Infralimbic cortex activation and motivated arousal induce histamine release

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Appetitive behaviours occur in a state of behavioural and physiological activation that allows the optimal performance of these goal-directed behaviours. Here, we tested the hypothesis that histamine neurons under the command of the infralimbic cortex are important to provide behavioural activation. Extracellular histamine and serotonin were measured by microdialysis of the medial prefrontal cortex in behaving rats in parallel with a picrotoxin microinjection into the infralimbic cortex. The injection aroused the rats behaviourally, increased histamine release and decreased serotonin levels. Inhibition of the infralimbic cortex with muscimol produced the opposite effects on neurotransmitter release. The behavioural activation induced by motivating hungry rats with caged food was paralleled by an immediate histamine release, whereas awakening induced by tapping their microdialysis bowl increased serotonin, but not histamine levels. In conclusion, picrotoxin injection into the infralimbic cortex produces protracted appetitive behaviour that clearly separates this phase from consumption (Valdés *et al.*, 2005). We place a closed wire mesh cube filled with regular food pellets inside the rat's home cage; the rat can see and smell the food, but cannot reach it. Food-deprived rats show a behavioural activation together with histamine release; in a similar manner, induction of an appetitive state produced histamine release, likely related to increased behavioural activation characteristic of an appetitive behaviour. *Behavioural Pharmacology* 26:338–344 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Motivation provides a rational and structured explanation for the most complex adaptive behaviours of mammals, those crucial for the fate of the individual and its descendants. Motivated behaviours have direction (towards or away from reinforcers) and intensity, which is manifested by increased arousal (behavioural and vegetative activation), and is essential for the optimal performance of motivated behaviours (Hebb, 1955). A useful distinction between the appetitive and the consummatory phases of a motivated behaviour was advanced by Craig (1918). This distinction emphasized the arousal component that characterizes only the appetitive phase. The role of dopamine and the striatum in this arousal as well as in the modulation of effort to obtain reinforcers has been studied and reviewed extensively (Baldo and Kelley, 2007; Salamone, 2007); we will briefly discuss how histamine and dopamine may participate in similar motivational functions.

We have studied motivation for food (Valdés *et al.*, 2005; Riveros *et al.*, 2014), which is induced by food restriction and increased when hungry rats are tempted with very palatable food such as salami (Valdes *et al.*, 2010). To study the intensity component of feeding-related appetitive behaviour, we have published a protocol of protracted appetitive behaviour that clearly separates this phase from consumption (Valdés *et al.*, 2005). We place a closed wire mesh cube filled with regular food pellets inside the rat's home cage; the rat can see and smell the food, but cannot reach it. Food-deprived rats show a marked increase in motor activity directed towards opening the cube and obtaining food (see video in Valdes *et al.*, 2010), as well as an increase in core temperature, locomotor activity (both used here as an indication of increased arousal) and active waking measured polysomnographically (Valdés *et al.* 2005). If the wire mesh cube is filled with salami and regular pellets, hungry rats show even higher increases in motor activity and temperature. In contrast, fed rats show no increase in motor activity or temperature when they are presented with the pellet-filled cube. Using this behavioural protocol, we have mapped with immediate early gene expression the neural activation of the arousal nuclei (Jones, 2003) potentially involved in the increased vigilance of appetitive behaviour. We found that the histaminegic tuberomamillary nucleus (TMN) became active before other aminergic nuclei and that a lesion of either the TMN (Valdes *et al.*, 2010) or the infralimbic cortex (IL) (Valdés *et al.*, 2006) prevented the expression of this appetitive behaviour. However, a mechanistic account for the relationship between TMN and IL activity and appetitive behaviour is lacking.

We hypothesized here that a corticohypothalamic axis that involves the TMN and its main cortical input, the IL

(Ericson *et al.*, 1991), is important for the intensity component, or the energizing, of appetitive behaviour. In support of this hypothesis, picrotoxin injection into the IL or food enticement to hungry rats increases body temperature and locomotor activity and both effects are reduced by pretreatment with the H1 receptor reverse agonist pyrilamine (Riveros *et al.*, 2014). However, a more direct test of the idea that IL might participate in a pathway that leads to the release of histamine during appetitive behaviour is still lacking.

In this study, we addressed our hypothesis by testing the specificity of the relationship between IL–TMN activity and appetitive behaviour (does any arousal-promoting stimulus release histamine?) by comparing histamine and serotonin release [as both neurotransmitters can promote arousal (Jones, 2003)] in a behavioural protocol that involved arousal during appetitive behaviour or arousal induced by knocking on the rat's home cage in the daytime when they typically sleep. Arousal was not assessed here because increased behavioural and vegetative activation was always present during enticement in our previous studies (Valdés *et al.*, 2005, 2010). To test whether IL activity is related to increased histamine release during appetitive behaviour, we pharmacologically activated or inhibited IL and measured histamine and serotonin levels in microdialysis samples from the medial prefrontal cortex (mPFC, which includes IL, prelimbic and rostral cingulate cortices).

Methods

All experiments were conducted in accordance with the NIH (USA) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). Our local institutional Bio Safety and Ethical Committee approved these experimental protocols, which minimized the number of rats used and their suffering.

Subjects

We used male adult Sprague–Dawley rats from the Pontificia Universidad Católica de Chile Animal Care Facility weighing 270–350 g. They were individually housed in a controlled environment at 23°C, with a 12/12 lights on/off schedule (lights off at 19:00 h), and, except when indicated, had permanent access to water and food pellets.

Surgery

The rats were anaesthetized with 100 mg/kg intraperitoneally of ketamine (Imalgene; Rhodia Merieux, Santiago de Chile, Chile) plus 20 mg/kg of xilazine (Rompun; Bayer, Santiago de Chile, Chile), and placed in a stereotaxic frame (S1600 model; Stoelting Co., Wood Dale, Illinois, USA). The head was fixed and positioned so that the bregma and the lambda were in the same horizontal plane. Guide cannulae for microdialysis or microinjection were implanted bilaterally into the IL at

the following coordinates (Swanson, 1998): 3.0 mm anterior to the bregma; 0.5 mm lateral and 3.6 mm below the dura mater. The guide cannulae (MD2251; BASi, West Lafayette, Indiana, USA), consisting of a guide head and an occluder, were fixed to the skull with dental glue (Marche; Felix Martin y Cia Ltda, Santiago, Chile), screws and a plastic support. We implanted a guide cannula in one IL at the above coordinates and another in the contralateral IL using a lateral angle of 24°. After guide cannula implantation, animals were individually housed for 7 days before brain microdialysis. To prevent infection and pain, we administered Enrofloxacin 5% (19 mg/kg intraperitoneally; Bayer) and Ketophen (0.2 mg/kg intraperitoneally; Rhodia Merieux) at the end of surgery.

Active versus passive induction of arousal

We compared, in the same microdialysis samples from the mPFC, the changes in extracellular histamine and serotonin concentration in response to two different situations where arousal increases. To distinguish between arousal related to appetitive motivation (active/self-initiated arousal) and arousal in terms of wakefulness, we performed the following steps: active arousal was elicited after 24 h of food deprivation, when the rats were enticed with a wire mesh box full of rat chow pellets (see below and video in Valdes *et al.*, 2010). To contrast with self-initiated arousal, we kept rats awake for 20 min by tapping repeatedly on their home cages or the microdialysis bowl (~2 taps/s) with an acrylic stick to produce a loud noise (91.6 ± 2.1 dB), while ensuring that the rats were awake all the time. The two manipulations were counterbalanced so that food presentation was first in half of the rats.

Pharmacological manipulations

To inhibit IL, we microinjected a selective $GABA_A$ receptor agonist, muscimol (Sigma-Aldrich Co., Saint Louis, Missouri, USA) (100 ng/1 μl) (Edeline *et al.*, 2002; Amat *et al.*, 2005), into both IL with the needle of a combination infusion and a microdialysis probe (BAS MD2262) attached to a 10 μl Hamilton syringe by polyethylene tubing (0.58 mm ID; 0.965 OD) 20 min before the onset of an experiment.

To disinhibit IL neurons, 1 μl of 120 ng of picrotoxin (Sigma-Aldrich Co.) (Berretta *et al.*, 2005) – a noncompetitive $GABA_A$ receptor antagonist – was injected into the IL ipsilateral to the microdialysis probe (BAS MD2262). To ensure a counterbalanced order, IL picrotoxin and muscimol injections were administered in the same group of animals, half of them receiving the muscimol injection first and the other half receiving picrotoxin first.

Histamine and serotonin monitoring in the medial prefrontal cortex of freely moving rats

We performed microdialysis to measure the extracellular concentration of these neurotransmitters every 10 min. On day 8 after surgery, a concentric microdialysis probe

of 2 mm length, 0.5 mm outer diameter, cut-off 30 000 kDa (BASi) or a combination probe (MD2262; BASi), which allowed substances to be microinjected in addition to performing microdialysis, was lowered 2 mm below the guide cannula tip into the IL. The probe was perfused continuously at a rate of 2 μl/min with ACSF using a microperfusion pump (KDS100L model from KD Scientific Inc., Holliston, Massachusetts, USA). The composition of the ACSF solution was as follows: 147 mmol/l NaCl; 1.2 mmol/l CaCl$_2$; 4 mmol/l KCl; 2 mmol/l Na$_2$HPO$_4$; 0.2 mmol/l NaH$_2$ PO$_4$; and pH 7.4, freshly prepared with sterile-distilled water. After a stabilization period (120 min), three basal samples were collected in 2.0 μl of 0.2 N perchloric acid before the experiments began.

Analysis of dialysate by HPLC and fluorometric detection

Twenty microliters of perfusate was mixed with 10 μl of borate buffer 0.5 mol/l and 5 μl of fresh solution [4 mg ophtalaldehyde (OPA) and 2 μl of β-mercaptoethanol in 1 ml of methanol] for derivatization. The mixture was agitated for 30 s and injected 1 min later in the HPLC column (LiChroCART 250-4 Purospher STAR RP-18 endcapped, particle size 5 μm; Merck KGaA, Darmstadt, Germany).The mobile phase consisted of phosphate buffer (0.1 mol/l KH$_2$PO$_4$) with an acetonitrile gradient from 25 to 50% at a flux of 1 ml per minute. Acetonitrile and phosphate buffer were mixed by the pump (Elite labChrom L-2130; Hitachi, Chiyoda, Tokyo, Japan) in an automatized manner. The fluorescent derivatives were analysed using the integrated fluorometric detector (Elite labChrom L-2480; Hitachi). Excitation/emission wavelengths used were 330/450 nm. The calibration curve was constructed using histamine and serotonin (from Sigma-Aldrich Co.) standards dissolved in artificial CSF in a concentration range from 0.06 to 0.25 pmol/20 μl, which yielded a linear correlation between neurotransmitter concentration and peak area.

Histology

We used Nissl staining to evaluate the positioning of probes and injection cannulae. The location of the probes was confirmed to be in the mPFC and the injection tips in the IL. Animals with misallocated cannulae were discarded.

Statistical analysis

We used GraphPad Prism software (GraphPad Software Inc., La Jolla, California, USA). The variables under different conditions were analysed by Kruskal–Wallis one-way analysis of variance (ANOVA), followed by Dunn's multiple pairwise comparison method. Temporal changes in locomotor activity were analysed by two-way repeated measures ANOVA, followed by the Bonferroni multiple comparison or Holm–Sidak post-hoc tests. We considered a difference as significant when P less than 0.05. Data are expressed as mean ± SEM.

Results

Location of cannula placements and injection sites

Figure 1 shows representative examples of the histological reconstructions of the locations in the mPFC of the microdialysis probe active membrane and the injection sites aimed at the IL. The probes and injection sites were located at an intermediate anteroposterior level of the mPFC. Animals with misplaced probes were discarded from the present study.

Arousal induced by motivating the rat with food versus awakening the rat with a loud noise

We compared histamine and serotonin release in the mPFC during these two situations. Enticement kept the hungry rats awake for the 20 min of the procedure, as evidenced by the attempts to open the box filled with food. In contrast, the rats with free access to food quickly fell asleep, as shown previously using polysomnographic analysis (Valdés et al., 2005). The enticement was performed only once and in the rat's home cage to avoid habituation and stress, respectively. Food motivation resulted in a significant increase (Fig. 2a) in the extracellular histamine level in the mPFC (one-way ANOVA: $F_{13,27} = 26$, $P < 0.001$); in contrast, the extracellular concentration of serotonin did not change.

In contrast, keeping the rats awake for 20 min by tapping the rat's microdialysis bowl (Fig. 2b) induced a marked increase in serotonin (one-way ANOVA: $F_{10,52} = 20$ $P < 0.005$) but not histamine level.

Pharmacologically activating and inactivating the infralimbic cortex

We used a pharmacological approach to directly test for a causal relationship between IL activity and histamine release. We used the local microinjection of picrotoxin to increase, and muscimol to decrease, IL activity.

Picrotoxin microinjection into the IL increased the histamine level by almost 110% and induced a significant decrease in the serotonin level, which then reverted to basal levels (Fig. 3a). ANOVA showed a significant effect of neurotransmitter ($F_{1,72} = 17.34$, $P < 0.002$) and a significant neurotransmitter × time interaction ($F_{6,72} = 7.13$, $P < 0.001$). The control microinfusion of vehicle into the IL did not induce significant changes in histamine ($n = 6$, $P = 0.57$) or serotonin ($n = 6$, $P = 0.17$) release (data not shown) in the mPFC.

Muscimol microinfusion, which presumably inhibited IL neural activity, induced a slow decrease in mPFC histamine levels that became significant after 20 min, and a transient 60% increase in the serotonin level that peaked after 20 min (Fig. 3b). ANOVA showed a significant effect of neurotransmitter ($F_{1,105} = 28.81$, $P < 0.001$) and a significant neurotransmitter × time interaction ($F_{7,105} = 9.32$, $P < 0.001$). These pharmacological results show a double dissociation of the effects of IL activity on histamine and serotonin release.
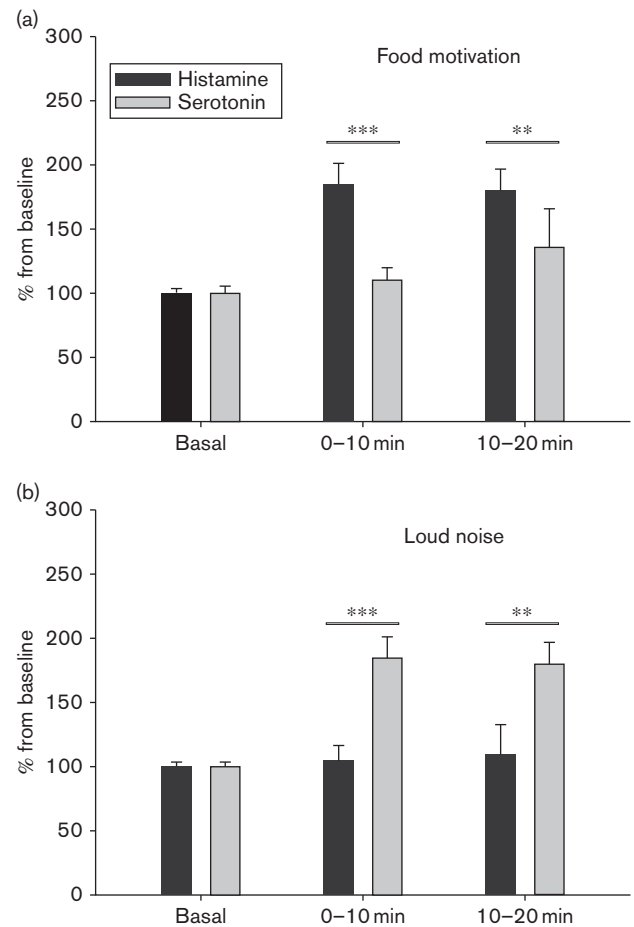
Fig. 1
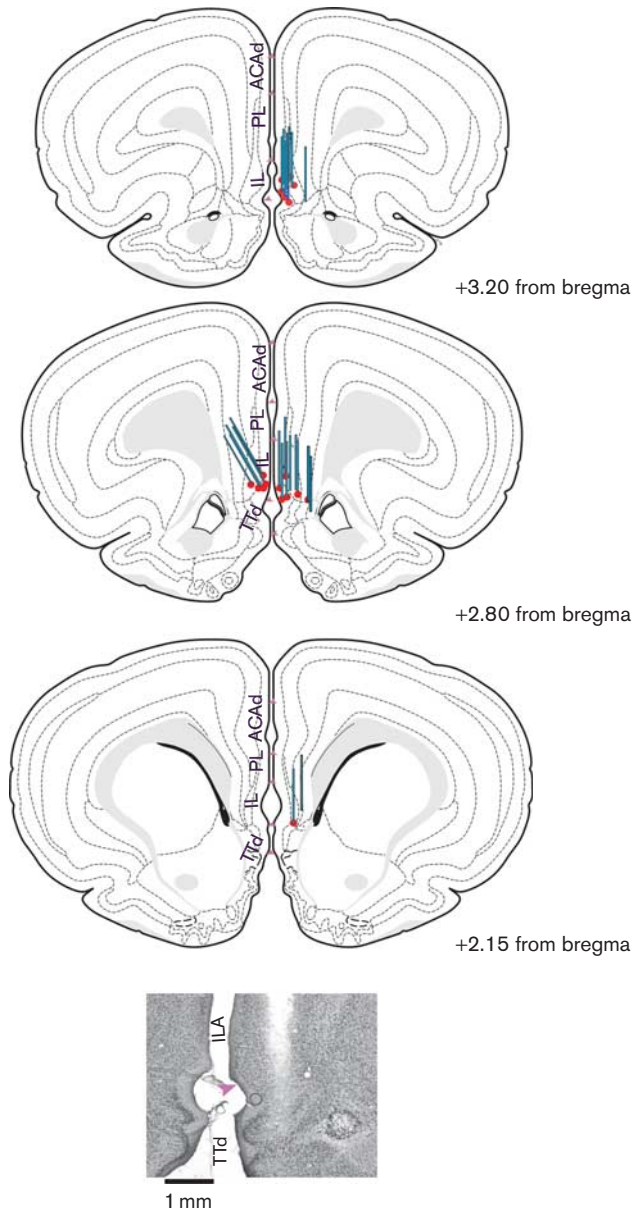
Representative examples of the microdialysis membranes and the injection sites aimed at the IL. Red dots represent injection sites and green lines represent the 2 mm extension of the dialysis membrane. ACAd, anterior cingulate cortex anterior dorsal; IL infralimbic cortex; PL, prelimbic cortex; TTd, tenia tecta dorsal.

Fig. 2

Histamine and serotonin neurons in the medial prefrontal cortex responded differentially to two ways of inducing arousal. (a) Food motivation with caged food increased the extracellular histamine, but not the serotonin levels. (b) Awakening with a loud noise stimulus increased extracellular serotonin, but not histamine levels. Extracellular levels of histamine and serotonin were measured in the same dialysis samples and are expressed as the mean ± SEM of the percentage of the basal neurotransmitter level; the basal level shown corresponds to the average of three consecutive 10-min samples taken after a 2 h stabilization period. The data were obtained from eight rats fasted 24 h before food motivation. $**P < 0.01$, $***P < 0.001$.

Discussion

Here, we report new evidence that supports the hypothesis that the IL–TMN axis contributes significantly towards the intensity or arousal component of motivation. First, histamine but not serotonin was released during the appetitive approach to caged food during enticement, whereas serotonin but not histamine was released during externally induced waking up. Second, the picrotoxin injection into the IL released

brain histamine while decreasing serotonin release in the mPFC.

A previous study from our laboratory (Riveros *et al.*, 2014) showed that picrotoxin infused by reverse microdialysis (to prevent manipulation that might awaken the rats) results in behavioural arousal, assessed by increased motor activity and vegetative arousal, evaluated by an increase in temperature (Meynard *et al.*, 2005). The present results suggest that this arousal induced by picrotoxin injection into the IL may result from increased histamine activity induced by mPFC activation.

The present evidence, together with our previous findings on the effect of either IL lesion (Valdés *et al.*, 2006),

Fig. 3
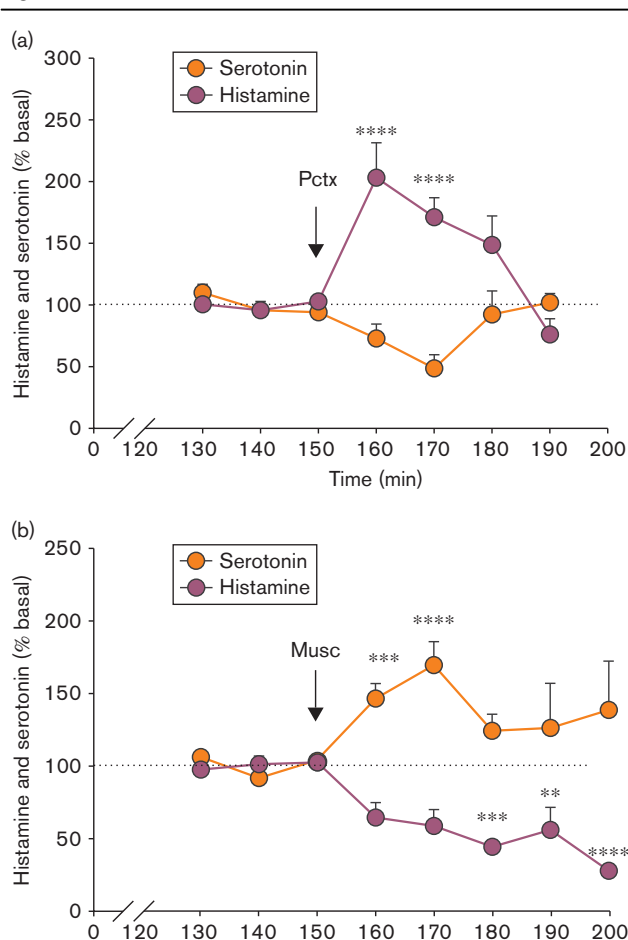
Double dissociation of the effects of injections of muscimol (Musc) or picrotoxin (Pctx) into the infralimbic cortex (IL) on histamine and serotonin release. (a) A single injection of 1 µl of 2 mmol/l Pctx into the IL induced a rapid increase in histamine and a slower decrease in the serotonin level that lasted 20–30 min. Pctx was injected (arrow) at noon to detect an increase in arousal. (b) A single injection of muscimol into the IL increased the serotonin level in the medial prefrontal cortex for 20 min, whereas the histamine level decreased significantly. Muscimol was injected (arrow) 30 min after lights off to detect a possible decrease in arousal. The data are expressed as the mean ± SEM of basal levels (%). **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ relative to baseline levels.

IL activation (Meynard *et al.*, 2005) or TMN lesion (Valdes *et al.*, 2010), shows the importance of an intact IL–TMN axis for energizing appetitive behaviour to obtain food.

Technical considerations

In the present study, we sampled histamine and serotonin from the mPFC, whereas we administered drugs or vehicle into the IL rather than to the whole mPFC. The mPFC that we sampled with the 2 mm long microdialysis probes corresponded to the IL, prelimbic and anterior cingulate cortices as the probe tip was usually placed in the ventral border of the IL.

Microinjections were centred at the IL, but we did not systematically evaluate the spread of the injections. However, we have shown in a previous study using the same rat strain that the expression of Fos-ir after the injection of picrotoxin in the IL was restricted to the contralateral IL, (adjacent areas did not show Fos-ir),which indicates that the spread of the picrotoxin injection was restricted to IL (Riveros *et al.*, 2014). Despite this uncertainty, we considered that a substantial part of the direct effects of these injections on the TMN should arise from changes in the activity of IL, rather than the whole mPFC, as the IL is by far the strongest cortical input to the TMN (Ericson *et al.*, 1991, and our unpublished observations). Our previous behavioural (Meynard *et al.*, 2005) and present physiological results with IL inhibition basically agree with the effects of IL lesion (Valdés *et al.*, 2006). The decrease in serotonin after a picrotoxin microinjection may indicate a spread to the prelimbic cortex because this cortex seems to be more important than IL in the regulation of the serotoninergic dorsal raphe responses to stressors (Baratta *et al.*, 2009).

Picrotoxin, a $GABA_A$ receptor blocker, likely increased the neural activity of IL neurons, including those that project to the TMN (Berretta *et al.*, 2005), to indirectly induce the release of histamine from TMN axons, rather than acting directly on these histaminergic axons in the mPFC to induce them to release histamine. The fact that serotonin levels decreased in response to picrotoxin in the same mPFC samples argues in favour of the idea that picrotoxin does not exert a local action at the site of administration that might have induced transmitter release in a generalized manner.

The local administration into IL of muscimol, a specific antagonist of $GABA_A$ receptors, exerted effects opposite to those of local picrotoxin. Extracellular levels of histamine decreased and serotonin levels increased in the mPFC, further supporting the idea that we have been affecting neural circuits that include IL, TMN and raphe nuclei rather than affecting histamine or serotonin axon terminals in the mPFC. However, the question still remains whether the changes in histamine and serotonin following pharmacological manipulations of IL are because of the existing direct projections of IL or because of indirect pathways.

The literature indicates that the intra-IL microinjections of muscimol at the dose used in the present study decreased neural activity for more than 2 h, whereas the diffusion was no further than 3 mm from the injection site (Edeline *et al.*, 2002). The effect of muscimol starts after 2 min, reaches a maximum after 20 min and remains there for at least 2 h (Edeline *et al.*, 2002).

Arousal induced by food motivation versus loud noise awakening

We compared histamine with serotonin release to evaluate the specificity of histamine release for appetitive

behaviour as both transmitters are known to promote arousal (Jones, 2003) and can be measured in the same microdialysis sample. The release of histamine in the mPFC (present results) and in the posterior hypothalamus (Valdes *et al.*, 2010) during food motivation may simply reflect a higher level of arousal and not be specific to what we have called voluntary arousal. In fact, the behavioural observations that we made suggested a higher level of arousal during food motivation than the passive awakening induced by tapping. However, the opposite response of serotonin to food motivation and tapping indicates that the higher release of histamine during food motivation is not related to qualitative differences in arousal, but rather to specific responses of both transmitters to the arousal-promoting protocol. In accordance with a specific role for histamine in appetitive behaviour, we have found that the TMN is the first arousal-promoting nucleus to express the immediate early gene protein Fos during food motivation (Valdés *et al.*, 2005) or during anticipatory awakening in a restricted feeding schedule protocol (Inzunza *et al.*, 2000; Angeles-Castellanos *et al.*, 2004; Meynard *et al.*, 2005).

Motivation, the energizing of behaviour and the reinforcement of adaptive behaviours, such as instrumental goal-seeking behaviour, have been associated extensively with the mesocorticolimbic dopamine systems (Robbins *et al.*, 1989; Berridge and Robinson, 1998). In fact, the relevance of arousal (or behavioural activation) to effort-related functions has been addressed extensively by Salamone *et al.* (2007) in relation to dopamine and nucleus accumbens.

A simple way of linking the motivational functions of the mesocorticolimbic systems with our hypothesis that the TMN and histamine are also key elements in motivation is to consider the IL–TMN axis as an integral part of the motivation circuit. The IL, as one component of the prefrontal cortex in rats, participates in the prefrontal cortex–basal ganglia–thalamus loops involved in goal-directed behaviours (Alexander *et al.*, 1986; Groenewegen *et al.*, 1990; Pierce and Kalivas, 1997; Zahm, 2000; Koob and Le Moal, 2008). In addition, there are interactions between histamine and dopamine at the neurochemical level that merit more attention (for a review, see Brabant *et al.*, 2010). These interactions are intricate as, for example, histamine can both stimulate and inhibit dopamine release depending on the site of action. However, the anatomical and functional relationship between the histaminergic system and the dopaminergic input to the nucleus accumbens to induce arousal and to modulate effort-based decisions (Salamone, 2007) remains to be explored. Within this framework, the IL–TMN axis specifically contributes towards motivation by increasing arousal and sympathetic activity (Valdés *et al.*, 2006) to prepare the brain and body for appetitive behaviour (Torrealba *et al.*, 2013).

Conclusion
Functional as well as pharmacological activation of the IL promotes the activation of histaminergic TMN neurons, leading to histamine release that, probably acting in many brain regions, increases arousal and allows the optimal unfolding of appetitive behaviour.

Conflicts of interest
There are no conflicts of interest.

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