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NEURAL MECHANISMS UNDERLYING THE ACTION OF PRIMER PHEROMONES IN MICE

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Abstract—Our electrophysiological experiments in female mice have provided evidence that electrical stimulation of the accessory olfactory bulb orthodromically excites a subpopulation of tuberoinfundibular arcuate neurons by way of the amygdala. The present study shows that half of such neurons are identified as dopaminergic by examining the effectiveness of infusing 6-hydroxydopamine and 5,7-dihydroxytryptamine locally into the median eminence in blocking their antidromic response. Further attention is focused on excitatory amino acid receptors within the amygdala and the amygdaloid pathway that mediate the accessory bulb-induced excitation of tuberoinfundibular arcuate neurons. The excitatory transmission was reversibly blocked by intra-amygdala infusion (3 nmol) of the excitatory amino acid antagonists kynurenic acid, D,L-2-amino-5-phosphonovalerate, γ-D-glutamylaminomethylsulphonate and D,L-2-amino-4-phosphonobutyrate. Intra-amygdala infusions (3 nmol) of *N*-methyl-D-aspartate and kainate markedly enhanced the firing activity of tuberoinfundibular arcuate neurons with excitatory inputs from the accessory bulb, whereas similar infusions of quisqualate were without effect. Intra-stria terminalis infusions of the local anaesthetic lignocaine completely abolished the excitatory transmission in all the cells tested. Furthermore, tuberoinfundibular arcuate neurons stimulated from the accessory bulb were also orthodromically stimulated from the stria terminalis with a shorter latency.

These studies demonstrate that the projections of the accessory olfactory bulb activate excitatory amino acid receptors within the amygdala and subsequently the stria terminalis route, thereby causing excitation of tuberoinfundibular dopaminergic arcuate neurons. This functional pathway can account for the reproductive effects so far described as a consequence of vomeronasal chemoreception.

The accessory olfactory system originating in the vomeronasal organ is important in a variety of chemosensory primer effects including the acceleration of puberty, induction of oestrus and pregnancy block in female mice following exposure to male urinary odours (pheromones).[11] A considerable body of circumstantial evidence has accumulated showing that chemosignals received via the vomeronasal organ may be finally transmitted to tuberoinfundibular (TI) dopaminergic neurons which, in turn, regulate prolactin secretion from the pituitary. Injections of the dopamine agonist bromocriptine reproduced the actions of male pheromones in female mice with identical timing.[2,15,20] Conversely, the blocking of dopaminergic transmission by pimozide prevented the pheromonal action of a strange male in newly mated female mice.[16]

The principal output neurons of the accessory olfactory bulb (AOB), the mitral cells, which project directly to the ipsilateral corticomedial nucleus of the amygdala,[22] may utilize glutamate or a closely related substance as a transmitter by analogy with main olfactory bulb mitral cells.[3,9]

From the amygdaloid complex, fibres travel via the stria terminalis to the ventromedial hypothalamus, medial preoptic area and bed nucleus of the stria terminalis.[6,12] There is evidence for a component also travelling via the ventral amygdaloid pathway.[13] This ventral pathway appears to be the essential core of copulating behaviour which is dependent on the vomeronasal and olfactory chemoreception in the male hamster.[28]

Our own studies[14] have shown that accessory olfactory information is transmitted via the amygdala to TI arcuate neurons identified as projecting to the median eminence. The purpose of the present study was three-fold: firstly, to examine if TI arcuate neurons with AOB inputs are dopaminergic; secondly, to explore the involvement of excitatory amino acid receptors within the amygdala in this neural transmission; thirdly, to determine whether this information is channelled through the stria terminalis or a non-strial pathway.

EXPERIMENTAL PROCEDURES

Animals

Details and procedures have been previously described.[14] A total of 65 Balb/c female mice was used. These animals were ovariectomized for at least four weeks prior to being implanted subcutaneously with silastic capsules containing 0.5 μg oestradiol. Unit recordings were made five to 14 days after capsule implantation, which has been shown to increase the percentage of TI arcuate neurons responding to AOB stimulation.[14]

Abbreviations: AOB, accessory olfactory bulb; AP4, D,L-2-amino-4-phosphonobutyric acid; AP5, D,L-2-amino-5-phosphonovaleric acid; 5,7-DHT, 5,7-dihydroxytryptamine; GAMS, γ-D-glutamylaminomethylsulphonic acid; NMDA, *N*-methyl-D-aspartate; 6-OHDA, 6-hydroxydopamine; TI, tuberoinfundibular.

Electrophysiological methods

Anaesthesia, stimulation, recording, microinfusion and histological techniques have been previously described.[14] Briefly, animals were anaesthetized with chloral hydrate (400 mg/kg) and mounted in a stereotaxic instrument. Coaxial bipolar stimulating electrodes were placed in the left AOB (coordinates: 0 mm anterior to the rhinal fissure, 1.0 mm lateral to the midline, 1.3 mm ventral to the brain surface), and the left half of the median eminence pituitary stalk junction exposed by a transpharyngeal approach. For experiments examining the effects of neurotoxins on axonal conduction of TI arcuate neurons, a glass micropipette (tip diameter of 30–50 μm) was placed in the median eminence between recording and stimulating electrodes. For analysis of excitatory amino acid receptors, a glass micropipette was placed just lateral to the medial nucleus of the amygdala (coordinates: 2.0 mm anterior to the interaural line, 2.5 mm lateral to the midline, 0.8–1.2 mm dorsal to the brain surface). For analysis of the pathway from the amygdala to TI arcuate neurons, another stimulating electrode or a glass micropipette was lowered into the left stria terminalis (coordinates: 0.7 mm caudal to the bregma, 1.8 mm lateral to the midline, 2.8 mm ventral to the brain surface). Single unit extracellular recordings were made with glass micropipettes filled with 0.5 M sodium acetate containing Pontamine Sky Blue dye. The left arcuate nucleus was systematically explored for the presence of cells which were stimulated antidromically from the median eminence and orthodromically from the AOB. Criteria for antidromic invasion have been previously described.[14] Balanced negative–positive biphasic rectangular pulses (duration of each pulse = 0.2 ms, separation of rectangular pulses within each biphasic pair = 0.1 ms, intensity = 0.5 mA) were delivered to the AOB or stria terminalis at 0.33 Hz.

Table 1. Effectiveness of neurotoxin infusions into the median eminence in blocking axonal conduction of tuberoinfundibular arcuate neurons with excitatory inputs from the accessory olfactory bulb

6-Hydroxydopamine	3/6 (50)
5,7-Dihydroxytryptamine	0/4 (0)

Fractions show the number of cells whose antidromic responses to median eminence stimulation were blocked over the number tested. Values in parentheses are percentages.

Drugs

The following drugs were infused locally into the specific brain areas: 6-hydroxydopamine (6-OHDA) hydrobromide, 5,7-dihydroxytryptamine (5,7-DHT) creatinine sulfate, kynurenic acid, *N*-methyl-D-aspartate (NMDA), kainic acid, quisqualic acid, D,L-2-amino-5-phosphonovaleric acid (AP5), γ-D-glutamylaminomethylsulphonic acid (GAMS), D,L-2-amino-4-phosphonobutyric acid (AP4) and 0.5% lignocaine hydrochloride (Fujisawa, Osaka). The 6-OHDA and 5,7-DHT were dissolved in saline with ascorbic acid (0.1 mg/ml) added to protect against oxidation; 4 μg (calculated as base) in 0.4 μl was infused over 15 min with a microinfusion apparatus (Summit Medical, Tokyo). Excitatory amino acid agonists and antagonists were dissolved in saline; their pH was neutralized with NaOH; 3 nmol in

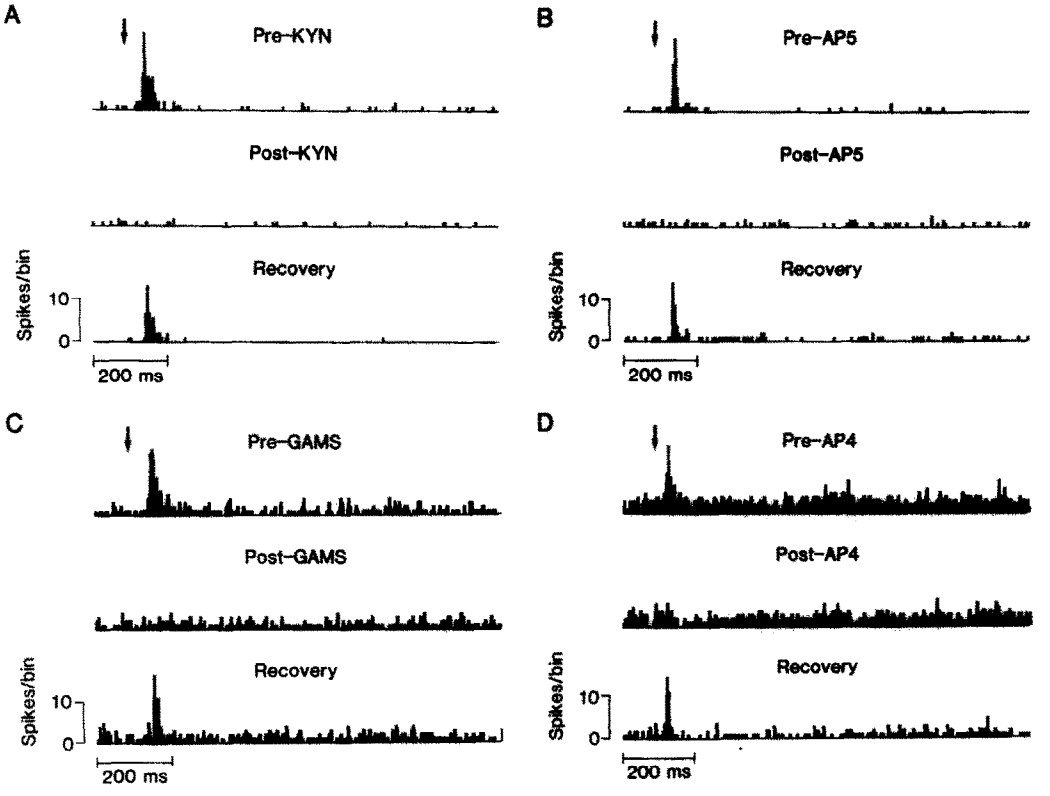

Fig. 1. Blockade of accessory olfactory bulb-induced excitation of TI arcuate neurons by intra-amygdala infusions of kynurenic acid (KYN, A), AP5 (B), GAMS (C) and AP4 (D). Peristimulus time histograms for each of three preparations were constructed before (top), 10 min (middle) and 50–90 min (bottom) after drug infusion. Each histogram (bin width, 5 ms) is computed from 70 to 150 stimulus repetitions. Arrows indicate time of stimulation of the accessory bulb.

0.3 μl was infused. Unless otherwise noted, all the drugs were obtained from Sigma Chemical Co.

Statistical analysis

Data were analysed using information statistics.[21] This procedure allowed the analysis of data with small cell frequencies which cannot be analysed using Chi-square. Information statistics have an identical distribution to Chi-square and hence these tables may be used to assess confidence limits.

RESULTS

Data are presented for 65 histologically verified arcuate neurons which were antidromically stimulated from the median eminence and also orthodromically stimulated from the AOB.

Effect of neurotoxins on axonal conduction

6-OHDA has been demonstrated to be effective in blocking the axonal conduction of catecholaminergic neurons but not other types of neurons after acute administration into the medial forebrain bundle[8,10,23] or the dorsal noradrenergic bundle.[1] A crucial test for determining if TI arcuate neurons with excitatory inputs from the AOB are dopaminergic, therefore, is to infuse 6-OHDA directly into the median eminence during the antidromic stimulation experiment. Antidromic responses of TI arcuate neurons with the AOB inputs to median eminence stimulation were tested before infusion of 6-OHDA or 5,7-DHT, and up to 1 h later. Results are shown in Table 1. The antidromic responses of three out of six cells tested could no longer be obtained within 1 h after 6-OHDA infusions. Infusions of 5,7-DHT had no obvious effect on any antidromic response in the four cells tested. The difference in the proportion of antidromic responses blocked between the two neurotoxins were statistically significant ($\uparrow = 1.95$, $P < 0.05$).

Effect of intra-amygdala infusions of excitatory amino acid agonists and antagonists

We examined the ability of excitatory amino acid antagonists infused into the amygdala to block the excitatory response of TI arcuate neurons to AOB stimulation. Four drugs were tested: the broad-spectrum antagonist kynurenic acid,[19] specific NMDA antagonist AP5,[27] preferential non-NMDA antagonist GAMS,[17] and AP4. Kynurenic acid, AP5, GAMS and AP4 reversibly blocked AOB-induced

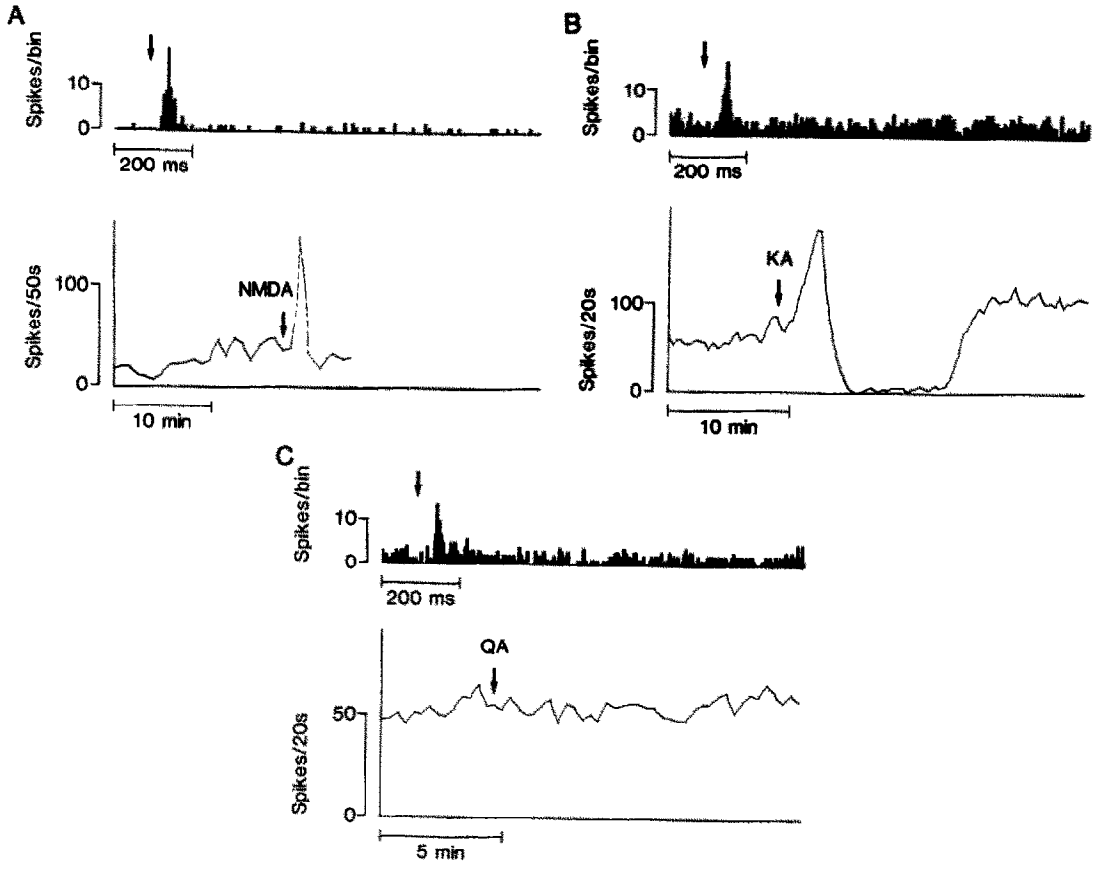

Fig. 2. Effect of intra-amygdala infusions of NMDA (A), kainate (KA, B) and quisqualate (QA, C) on firing activity of TI acuate neurons with excitatory inputs from the accessory olfactory bulb. Upper panels: peristimulus time histograms are presented showing excitatory responses of TI arcuate neurons to stimulation of the accessory olfactory bulb. Lower panels: ratemeter records showing excitatory or no effects of intra-amygdala infusions of the drugs on same three neurons from which peristimulus time histograms were obtained.

excitation in all six TI arcuate neurons tested (Fig. 1A–D, middle). The AOB-induced excitation was totally blocked within 2 min after each drug infusion. There were no detectable changes in the firing activity of TI arcuate neurons following any of the drug infusions. The AOB-induced responses that were abolished reappeared 50–90 min after drug infusion in all the neurons from which long-lasting recordings were made (three cells for each of kynurenic acid, AP5, GAMS and AP4; Fig. 1A–D, bottom).

To further characterize the excitatory amino acid receptors involved, we examined the ability of excitatory amino acid agonists infused into the amygdala to change the firing activity of TI arcuate neurons with excitatory inputs from the AOB. Both NMDA and kainate markedly enhanced the activity of all six neurons tested, whereas quisqualate was without effect in any of three neurons tested (Fig. 2).

Analysis of the amygdaloid pathway mediating accessory olfactory bulb-induced excitation

To determine whether and to what extent the excitatory response of TI arcuate neurons to AOB stimulation is via the stria terminalis, we investigated the effectiveness of lignocaine infusions into the stria terminalis in blocking the AOB-induced excitation, and that of stria terminalis stimulation in evoking a response with a shorter latency. Lignocaine infused into the stria terminalis completely blocked the AOB-induced excitation of all seven TI arcuate neurons (Fig. 3A, middle). The AOB-induced excitation that was blocked reappeared 40 min after infusion of the local anaesthetic in all the cells tested (Fig. 3A, bottom). Saline infused in the same manner was without effect in any of three cells tested (Fig. 3B). All six TI arcuate neurons orthodromically stimulated from the AOB were also orthodromically stimulated from the stria terminalis with a shorter latency (Fig. 4).

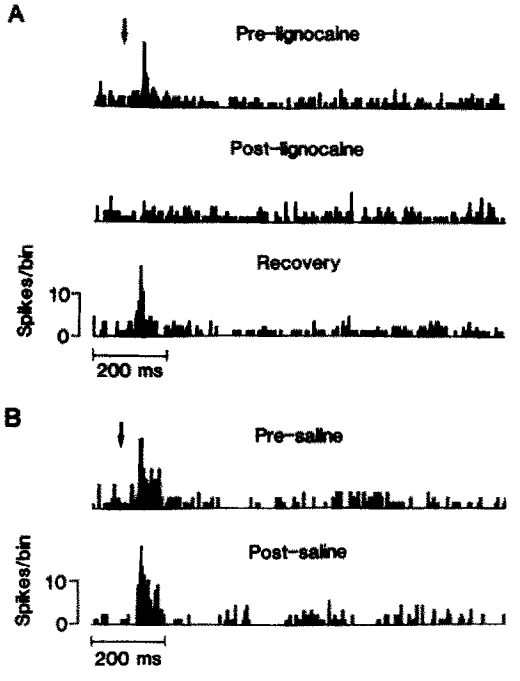

Fig. 3. Blockade of accessory olfactory bulb-induced excitation of TI arcuate neurons by lignocaine (A), but not by saline (B), infused into the stria terminalis. Peristimulus time histograms for each of two preparations were constructed before (top), 10 min (middle) and 40 min (bottom, shown only for the cell in A) after drug infusion. Stimulus (100 in each histogram) at arrows.

DISCUSSION

Electrophysiological techniques have served as powerful tools for estimating the activity of mesotelencephalic dopaminergic neurons.[4] However, there have been some difficulties in making similar estimations in TI dopaminergic neurons. One reason for this is that perikarya of TI dopaminergic neurons are not as densely packed as those in the substantia nigra, and they are dispersed among non-dopaminergic cells.[18] In an attempt to identify TI dopaminergic as opposed to other types of TI arcuate neurons, we examined the effectiveness of 6-OHDA infusions into the median eminence in interrupting impulse flow. The 6-OHDA disrupted the antidromic responses of half of the TI arcuate neurons with excitatory inputs from the AOB, whereas 5,7-DHT did not. The specificity of these changes is indicated by the fact that 5,7-DHT but not 6-OHDA has been found to block propagation in serotoninergic axons.[24,25] The findings presented here therefore clearly show that accessory olfactory information is transmitted to TI dopaminergic neurons. Although the administration of 6-OHDA eliminated 50% of the TI arcuate neurons that responded to AOB stimulation, this may be a conservative estimate of those which are dopaminergic, because such infusions are rarely 100% effective.

There are three distinct subtypes of postsynaptic receptors for excitatory amino acids based on their selective activation by the agonists kainate, quisqualate, or NMDA.[26] However, since a particular limitation, at present, is the absence of specific antagonists that distinguish kainate- from quisqualate-mediated responses, these two receptor subtypes have been collectively called non-NMDA receptors.[17] A fourth subtype of excitatory amino acid receptor has been proposed based on the antagonist action of L-AP4 which has been shown to act at a presynaptic site to inhibit transmitter release.[5,7] Our finding that intra-amygdala infusions of the broad spectrum antagonist kynurenic acid abolished AOB-induced excitation of TI arcuate neurons suggest that a glutamate-related substance is the transmitter of AOB mitral cells which project to the amygdala. The AOB-induced excitations were antagonized, in addition, by the specific NMDA antagonist AP5, the preferential non-NMDA antagonist GAMS, and

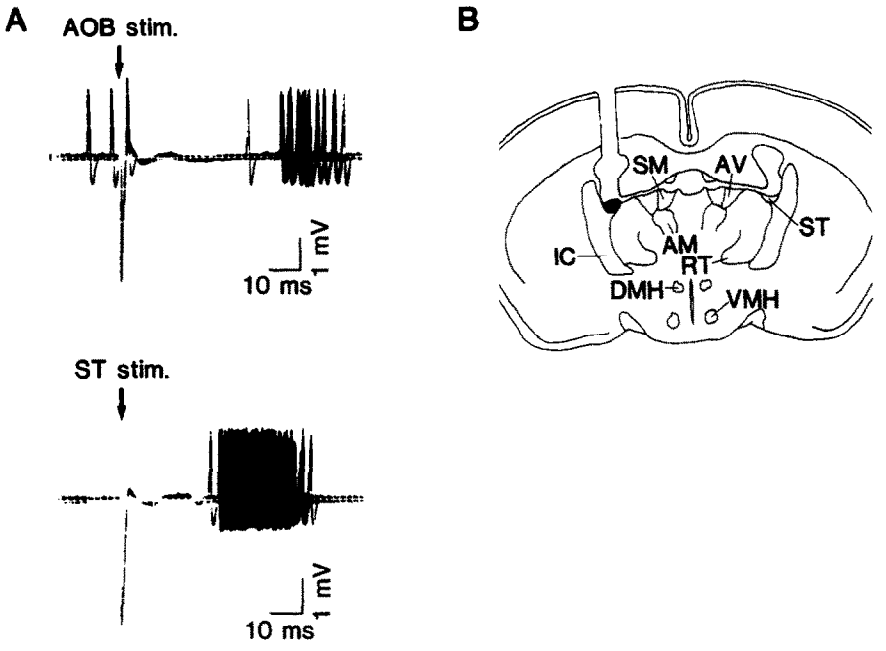

Fig. 4. (A) Fifty superimposed oscilloscope traces showing a TI arcuate neuron orthodromically stimulated from the AOB and also orthodromically stimulated with a shorter latency from the stria terminalis (ST). (B) Circles superimposed on a schematic coronal section of the brain depict the location of electrode tips in the ST where stimulation evoked orthodromic excitation of TI arcuate neurons with excitatory inputs from the AOB. AM, anteromedial thalamic nucleus; AV, anteroventral thalamic nucleus; DMH, dorsomedial hypothalamic nucleus; IC, internal capsule; RT, reticular thalamic nucleus; SM, stria medullaris; VMH, ventromedial hypothalamic nucleus.

AP4, elaborating mediation by excitatory amino acid receptors within the amygdala. The ability of NMDA and kainate to enhance the firing activity of TI arcuate neurons showing AOB-induced excitation offers further evidence for the involvement of excitatory amino acid receptors in this response. The failure of intra-amygdala infusion of quisqualate to produce changes in the firing activity of TI arcuate neurons suggests that amygdaloid quisqualate receptors are not involved in the AOB-induced excitation of these neurons and that the results obtained with the same dose of NMDA and kainate are unlikely to be due to some non-specific effect.

Taken together, our findings lead us to the tentative conclusion that multiple receptor subtypes, namely NMDA, kainate and AP4 receptors within the amygdala may be implicated in the AOB-induced excitation of TI arcuate neurons. However, this conclusion must be taken cautiously since the effectiveness and specificity of excitatory amino acid agonists and antagonists, like other pharmacological agents, is a function of their concentration at the receptor. A question may be raised why GAMS completely blocked AOB-induced excitation of TI arcuate neurons when NMDA-mediated responses are still available if GAMS is a non-NMDA antagonist. Likewise for AP5 when non-NMDA-mediated responses are available. Further experiments will be necessary to answer this question.

Intra-stria terminalis infusions of lignocaine completely abolished the AOB-induced excitation of all the TI arcuate neurons tested, indicating that the excitatory transmission from the AOB to TI arcuate neurons is mediated entirely via the stria terminalis. This conclusion is substantiated by the finding that TI arcuate neurons responding to AOB stimulation also responded with a shorter latency to stria terminalis stimulation.

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