from baseline) and TTail decreased (down to 25.1 °C and 23.6 °C) as was observed after saline injection. This suggests that the effects we observed were restricted to the midline region. These 2 sites were not injected with kynurenic acid.

One animal was re-injected with muscimol and re-tested after completing the kynurenic acid and saline tests. The response observed was the same as in the first test, with a 64 % reduction of the tachycardic response and a complete abolition of the TTail response.

DISCUSSION

This study shows that neurons in the region of the rostral medullary raphe nuclei are involved in the expression of the contextual fear response because five of the six components we recorded, including autonomic and behavioral ones, could be significantly reduced by temporary blockade with either 0.2 mM muscimol or 0.1M kynurenic acid. Interestingly, the effects of the two drugs on these five components were different. 0.2mM Muscimol's main effect was cardiac and vascular: it reduced the tachycardia and abolished the tail vasoconstriction, but did not reduce

the freezing and vocalization responses. In contrast, the main effect of 0.1M kynurenic acid was on the somatic motor components of the response: it abolished ultrasonic vocalizations and reduced freezing but had no effect on any of the autonomic components recorded. This indicates that fear activates two types of synapses in RVM: glutamatergic synapses that mediate some of the behavioral components of the response, and non glutamatergic synapses that mediate some of its autonomic components.

Muscimol effect

The GABA-A receptor agonist muscimol is an effective inhibitory agent that could potentially block all neuronal activity at the site of injection, although this may depend on the dose. The dose we used here (80 pmol in 400 nl) completely abolished the tail vasoconstrictor response and significantly reduced the tachycardic response but the pressor and freezing responses were not significantly reduced. Blockade of the tail vasoconstrictor response was specific to fear because the tail response in handling controls was the same after muscimol and saline. Thus it was not due to a non specific vasodilation of tail cutaneous blood vessels. Reduction of the tachycardic response was also specific to fear since the tachycardia observed in handling controls was not significantly reduced by muscimol.

The complete blockade of the TTail response to fear is consistent with the work of Blessing and collaborators which shows that RVM plays a major role in the control of cutaneous blood flow (Blessing, 2003). For example, muscimol injections in RVM reduce alerting stimuli, cold stress and hypothalamic induced ear vasoconstriction in the rabbit (Nalivaiko and Blessing, 2001, Ootsuka and Blessing, 2005) as well as cold induced tail sympathetic activity in the anesthetized rat (Ootsuka et al., 2004). Our results support this view and show that RVM plays a major role in the tail vasoconstrictor component of the fear response. Our results also indicate that RVM is not involved in the milder tail vasoconstriction of the handling control, nor in the control of tail

basal vasoconstrictor tone. This may appear at odds with the reports of Blessing and collaborators that RVM mediates the tail response to mild and transient stressors such as alerting stimuli. However, blockade of alerting stimuli from RVM has only been reported in the rabbit (Ootsuka and Blessing, 2005). We are not aware of any report showing that RVM blockade prevents the tail vasoconstriction associated with alerting stimuli in the rat. Furthermore, the dose of muscimol required to block alerting stimuli in the rabbit was high (3 nmol in 300 nl), 37.5 times greater than the one we used (80 pmol in 400 nl). It will be important to clarify these species-specific and dose issues. Meanwhile, our observations in the handling controls suggest that the control of tail vasoconstrictor tone is more complex and not solely dependant on RVM (see also Ootsuka and McAllen, 2005).

RVM also appears to play a significant role in the tachycardic response of fear although it was only reduced by half. This is consistent with a previous report by Zaretsky et al. (2003b) in the conscious rat, in which RVM muscimol caused a similar reduction of the tachycardic response to air-jet stress with no effect when the animal was at rest (Zaretsky et al., 2003a, Zaretsky et al., 2003b).

The pressor response was not significantly reduced by muscimol. Zaretsky et al, (2003b) made a similar observation in response to air jet stress using the same dose of muscimol (80 pmol also, but in 100 nl). However, this dose is relatively small and it could be argued that the noticeable reduction we observed could have reached significance with a larger dose. In fact, studies conducted under anesthesia give mixed results concerning the role of RVM in mediating pressor responses. In some cases, the pressor response was also found not to be significantly reduced, as in response to skin cooling (Nakamura and Morrison, 2007) or dorsomedial hypothalamus (DMH) activation (Samuels et al., 2002, Cao et al., 2004), while in other cases, it was significantly reduced, as in response to icv prostaglandin (Morrison, 2003), icv CRF (Cerri and Morrison, 2006) or activation of DMH (Nalivaiko and Blessing, 2001, Horiuchi et al., 2004).

Thus, while it appears that RVM does not play a major role in the pressor response of fear, at least compared to the tail vasoconstrictor and tachycardic responses, we should not completely rule out any involvement. After all, the tail vasoconstriction and tachycardia that RVM mediates would be expected to indirectly contribute to the rise in blood pressure by increasing peripheral resistance and cardiac output respectively.

TBody was only recorded in 2 animals during fear and in one handling control, precluding any statistical analysis. However, in both conditions, TBody droped below baseline, indicating an effect on baseline function rather than a specific blockade of the hyperthermic response of fear. The same effect has been reported by Zaretsky et al (2003a) in conscious rats tested at rest. This confirms that RVM plays an important role in maintaining body temperature in the conscious animal. Fear could enhance this tonic function, however, the present experiment cannot resolve this issue.

Muscimol had little effect on freezing, ultrasonic vocalizations and activity. Taken at face value, this result suggests that RVM plays little role in the control of the somatic motor response of fear, but this also should be interpreted with caution, especially since kynurenic acid did reduce these components significantly. A number of studies show that RVM can control somatic motor responses. Thus muscle tone in hindlimbs (Nason and Mason, 2004) is increased by bicuculine disinhibition while fusimotor activity evoked by skin cooling, a response thought to lead to increased muscle tone and shivering, is prevented by glycine inhibition (Tanaka et al., 2006). More importantly, a recent study in the hamster shows that tonic immobility - a characteristic defensive response in this species- is reduced by 80% with a dose of muscimol 6 times ours (da Silva and Menescal-de-Oliveira, 2007). Thus it may be that higher doses of muscimol are required to significantly reduce the somatic motor components of the fear response. Perhaps output neurons relaying the somatic motor components have fewer GABA-A receptors or are under a particularly strong activation that would require higher doses of muscimol to offset.

They could also be located at a distance from the center of injection, further than those that control tail vasoconstrictor tone and heart rate.

Kynurenic acid.

Kynurenic acid is a broad spectrum antagonist of glutamate ionotropic receptors that should block any glutamatergic activation during fear. An important finding of this study is that none of the autonomic components we recorded were affected by this glutamatergic blockade. Thus, although the muscimol effects show that RVM contributes to the expression of some of these components (the tail vasoconstriction and the tachycardia), this function is not mediated by a local glutamatergic synapse. For the tail vasoconstrictor effect, this is consistent with a report by Blessing and Nalivaiko (2000) showing that the ear vasoconstriction evoked by stimulation of the spinal trigeminal tract or pinching of the lip in the rabbit is reduced by muscimol but not by kynurenic acid in RPa. For the tachycardia, results from previous studies are mixed. The tachycardic response evoked from the DMH is not reduced either by kynurenic acid (Cao and Morrison, 2006) but the tachycardic response to skin cooling is (Nakamura and Morrison, 2007) (both with 0.1M dose, same as in the present study), as is the tachycardic response to lateral hypothalamus stimulation after injection of a glutamate antagonist cocktail in RPa (Cerri and Morrison, 2005). Thus, in some conditions a glutamatergic transmission can mediate a tachycardic response from RVM. However, this does not appear to be the case during fear.

In contrast to autonomic variables, kynurenic acid had a marked effect on the somatic motor components characteristic of the fear response. Freezing immobility was significantly reduced by 40%. This is consistent with a previous report showing that RVM injection of kainic acid produces immobility in conscious unrestrained rats (Morgan and Whitney, 2000). Ultrasonic vocalizations were practically abolished, suggesting that they too are under glutamatergic control in the RVM during fear. This glutamatergic activation may be specifically targeted at

neurons that control the postural muscles of the back and abdominal wall and the constrictor muscles of the larynx. Tonic activation of these muscles would in turn lock the animal in a frozen posture and cause vibrations of the larynx during expiration.

Interestingly, the reduction in freezing immobility appears to have unmasked an underlying state of increased activity, mainly of a locomotor nature, because activity measured by the probe increased four-fold at the same time. This increase in activity was clearly fear-related because it was not observed when the animals were returned to their home box (i.e., it was not different to saline). Thus the glutamatergic activation of RVM neurons that imposes the immobile freezing posture also acts to prevent the expression of an increase in somatic motor activity. This state of increased activity would be another component of the fear response, and would be mediated by a different, parallel pathway, as we have previously suggested (see also discussion in Walker and Carrive, 2003).

Role of RVM in the neural network of fear

RVM is an important premotor center. Its dense projections to the spinal cord (Holstege and Kuypers, 1982) target not only the dorsal horn where they modulate sensory input but also the lateral and ventral horns where they modulate sympathetic outflow and motor output, respectively (Kerman et al., 2003, Nason and Mason, 2004, Mason, 2005). These projections may be premotor links in the descending motor system mediating the fear response.

With respect to modulation of sympathetic outflow, tracing studies with viruses reveal transynaptic retrograde labeling in RVM at short survival time after injection in many sympathetic targets, including tail artery (Smith et al., 1998, Toth et al., 2006), heart and stellate ganglion (Strack et al., 1989a, Jansen et al., 1995b, Ter Horst et al., 1996) adrenal medulla (Strack et al., 1989b, Jansen et al., 1995a, Kerman et al., 2003), kidney (Ding et al., 1993, Schramm et al., 1993, Huang and Weiss, 1999), brown adipose tissue (Cano et al., 2003,

Nakamura et al., 2004) and many other viscera (see Mason (2005) for review), indicating that this area is an important premotor sympathetic center. Our finding that RVM blockade abolished the tail vasoconstrictor component of the response associated with fear strongly suggests that those premotor sympathetic neurons controlling tail skin vasomotor tone are the main, possibly sole, premotor neurons for this component of the fear response, although it is known that premotor neurons in the vasopressor region of the rostral ventrolateral medulla can also affect skin vasoconstriction (Ootsuka and McAllen, 2005). For similar reasons, RVM may also be an important premotor sympathetic center for the tachycardic component of fear. However, because this component could only be reduced by half, it is likely that premotor sympathetic neurons located elsewhere also contribute.

Less is known about RVM modulation of somatic motor output. However, a series of virus studies by Yates and collaborators show transynaptic retrograde labeling in RVM from a number of muscles including, abdominal musculature, diaphragm, genioglossus and gastrocnemius (Billig et al., 1999, Yates et al., 1999, Billig et al., 2000, Billig et al., 2001, Kerman et al., 2003, Shintani et al., 2003). Interestingly, the neurons labelled at short survival time, i.e., those more likely to have direct spinal projection, are usually not located on the midline in RPa, but more laterally and dorsally in RVM. These somatic premotor neurons could be those mediating the postural changes that characterize the freezing response and associated vocalizations. Their location away from the midline could also explain why our relatively low dose of muscimol did not affect them as much as the autonomic ones because autonomic premotor neurons tend to have a more medial and ventral distribution, ie closer to our centers of injection (see (Kerman et al., 2003)).

On the afferent side, RVM receives direct inputs from the dorsal parts of the hypothalamus and from VLPAG as well as from limbic forebrain structures such as prefrontal cortex and bed nucleus of the stria terminalis (Hermann et al., 1997). Inputs driving RVM during fear would

have to come from these structures. In this respect, it is interesting to note the striking similarity between the effect of kynurenic acid in RVM and that of blocking VLPAG (with muscimol or kynurenic acid). In both cases ultrasonic vocalizations and freezing are markedly reduced while the tachycardic and pressor responses are not (Walker and Carrive, 2003). This raises the possibility that the direct projection from VLPAG to RVM is the pathway driving the glutamatergic synapse that controls freezing immobility from RVM. If this is the case, then the non glutamatergic synapse that controls the tail vasoconstrictor and tachycardic responses must be driven by separate inputs. The input mediating the tachycardic component could come from the hypothalamus since it projects directly onto RVM (Hermann et al., 1997, Samuels et al., 2004) and its lesion markedly reduces the tachycardic response of fear (Furlong and Carrive, 2007). Indeed, muscimol inhibition of RVM reduces the tachycardic effect evoked by disinhibition of the dorsal tuberal hypothalamus (Samuels et al., 2002, Cao et al., 2004, Cerri and Morrison, 2005). Muscimol inhibition of RVM also reduces the skin vasoconstrictor effect evoked by electrical stimulation of the hypothalamus, but it is not clear if the latter response is due to activation of neurons or passing fibers (Zhang et al., 1997, Nalivaiko and Blessing, 2001).

In other words, RVM could be mediating two groups of components of the fear response, each with their own inputs and via different synapses: a cardiac and skin vascular component driven from the hypothalamus or elsewhere via a non glutamatergic synapse and a somatic motor component driven from VLPAG via a glutamatergic synapse. Further work is needed to test this hypothesis.

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FIGURE LEGENDS

Figure 1. Distribution of the centers of injection at which muscimol (left) and kynurenic acid (right) were tested. The injection sites indicated by closed circles were included in the analysis. The 2 muscimol tested sites indicated by open circles were more than 0.5 mm from the midline and were not included in the analysis (see results). Plates were extracted from the atlas of Paxinos and Watson (2005). Abbreviations: 7, facial nucleus; GiA, gigantocellular nucleus pars alpha; ml, medial lemniscus; py, pyramidal tract; RMg, nucleus raphe magnus; RPa, nucleus raphe pallidus.

Figure 2. Changes in heart rate, mean arterial pressure, tail surface temperature, freezing, 22KHz ultrasonic vocalizations and activity during contextual fear evoked by re-exposure to the aversive context of the shock box after microinjection of saline (open circles) or 0.2mM muscimol (closed circles). No shock was given during the fear test. Mean ± SEM.

Figure 3. Changes in heart rate, mean arterial pressure, tail surface temperature and activity in animals returned to the safe context of their home box (Handling controls) immediately after microinjection of saline (open circles) or 0.2mM muscimol (closed circles). Mean ± SEM.

Figure 4. Changes in heart rate, mean arterial pressure, tail surface temperature, freezing, 22KHz ultrasonic vocalizations and activity during contextual fear evoked by re-exposure to the aversive context of the shock box after microinjection of saline (open circles) or 0.1M kynurenic acid (closed circles). No shock was given during the fear test. Mean ± SEM.

Figure 5. Infrared digital images captured during contextual fear showing the effects of saline, 0.2 mM muscimol and 0.1M kynurenic acid on body surface temperature during contextual fear. Note the low temperature of the tail after saline and kynurenic acid (24°C, close to ambient temperature) but not after muscimol (31°C).

Figure 1

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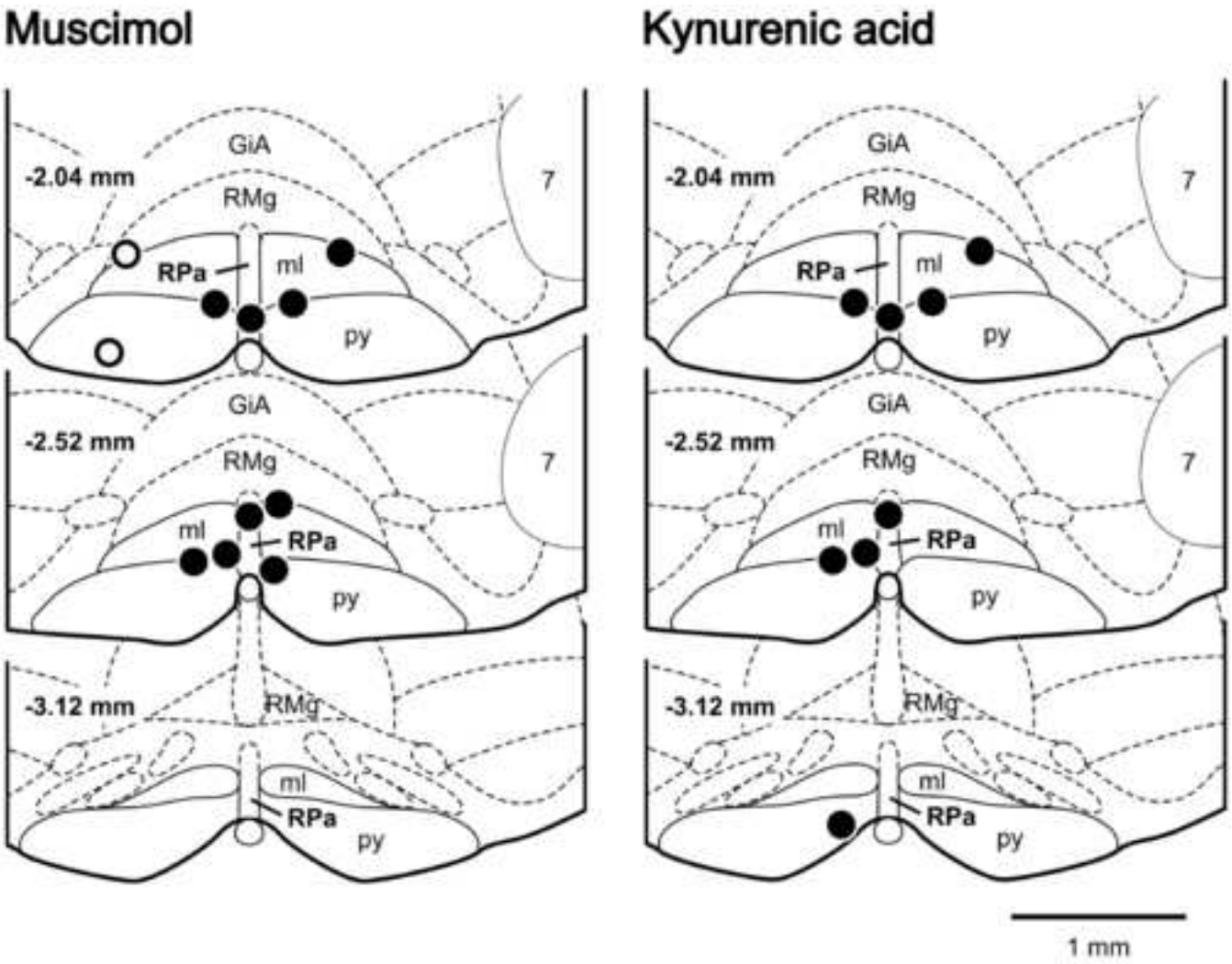

Figure 2

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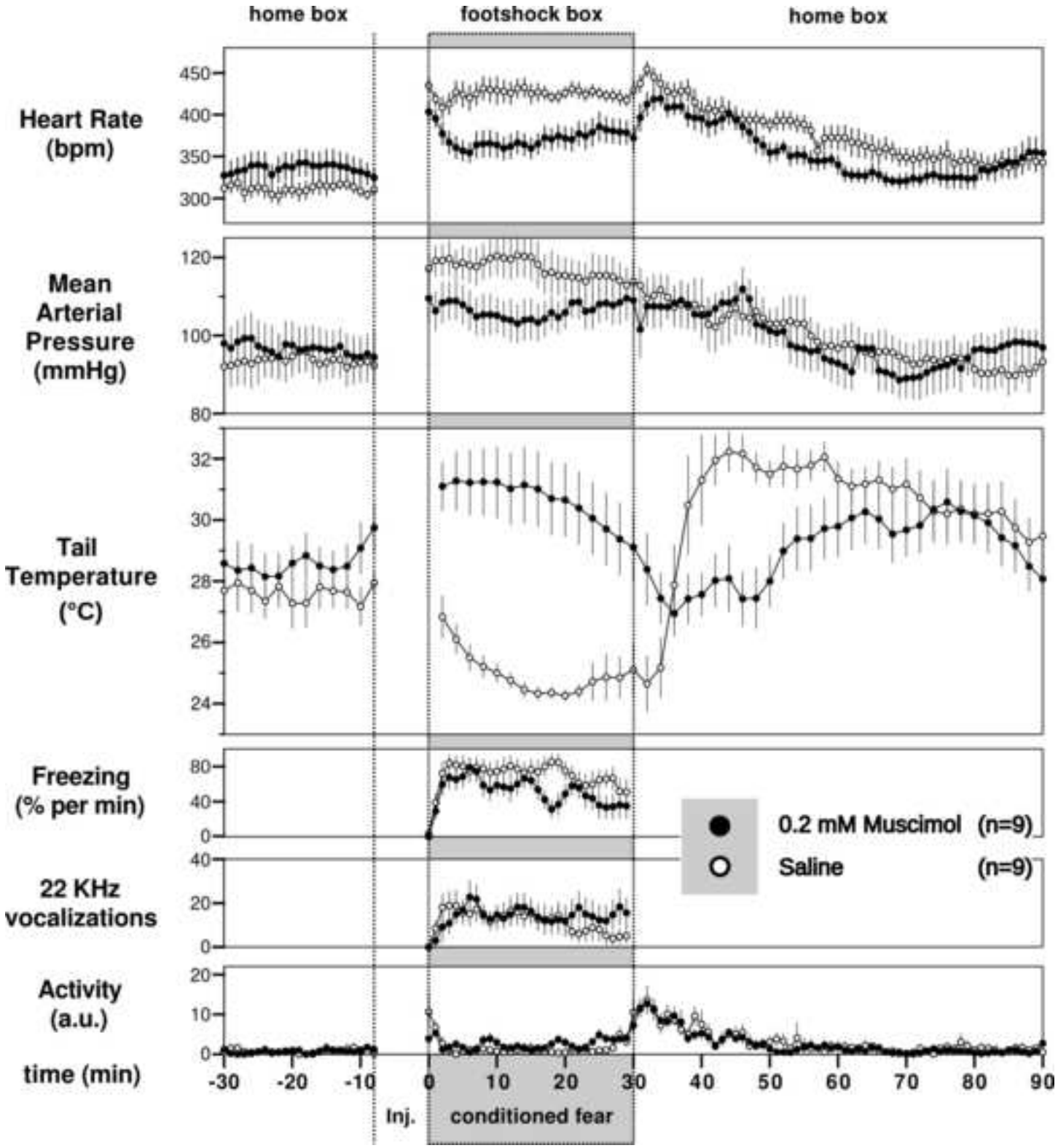

Figure 3

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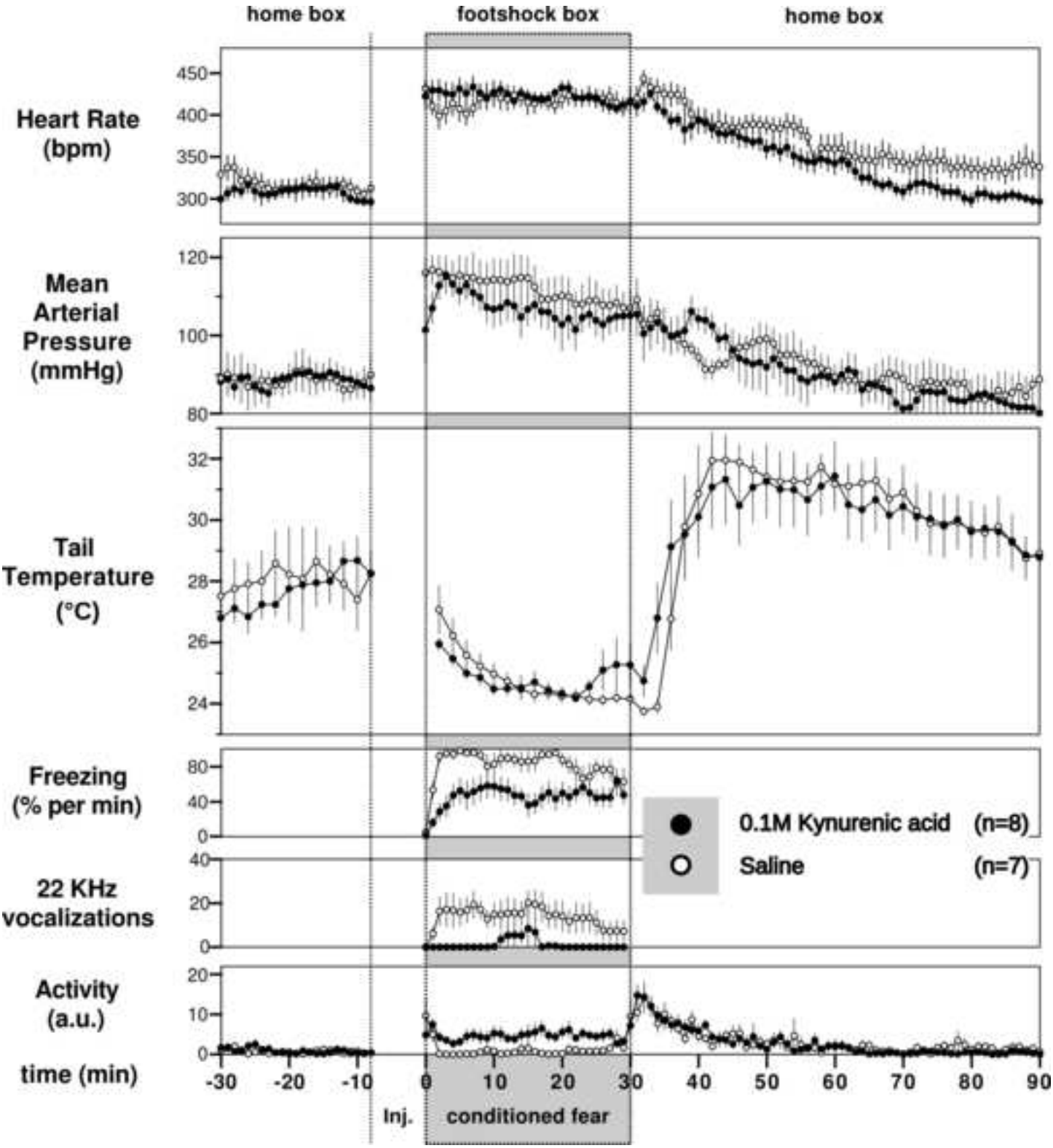

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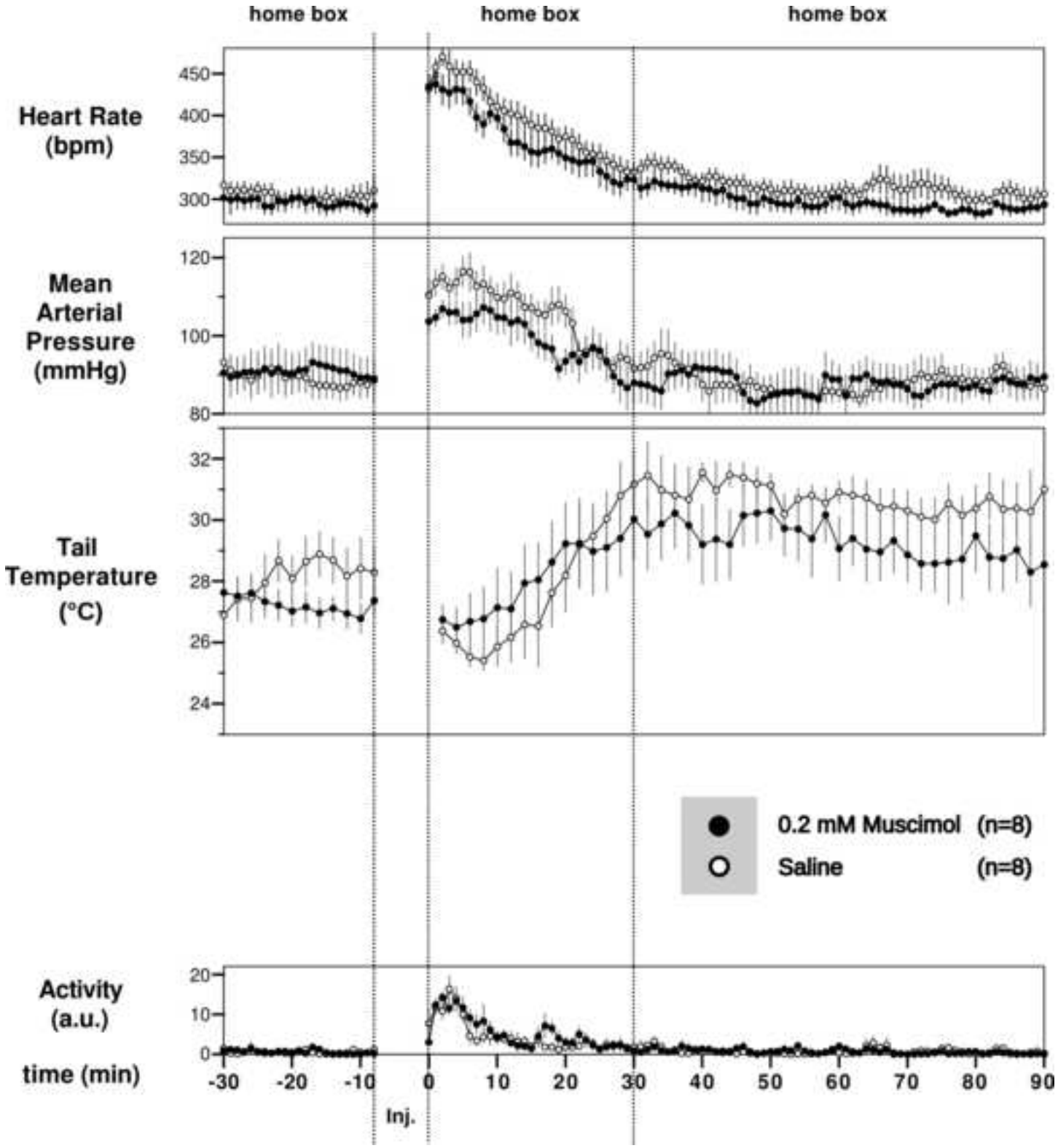

Figure 5

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