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# Constitutive activity of the recombinant and native histamine $H_3$ receptor

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**Abstract**

Although constitutive activity was shown to occur with many recombinant and/or mutated G-protein-coupled receptors, the physiological relevance of the process has remained debated. We have further explored this important issue with the histamine $H_3$ receptor ($H_3R$), a presynaptic receptor regulating histamine neuron activity in the brain. Constitutive activity of the recombinant receptor was studied using [$^3$H]arachidonic acid release, [$^{35}$S]GTPγ[S] binding and inhibition of cAMP accumulation. Evidence for constructive activity was obtained in these three functional assays with two isoforms of the rat $H_3$ receptor, as well as with the human $H_3$ receptor, expressed at physiological densities. Several standard $H_3$-receptor antagonists, such as thioperamide and ciproxifan, were in fact acting as potent inverse agonists. Proxyfan opposed both agonists and inverse agonists and was therefore identified as a neutral antagonist. Using these drugs, we show high constitutive activity of native receptors. [$^{35}$S]GTPγ[S] binding demonstrated constitutive activity of $H_3$ receptors expressed at a normal level in mouse or rat brain. Constitutive activity of presynaptic $H_3$ autoreceptors modulates histamine release from cortical synaptosomes in vitro and controls histamine neuron activity in vivo. This implies that inverse agonists rather than neutral antagonists may find therapeutic applications.


*Keywords:* Histamine; $H_3$ receptor; Arachidonic acid release; [$^{35}$S]GTPγ[S] binding; Neutral antagonist; Native receptors; Autoreceptors; Histamine release; Brain; In vivo constitutive activity; Proxyfan; Ciproxifan

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## 1. Introduction

During the last decade, numerous examples of constitutive activity were shown for G-protein-coupled receptors. Many compounds previously known as antagonists were found to act in fact as inverse agonists and this class of drugs received a large attention [1–5]. In contrast, it has proved difficult to identify neutral antagonists, i.e. drugs able to block both the effects of agonists and inverse agonists. Although it was evidenced for many recombinant receptors overexpressed and/or mutated, only indirect indications suggested that constitutive activity could occur for native receptors expressed at normal levels in cells or tissues, and the physiological relevance of the process has remained debated [6,7]. We have further explored this important issue with the histamine $H_3$ receptor.

In 1983, we characterized the histamine $H_3$ receptor ($H_3R$) as an autoreceptor controlling histamine synthesis and release in the brain [8]. Four years later, we designed its first potent and selective ligands [9]. Thereafter, the $H_3R$ was shown to inhibit the release of other neurotransmitters in brain and peripheral tissues [10,11]. The inhibition mediated by $H_3$ autoreceptors constitutes a major regulatory mechanism of the activity of histaminergic neurons in the brain [11].

The modulation of agonist binding by guanylnucleotides and the sensitivity of various $H_3R$-mediated responses to pertussis toxin [12,13] suggested that the $H_3R$ was a $G_i/G_o$-protein-coupled heptahelical receptor. This proposal was confirmed by Lovenberg et al. [14] in 1999 with the first cloning of the $H_3R$. Following this cloning, evidence was obtained for the existence of $H_3R$ isoforms generated by pseudo-intron retention/deletion in the third intracellular loop [15–18].

## 2. Constitutive activity of recombinant $H_3$ receptors

After cloning the rat $H_3R$, we noticed that the carboxy terminus of the third intracellular loop has a stretch of eight amino acids strikingly similar to the corresponding sequence of a mutated human $\beta_2$-adrenergic receptor in which the mutation confers a constitutive activity (CAMh$\beta_2$-AR in Fig. 1) that is absent in the native receptor [19]. We have, therefore, assessed the constitutive activity of two $H_3R$ isoforms ($H_{3(445)}$ and $H_{3(413)}$), expressed in CHO cells at increasing densities. The coupling changes generated by receptor expression were evaluated in two signalling pathways activated by histamine and involving $G_i/G_o$-proteins, i.e. adenylyl cyclase inhibition and phospholipase $A_2$ activation. In both pathways, constitutive activity of $H_3R$ isoforms was clearly evidenced [20]: [$^3$H]arachidonic acid release evoked by the $Ca^{2+}$-ionophore A23187 was enhanced (Fig. 2), whereas forskolin-induced cAMP accumulation was reduced when the receptor density was increased. These changes were largely attenuated by thioperamide, a prototypical $H_3$-receptor antagonist [9], appearing here as a potent inverse agonist (Fig. 2). Constitutive activity was slightly higher with the longer isoform $H_{3(445)}$, being already significant at 80 fmol $mg^{-1}$ protein [20], and more marked than with $H_{3(413)}$ at intermediate expression levels (Fig. 2).

These observations could be repeated using cells expressing the human $H_3R$ ($hH_3R$) instead of the rat $H_3R$ ($rH_3R$) [21]. We found that constitutive activity was of similar

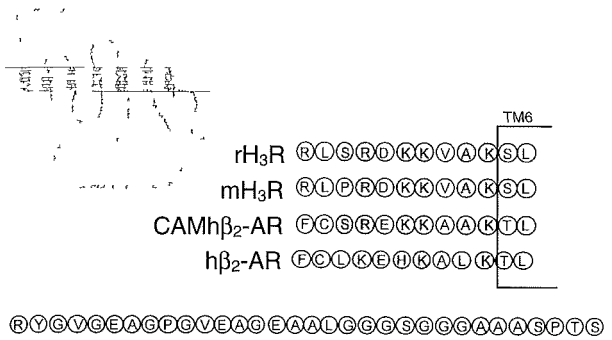


Fig. 1. Putative seven-transmembrane topography of the two rat $H_3$-receptor isoforms $H_{3(445)}$ and $H_{3(413)}$, differing by a 32 amino acid insertion in the third intracellular loop. This loop C-terminal sequence is also compared to that of the mouse ($mH_3R$), the native human $\beta_2$-adrenergic receptor ($h\beta_2$-AR) and a constitutively active mutant (CAM$h\beta_2$-AR) [19]. Reproduced with permission from Ref [20], Copyright 2000 Macmillan Publishers.

amplitude in the two species when arachidonic acid release was monitored and ciproxifan, another standard antagonist [22], used as an inverse agonist (Fig. 3A). Wieland et al. [23] found that it was even more pronounced for the $hH_3R$ when forskolin-induced cAMP formation was monitored in SK-N-MC cells. However, using $H_3R$-mediated [$^{35}$S]GTPγ[S] binding [12], we observed that the human receptor

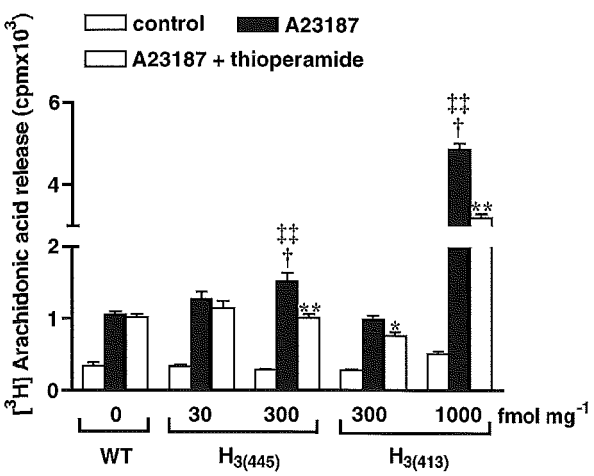


Fig. 2 Constitutive activity of rat $H_{3(445)}$- and $H_{3(413)}$-receptor isoforms expressed in CHO cells. Effects of thioperamide on [$^3$H]arachidonic acid release evoked by 2 μM A23187 in CHO cells expressing various densities of the two $H_3$-receptor isoforms. *$P<0.05$, **$P<0.001$ vs A23187 alone; $^{\dagger}P<0$ 001 vs wild-type (WT) cells; $^{\ddagger\ddagger}P<0.001$ vs CHO($H_{3(413)}$) cells expressing 300 fmol mg$^{-1}$ protein. Reproduced with permission from Ref. [20], Copyright 2000, Macmillan Publishers.

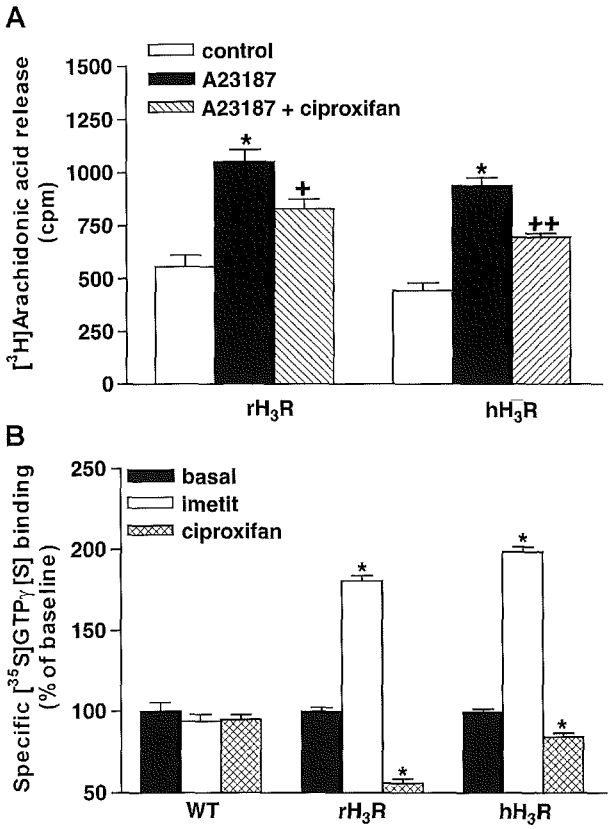


Fig. 3. Effects of $H_3$-receptor ligands on two responses mediated by the recombinant rat and human $H_3$Rs. (A) Effect of ciproxifan on A23187-evoked [$^3$H]arachidonic acid release from CHO cells expressing ~ 200–300 fmol mg$^{-1}$ protein of rat ($rH_3R$) or human ($hH_3R$) receptors. *$P<0.001$ vs the corresponding control; $^{+}P<0.01$, $^{++}P<0.001$ vs A23187. (B) Effects of $H_3$-receptor ligands on specific [$^{35}$S]GTPγ[S] binding to membranes of wild-type CHO cells (WT), CHO($rH_3R$) and CHO($hH_3R$) cells. *$P<0.001$ vs the corresponding basal.

displayed lower constitutive activity (Fig. 3B). Whereas imetit, a selective $H_3R$ agonist [24], similarly increased [$^{35}$S]GTPγ[S] binding to membranes of CHO($rH_3R$) and CHO($hH_3R$) cells, the effect of ciproxifan and the increment of basal binding (20 and 5 fmol mg$^{-1}$ protein, respectively) associated with receptor expression were lower for the $hH_3R$ (Fig. 3B).

As shown for the recombinant $rH_3R$ [20], constitutive activity of the recombinant $hH_3R$ was correlated to receptor density (Fig. 4). The increment of [$^{35}$S]GTPγ[S] binding due to the transfected receptor was fourfold greater (~ 20 and 5 fmol mg$^{-1}$ protein, respectively) at ~ 700 vs ~ 300 fmol mg$^{-1}$ protein of $hH_3R$ [21]. However, the maximal increase in [$^{35}$S]GTPγ[S] binding observed at the highest densities of $hH_3R$ remained substantially lower than the maximal response produced by the agonist

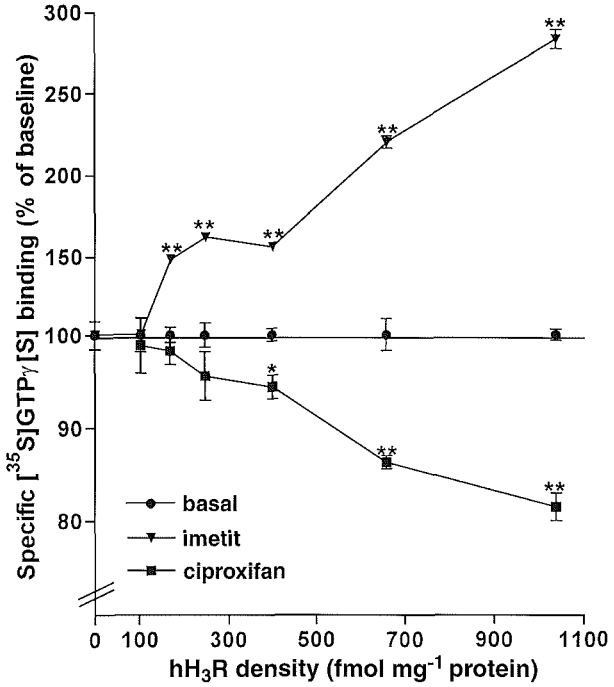


Fig. 4. Effects of $H_3$-receptor ligands on specific [$^{35}$S]GTPγ[S] binding to membranes from CHO cells expressing various densities of the human $H_3$ receptor ($hH_3R$). Membranes were incubated with 0.1 nM [$^{35}$S]GTPγ[S] and, when required, imetit or ciproxifan (1 μM). *$P<0.01$ and **$P<0.001$ vs the corresponding basal. Reproduced with permission from Ref. [21], Copyright 2002, Nature Publishing Group.

imetit, suggesting that the amount of receptor may be limiting for constitutive activity of the $hH_3R$.

Constitutive activity of the $hH_3R$ was also evidenced using inhibition of [$^{35}$S]GTPγ[S] binding by unlabelled GTPγS (Fig. 5). The expression of the $hH_3R$ (300 fmol $mg^{-1}$ protein) generated a high affinity binding for GTPγS ($pIC_{50}=8.2$), which was not observed in wild-type cells. The capacity of this binding site was similar to the total increment of binding generated by the transfected receptor. It was further increased by the agonist imetit. Since it represented ~ 20 vs ~ 5 fmol $mg^{-1}$ protein in the presence and absence of imetit, respectively (Fig. 5), it can be calculated that ~ 25% of $hH_3Rs$ exist in a precoupled state. This level of precoupling is similar to that found at higher $hH_3R$ densities and is in the same range as that previously reported for other $G_i$-protein-coupled receptors [21]. Although it may be dependent on the receptor density and G protein subtypes, the high affinity binding of GTPγS may allow to quantify the degree of constitutive activity and, therefore, the intrinsic activity of inverse agonists [25]. According to this model, ciproxifan, which attenuated but did not abolish totally the high affinity component, would be acting as a partial inverse agonist at the $hH_3R$ (Fig. 5).

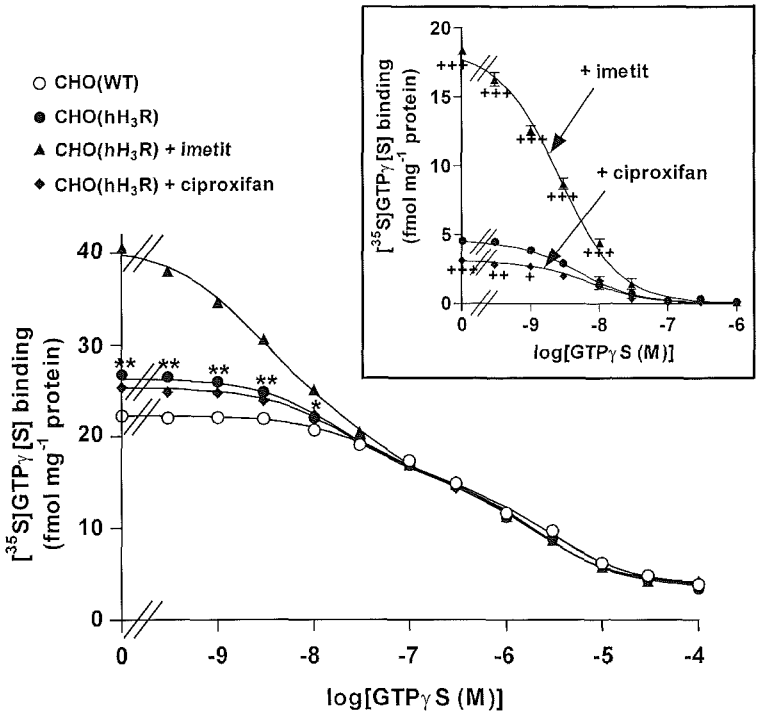


Fig. 5. Inhibition of [$^{35}$S]GTPγ[S] binding to membranes of CHO(WT) and CHO(hH$_3$R) cells by GTPγS. Membranes of CHO(WT) or CHO(hH$_3$R) cells expressing ~ 300 fmol mg$^{-1}$ protein were incubated with [$^{35}$S]GTPγ[S] and increasing concentrations of GTPγS, in the presence, when required, of 1 μM imetit or ciproxifan. The inset shows the same data after subtraction of [$^{35}$S]GTPγ[S] binding to membranes of CHO(WT) cells. $^{*}P<0.01$, $^{**}P<0.001$ vs CHO(WT) cells, $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ vs CHO(hH$_3$R) cells in the absence of ligand. Reproduced with permission from Ref. [21], Copyright 2002, Nature Publishing Group.

All these findings show that constitutive activity of the rH$_3$R and hH$_3$R is already significant at moderate densities, i.e. consistent with natural cellular levels of receptors. This suggests that constitutive activity of the native H$_3$R may occur in the brain.

## 3. Identification of a neutral antagonist: proxyfan

To assess this possibility, we needed to identify ligands displaying well-defined agonist, inverse agonist and neutral antagonist properties in cell lines, and then to determine the effects of these probes at the native cerebral receptor. Neutral antagonists are not easily identified because, theoretically, they correspond to ligands displaying equal affinity for the active and inactive receptor conformations, a condition obviously difficult to achieve [6,26]. In agreement, a large number of tested H$_3$R antagonists (defined by their ability to block the histamine response at the autoreceptor inhibiting [$^{3}$H]histamine release from synaptosomes) behaved as potent inverse agonists [20]: they decreased [$^{3}$H]arachidonic

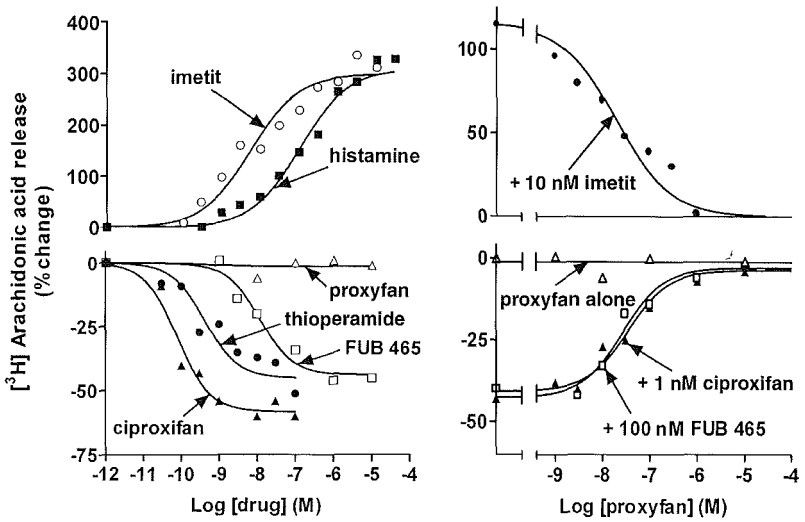


Fig. 6. Effects of $H_3$-receptor ligands on A23187-evoked [$^3$H]arachidonic acid release from CHO cells expressing the rat $H_{3(413)}$-receptor isoform (500 fmol $mg^{-1}$ protein). Proxyfan antagonized the effects of imetit, an agonist, and ciproxifan or FUB 465, two inverse agonists. Reproduced with permission from Ref. [20], Copyright 2000, Macmillan Publishers.

acid release from CHO cells with a potency up to two orders of magnitude higher than that they displayed as antagonists at the $H_3$ autoreceptor (Fig. 6).

After testing a large variety of compounds, we identified proxyfan (3-(1*H*-imidazol-4-yl)propyl-phenylmethyl ether) as a potent (Ki ~ 10 nM) neutral antagonist [20]. In CHO($rH_3R$) cells with moderate expression, proxyfan inhibited the effects of the agonist imetit as well as those of the inverse agonists ciproxifan and FUB 465, without affecting [$^3$H]arachidonic acid release alone, even at a 10 μM concentration (Fig. 6). As expected, however, the pharmacological profile of proxyfan depended on the test system, i.e. on the equilibrium between the active and inactive conformations of the receptor and/or the stoichiometric ratio of the receptor to the various G proteins, indicating that the compound can be used as a neutral antagonist only after careful assessment of its effect on the test system that is selected [20].

## 4. Constitutive activity of the native $H_3R$ studied in vitro

[$^{35}$S]GTPγ[S] binding demonstrated the constitutive activity of native $H_3$ receptors expressed at a normal level in mouse or rat brain [20,21]. Thus, specific [$^{35}$S]GTPγ[S] binding to mouse cerebral cortical membranes was reduced by FUB 465, ciproxifan or thioperamide, which were acting as inverse agonists as their effects were blocked by 1 μM proxyfan. Proxyfan also blocked the increase in binding elicited by imetit but did not itself significantly affect binding, and was therefore acting again as a neutral antagonist in this test system (Fig. 7). This constitutive activity of the native $H_3R$ was also observed in all

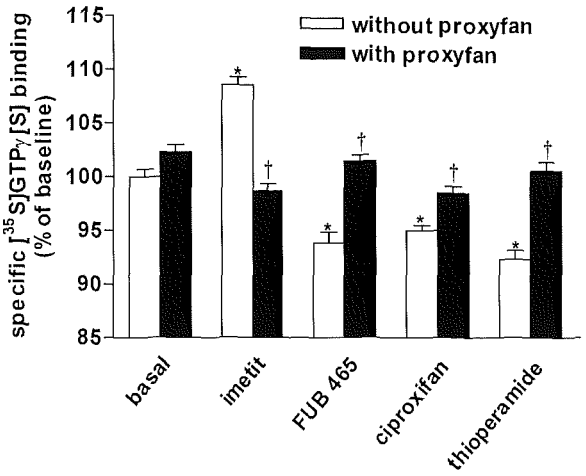


Fig. 7. Effects of $H_3$-receptor ligands (10 nM) on [$^{35}$S]GTPγ[S] binding to mouse cerebral cortical membranes in the presence or absence of 1 μM proxyfan. *$P<0.001$ vs basal, $^{\dagger}P<0.01$ vs without proxyfan. Reproduced with permission from Ref. [20], Copyright 2000, Macmillan Publishers.

regions of the rat brain. Moreover, it appeared to be one of the highest among G-protein-coupled receptors. Although an apparent lack of inverse agonism does not furnish definitive evidence for the absence of constitutive activity, it is worth noting that several compounds classified as inverse agonists at overexpressed or mutated receptors failed to decrease [$^{35}$S]GTPγ[S] binding to membranes of rat cerebral cortex or striatum. However,

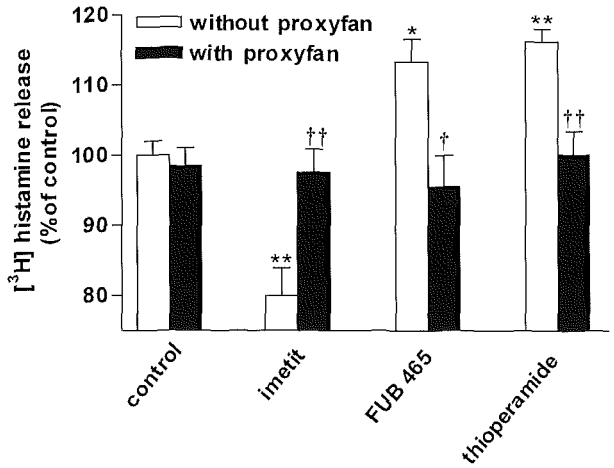


Fig. 8. Effects of $H_3$-receptor ligands (100 nM) on [$^3$H]histamine release induced by 55 mM $K^+$ from mouse cortical synaptosomes. When required, 10 μM proxyfan was added. *$P<0.01$, **$P<0.001$ vs control; $^{\dagger}P<0.01$, $^{\dagger\dagger}P<0.001$ vs without proxyfan. Reproduced with permission from Ref. [20], Copyright 2000, Macmillan Publishers.

we observed that besides $H_3$Rs, native $A_1$ adenosine receptors display high constitutive activity in rat brain [21].

The constitutive activity of the $H_3$ autoreceptor controlling [$^3$H]histamine release could be evidenced in cortical synaptosomes submitted to a strong depolarizing stimulus (40–55 mM $K^+$) in the mouse or rat [20]. In agreement, both thioperamide and FUB 465 behaved as inverse agonists enhancing significantly the amine release, release being on the contrary reduced by the agonist imetit. Proxyfan, which alone did not affect [$^3$H]histamine release, blocked the opposite effects of either thioperamide and FUB 465 or imetit, therefore acting again as a neutral antagonist (Fig. 8). Blockade of the $H_3$ autoreceptor stimulation by endogenous histamine does not significantly contribute to the releasing effect of thioperamide and FUB 465. This is shown by the lack of releasing effect of proxyfan, a potent neutral antagonist, and by the potent releasing effect of FUB 465, a potent inverse agonist but weak neutral antagonist (Fig. 8).

## 5. Constitutive activity of the native $H_3$R in brain in vivo

The inverse agonists markedly enhance cerebral histamine neuron activity in vivo, increasing by ~ 80% at maximum the levels of the histamine metabolite *tele*-methylhistamine (*t*-MeHA), a reliable marker of this activity (Fig. 9). This effect reflects an inverse agonist rather than the antagonist activity (towards endogenous histamine) of these ligands, as assumed so far [9,27]. In agreement, the effect of FUB 465 obtained at a low dose ($ED_{50}$ ~ 1 mg/kg, p.o.) was more consistent with its nanomolar potency as an inverse agonist than its micromolar potency as an antagonist. Moreover, the increases in *t*-MeHA level by FUB 465 and ciproxifan were competitively antagonized by proxyfan given at doses of ~ 2 mg/kg, which also blocked the decrease in *t*-MeHA level induced by the agonist imetit. At these doses, proxyfan administered alone failed to affect significantly *t*-MeHA levels, indicating that it was acting as a neutral antagonist in vivo on a system regulated by $H_3$Rs displaying constitutive activity (Fig. 9). The small but significant

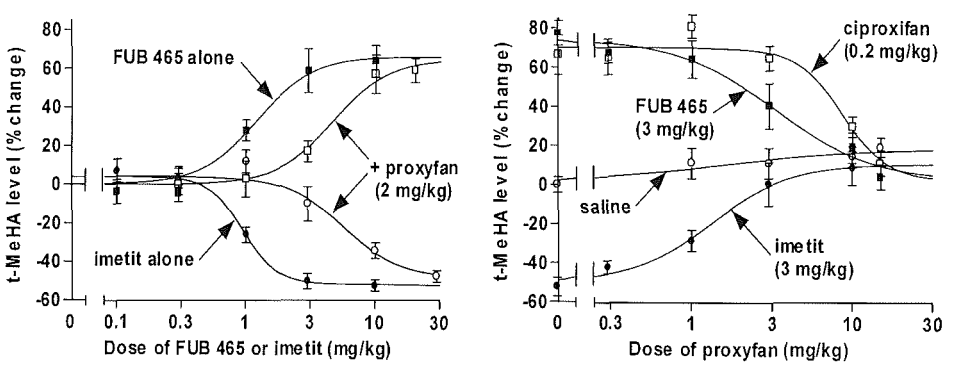


Fig. 9. Changes in brain *tele*-methylhistamine (*t*-MeHA) levels in mice receiving $H_3$-receptor ligands (p.o.). Values are expressed as percent change of values obtained in vehicle-treated mice (119+4 ng/g). Reproduced with permission from Ref. [20], Copyright 2000, Macmillan Publishers.

increase (by ~ 20%) observed with proxyfan in doses above 10 mg/kg might reflect its antagonist activity towards endogenous histamine. Recently, Fox et al. [28] reported similar observations in vivo on drinking behavior. An $H_3R$ agonist induced dipsogenia and this effect was blocked by ciproxifan. Proxyfan partially attenuated both the effect of the agonist and the effect of ciproxifan, suggesting that it was again acting as a neutral antagonist in this system.

## 6. Conclusion

The present findings show that native $H_3Rs$ present in rat brain display high constitutive activity. The human $H_3R$ also displays constitutive activity. The latter is easily detected at moderate densities of the recombinant receptor, suggesting that it occurs in human brain. This observation may have important implications in terms of drug development. The role of constitutive activity of $H_3$ autoreceptors in the regulation of histamine neurons in vivo further suggests that inverse agonists should find therapeutic applications in neuropsychiatric diseases. One of the best substantiated roles of histaminergic neurons is to promote arousal and attention, and to improve learning [11,29]. The strong enhancement of histamine neuron activity that they induce in brain suggests that $H_3R$ inverse agonists (rather than neutral antagonists) might be a new therapeutic approach in attentional and cognitive deficits, such as those encountered in attention-deficit hyperactivity disorders, schizophrenia or Alzheimer's disease [22,30–32].

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## Discussion 12

G. Milligan

Maybe a number of people in the room got the impression that you'd argued that the rat receptor was substantially more constitutively active than the human.

J.M. Arrang

Yes, apparently in the GTPγ[S] binding, but clearly not on other systems. Rob Leurs published that on cAMP formation, constitutive activity was even higher for the human receptor.

R. Leurs

Yes, if you express it in a neuronal cell line. We observed that with the same expression levels the human receptor seems to be somewhat higher in its constitutive activity compared with the rat. That's a bit in contrast to the GTPγ[S] data you have seen in CHO cells.

G. Milligan

So you've not, for example, looked at the sequence between the rat and the human and tried to mutate one to the other form.

J.M. Arrang

No, we did not perform mutagenesis studies to explore constitutive activity.

R. Leurs

Neither did we.

R. Bond

I noticed that you used 55 mM of potassium chloride to depolarise, and at the beginning, Ad IJzerman pointed out that Tommy Costa discovered constitutive activity by a substitution of potassium for sodium ions. I was just wondering whether neuronal receptors may have a higher predisposition to achieve a spontaneously active state just simply from the potassium.

J.M. Arrang

It is an interesting point. We were a bit surprised at the beginning of the study because we did not expect to need a high depolarisation to evidence the constitutive activity. Now, we want to further study this kind of relationship between constitutive activity and the depolarisation level.

G. Milligan

Is there any reason to believe that in neuronal cells there are, maybe, a greater proportion of lipid domains or rafts that are present? Because clearly we see more efficient signalling through receptors that are in rafts compared to those that are in the bulk membrane phase, and whether this therefore would lead to higher constitutive activity in a detergent-insensitive domain.

J.M. Arrang

It is a very interesting point. I really do not know but may be someone wants to comment on that.

T. Schwartz

It was related to that, or rather, to what we are going to hear a little later, about when we measure the activity in various cell lines. It may be pretty far away from where we really want to be with the right scaffolding and adapter proteins that may or may not be there, and depending on whether they recognise it, and whether the turnover and the internalisation may occur. But we just heard about that, Rob Leurs was also seeing these things in neuronal cells, as you discussed.