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# Independent regulation of $\beta_1$- and $\beta_2$-adrenoceptors

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1 The down-regulation of β-adrenoceptors has been postulated as a biochemical marker of antidepressant efficacy. Here we demonstrate that chronic treatment with desipramine down-regulates $\beta_1$-adrenoceptors in rat cerebral cortex and that β-adrenoceptor subtypes can be independently regulated by treatment with different β-adrenoceptor agonists.

2 Desipramine, (±)-clenbuterol, prenalterol, corwin (20 mg kg$^{-1}$ daily) and corwin (10 mg kg$^{-1}$ daily) were administered to male, Sprague-Dawley rats, over eight days, by means of osmotic Alzet pumps placed subcutaneously and removed 24 h before analysis. Control rats received vehicle only. The $\beta_1$- and $\beta_2$-adrenoceptor populations were measured in cerebral cortex by a modified (−)-[$^{125}$I]-pindolol receptor binding assay.

3 The conventional antidepressant, desipramine, preferentially down-regulated $\beta_1$-adrenoceptors whereas the non-selective β-adrenoceptor agonist (±)-clenbuterol preferentially down-regulated $\beta_2$-adrenoceptors. The $\beta_1$-selective partial agonist, prenalterol, up-regulated $\beta_1$-adrenoceptors perhaps acting more as an antagonist than as an agonist. Finally, neither dose of corwin had any significant effect on β-adrenoceptor number.

## Introduction

For many years, it has been widely postulated that the primary actions of antidepressant drugs are their acute actions at the presynaptic level, increasing the concentration of monoamines in the synaptic cleft. More recently, attention has focused on the chronic effects of antidepressant drugs at the postsynaptic level, in regulating the level of neurotransmitter receptors.

The two main classes of conventional antidepressants, tricyclic antidepressants and monoamine oxidase (MAO) inhibitors, both enhance monoaminergic function acutely (Banerjee *et al.*, 1977). Chronic, but not acute, treatment with antidepressants produces adaptive changes in neurotransmitter receptors, in particular a down-regulation of β-adrenoceptors (Banerjee *et al.*, 1977; Bergstrom & Kellar, 1979a) and 5-hydroxytryptamine$_2$ (5-HT$_2$) receptors (Bergstrom & Kellar, 1979a; Peroutka & Snyder, 1980). The time course for this change corresponds closely with the onset of clinical therapeutic effects (Sulser *et al.*, 1978; Sellinger-Barnette *et al.*, 1980). Receptor down-regulation with conventional antidepressants has been demonstrated in several brain regions (Sulser & Mobley, 1981) and in peripheral tissues (Honegger & Bickel, 1980). Also, electroconvulsive therapy treatment down-regulates β-adrenoceptors (Bergstrom & Kellar, 1979b) but not 5-HT$_2$ receptors. The down-regulation of β-adrenoceptors is associated with a subsensitivity of the receptor-coupled adenylate cyclase (Sulser & Mobley, 1981).

If down-regulation of CNS β-adrenoceptors is essential to the action of antidepressants, one could postulate that centrally active β-adrenoceptor agonists would rapidly down-regulate CNS β-adrenoceptors, and produce a fast onset antidepressant effect. In fact preliminary but unconfirmed clinical reports on the efficacy of salbutamol (Widlocher *et al.*, 1977; Lecrubier *et al.*, 1980) and of clenbuterol (Simon *et al.*, 1984) in the treatment of depression presented encouraging evidence for the potential antidepressant properties of β-adrenoceptor agonists. Also there is ample evidence that β-adrenoceptor agonist administration does result in a rapid down-regulation of total β-adrenoceptors at least in the periphery (Stiles *et al.*, 1984). Although conventional antidepressants are widely reported to down-regulate total β-adrenoceptors (Sulser *et al.*, 1978), probably due to selective actions on $\beta_1$-adrenoceptors (Minneman *et al.*, 1979a), the actions of β-adrenoceptor agonists on central β-adrenoceptor subtypes is not widely reported. In fact, using autoradiographic methods Frazer *et al.* (1986) suggest that the β-adrenoceptor agonist clenbuterol acts at $\beta_2$-adrenoceptors. Thus in this study we aimed to determine firstly whether the down-regulation of central β-adrenoceptors by conventional antidepressants and by β-adrenoceptor agonists was at the same β-adrenoceptor subtype. Secondly, we hoped to mimic

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the action of conventional antidepressants with CNS-active selective β-adrenoceptor agonists and hence combine increased efficacy with the fast acting properties of 'β-agonist type antidepressants'.

## Methods

Male Sprague-Dawley rats (starting weight 180–190 g) were used. The animals were housed in a room adjusted to a 12 h light/dark cycle (07 h 00 min–19 h 00 min), temperature 21–23°C and had free access to food and water. The drugs were administered at 20 mg $kg^{-1}$ daily (and also at 10 mg $kg^{-1}$ daily in the case of corwin) by use of osmotic Alzet pumps inserted subcutaneously. The rats were anaesthetized with pentobarbitone sodium and the pumps were inserted posterior to the scapulae, the wound being closed with wound clips. The control animals received vehicle only (propylene glycol:$H_2O$ 1:1). The pumps were removed, under anaesthesia, 8 days after implantation. Twenty four hours after the removal of the pumps the rats were killed by decapitation.

### *Membrane preparation*

The whole brain was removed and the cerebral cortex and cerebellum quickly dissected on ice, weighed and promptly transferred to 10–15 volumes (weight/volume) of ice-cold 0.32 M sucrose containing 1 mM EDTA-$Na_2$. The tissue was then homogenized with 10 strokes of a motor driven teflon/glass homogenizer (Janke & Kunkel) at 500 r.p.m. The homogenate was centrifuged for 10–15 min at 1,000 *g* at 4°C and the pellet was discarded. The supernatant was recentrifuged for 10–15 min at 20,000 *g* at 4°C. The supernatant was discarded and the pellet resuspended in 10–15 volumes of ice-cold 50 mM Tris/HCl/0.5 mM EDTA buffer (pH 7.8 at room temperature) and rehomogenized with 10 strokes of a motor driven teflon/glass homogenizer before being recentrifuged for 10–15 min at 20,000 *g* at 4°C. The pellet was washed, a further 3 times. Membranes prepared this way could be stored, at −20°C, for up to 5 weeks without affecting the specific binding of (−)-[$^{125}$I]-pindolol (IPIN). On the day of use the pellets were thawed, on ice, and resuspended in assay buffer (20 mM Tris HCl, 10 mM $MgCl_2$ containing 1 mM EDTA and 0.1 mM ascorbic acid, pH 7.8 at room temperature) at a weight:volume ratio of 1:75 for cerebral cortex and 1:50 for cerebellum.

### *(−)-[$^{125}$I]-pindolol binding experiments*

The IPIN saturation experiments were carried out as described previously (Beer *et al.*, 1987). The membranes, IPIN (2,200 Ci $mmol^{-1}$), NEN Corporation, Boston, MA) and drugs were prepared in assay buffer. A 150 μl aliquot of the membrane suspension (approximately 50 μg protein), was incubated at 37°C, in a shaking water bath, with 200 μM guanosine triphosphate (GTP), 150 μM phentolamine and IPIN over a range of approximately 10–800 pM (final volume = 250 μl). The phentolamine was included to avoid any possible interference at α-adrenoceptors without affecting specific binding. The reaction was begun by adding the membrane suspension and was terminated after 20 min by the addition of 4 ml assay buffer to each tube followed by rapid filtration through Whatman GF/C glass fibre filters using a Brandel cell harvester. Each tube was then washed three times with 4 ml assay buffer through the filters. The filters were then transferred to scintillation vials and the radioactivity determined, with a counting efficiency of 80.8%, in a Model 1272 Clinigamma LKB scintillation counter. (−)-Isoprenaline (200 μM) was used to define non-specific binding. Specific binding was found to represent 85–90% of total binding. Protein concentrations were determined by the method of Lowry using bovine serum albumin as a standard.

The above procedure gave a measure of total β-adrenoceptors and was modified to measure the $\beta_2$-adrenoceptor population by the inclusion of the highly selective $\beta_1$-adrenoceptor antagonist CGP 20712A (Dooley & Bittiger, 1984). This was added at a concentration of 100 nM for cortical and 300 nM for cerebellar membranes. These concentrations had been shown to block out 98% and 99% of the $\beta_1$-adrenoceptor sites and only 2% and 1% of the $\beta_2$-adrenoceptors in cortex and cerebellum respectively. The difference in these two populations gave a measure of the $\beta_1$-adrenoceptor density.

### *Data analysis*

Radioactivity measurements from the binding studies were converted to picomolar concentrations as described by Doyle *et al.* (1984). Scatchard analysis was used to estimate the equilibrium dissociation constant ($K_D$) and maximum number of binding sites ($B_{max}$). The resulting dissociation constants and binding capacities were summarized as the arithmetic means and were compared using the Dunnett's test following an analysis of variance.

### *Materials*

The following drugs were gifts: CGP 20712A (benzamide, 2-hydroxy-5-(2-[2-hydroxy-3-(4-((1-methyl-4-trifluoro-methyl) 1H-imidazol-2-yl)phenoxy) propyl)amino]ethoxyl)-, monoethane-sulphonate salt; Ciba-Geigy Ltd., Basle, Switzerland); corwin (Imperial Chemical Industries Ltd., Macclesfield,

Cheshire); prenalterol (Astra Pharmaceuticals Ltd., Kings Langley, Hertfordshire); (±)-clenbuterol (Boehringer Ingelheim Ltd., Bracknell, Berkshire). (−)-[$^{125}$I]-pindolol (2,200 Ci mmol$^{-1}$) was purchased from NEN Research Products.

## Results

The IPIN binding to β-adrenoceptors was saturable, of high affinity and with a low proportion, approximately 10%, of non-specific binding. Figures 1, 2 and 3 summarize the values of maximal binding capacities ($B_{max}$) and Table 1 the apparent dissociation constants ($K_D$) obtained in cortex and cerebellum, after the various drug treatments.

### *Effects of desipramine treatment*

The subcutaneous administration of the tricyclic antidepressant desipramine (DMI) resulted in a statis-

**Table 1** The effect of eight days treatment with vehicle, desipramine, clenbuterol, prenalterol and corwin on the dissociation constants for IPIN binding to rat cerebral cortex and cerebellum

| | $K_D$ (pM) *values for iodopindolol binding in rat cortex and cerebellum* | | | | | |
|---|---|---|---|---|---|---|
| *Treatment* | *Control* $n = 9$ | *Desipramine* 20 mg kg$^{-1}$.day$^{-1}$ $n = 7$ | *Clenbuterol* 20 mg kg$^{-1}$.day$^{-1}$ $n = 7$ | *Prenalterol* 10 mg kg$^{-1}$.day$^{-1}$ $n = 6$ | *Corwin* 20 mg kg$^{-1}$.day$^{-1}$ $n = 5$ | *Corwin* 20 mg kg$^{-1}$.day$^{-1}$ $n = 5$ |
| Cortex total | 250 ± 14.7 | 210 ± 12.3 | 372 ± 14.7* | 267 ± 18.3 | 241 ± 27.3 | 245 ± 7.8 |
| $\beta_2$ | 118 ± 9.5 | 124 ± 8.7 | 266 ± 33.4* | 116 ± 9.7 | 127 ± 17.2 | 111 ± 13.5 |
| Cerebellum total | 108 ± 6.0 | 110 ± 13.9 | 156 ± 21.3 | 136 ± 29.7 | 115 ± 21.6 | 94 ± 8.6 |
| $\beta_2$ | 113 ± 8.5 | 121 ± 14.5 | 125 ± 9.8 | 164 ± 19.9 | 116 ± 20.2 | 246 ± 6.5 |

The results are represented as mean values ± s.e.means. Significance of differences with respect to controls: *$P < 0.01$.

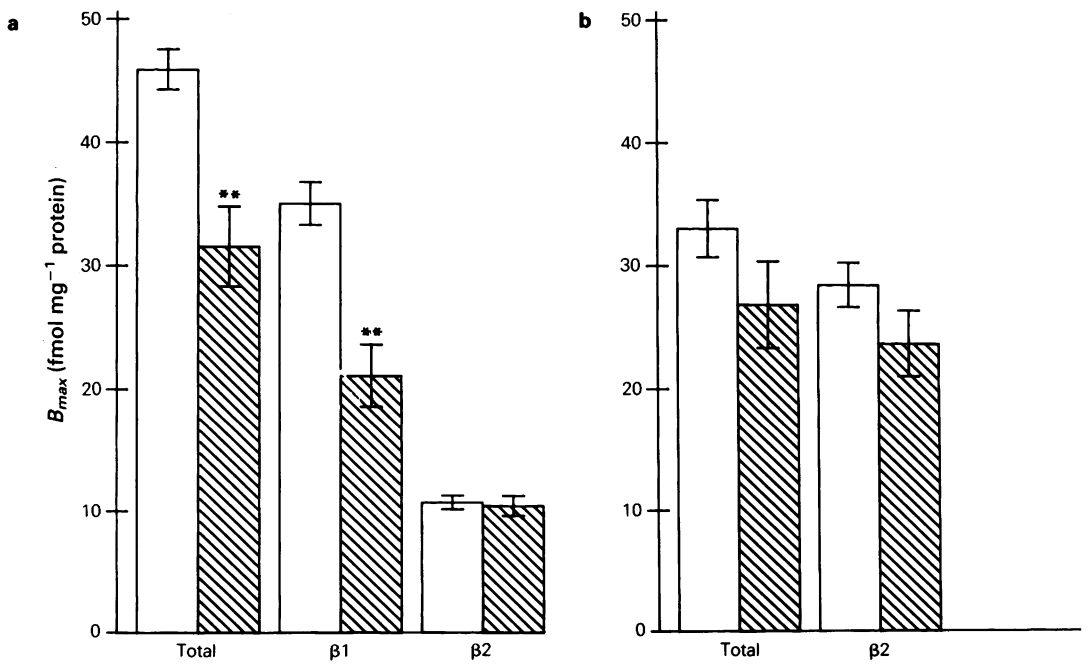


**Figure 1** Effects of eight days treatment with vehicle (open columns, $n = 9$) or desipramine (hatched columns, $n = 7$) on the maximal binding capacity of (−)-[$^{125}$I]-pindolol to total, $\beta_1$- and $\beta_2$-adrenoceptors in rat cerebral cortex (a) and cerebellum (b). Each column represents the mean value and the vertical lines show s.e.means. Significantly different from control: **$P < 0.01$.

tically significant ($P<0.01$) down-regulation of the total β-adrenoceptor population, to 68% of the control value in rat cortex (Figure 1a). A closer examination of the resolved $\beta_1$- and $\beta_2$-adrenoceptor subtypes revealed that this was due to a selective down-regulation of the $\beta_1$-adrenoceptor subtype, to 60% of the control value ($P<0.01$). The $\beta_2$-adrenoceptor subtype was not significantly affected. This lack of significant effect on $\beta_2$-adrenoceptors was confirmed by the results from cerebellum (Figure 1b) where the $\beta_2$-adrenoceptor population was similarly not significantly altered by DMI treatment. Administration of DMI had no effect on the $K_D$ values obtained for IPIN binding to total, $\beta_1$- or $\beta_2$-adrenoceptors in rat cortex or cerebellum.

*Effects of clenbuterol treatment*

The centrally non-selective β-adrenoceptor agonist (±)-clenbuterol given subcutaneously at 20 mg kg$^{-1}$ daily for eight days had no effect on total or $\beta_1$-adrenoceptor populations in rat cortex as can be seen in Figure 2a. However, both the total and $\beta_2$-adrenoceptor density in cerebellum (Figure 2b) and the $\beta_2$-adrenoceptor density in cortex were all significantly reduced, to 33%, 24% and 60% of the control values, respectively ($P<0.01$).

The affinity of IPIN binding was unchanged in all categories except for the binding to total and $\beta_2$-adrenoceptors in cortex, where it was significantly reduced as shown in Table 1 ($P<0.01$).

*Effect of prenalterol treatment*

The $\beta_1$-selective agonist prenalterol, which is known to act as a partial agonist in the periphery (Carlsson *et al.*, 1977) and to penetrate the blood brain barrier (Brunswick & Conway, 1984), which administered subcutaneously at 20 mg kg$^{-1}$ daily for 8 days caused a significant ($P<0.05$) up-regulation of total β-adrenoceptors in cortex, to 123% of the control value, but had no effect on total β-adrenoceptors in cerebellum (Figures 2a and 2b). A closer examination of the results from both cortex and cerebellum revealed that the up-regulation seen in total β-adrenoceptors in cortex can be accounted for by an alteration in the $\beta_1$-population, which was also significantly up-regulated ($P<0.05$) whereas the $\beta_2$-adrenoceptors were not significantly altered. This effect in cortex is perhaps not surprising if this compound also acts as a partial agonist in the CNS as we have shown it to possess a 13 fold selectivity for $\beta_1$- versus $\beta_2$-adrenoceptors in CNS binding studies (Beer *et al.*, 1987). Also a similar up-regulation of $\beta_1$-receptors has been demonstrated by

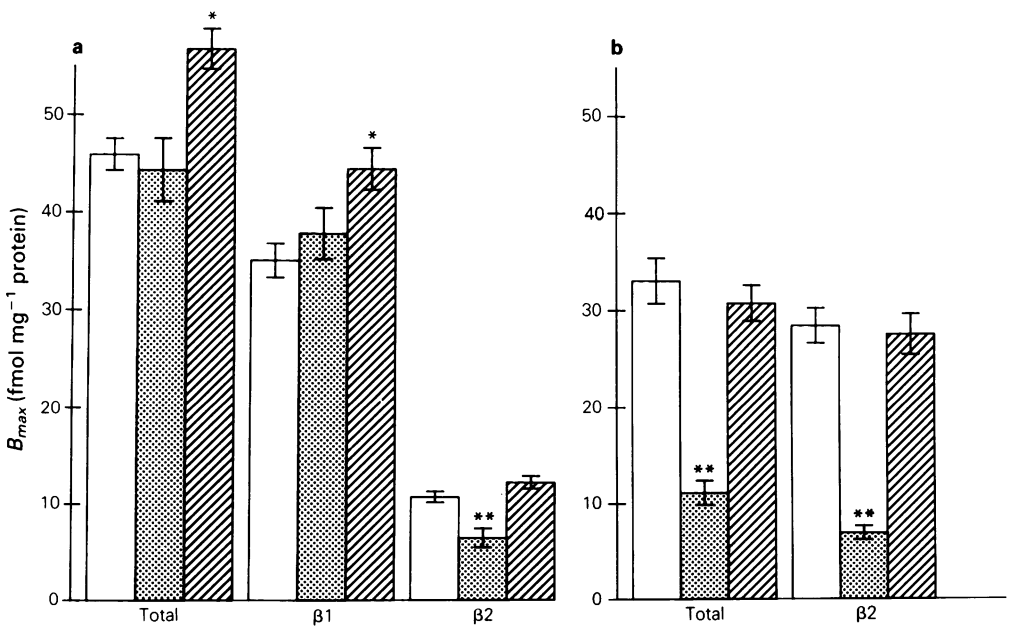


**Figure 2** Effect of eight days treatment with vehicle (open columns, $n = 9$), clenbuterol (stippled columns, $n = 7$) or prenalterol (hatched columns, $n = 6$) on the maximal binding capacity of (−)-[$^{125}$I]-pindolol to total, $\beta_1$- and $\beta_2$-adrenoceptors in rat cerebral cortex (a) and cerebellum (b). Each column represents the mean value and the vertical lines show s.e.means. Significantly different from control: $^*P<0.05$; $^{**}P<0.01$.

receptor blockade with the antagonist propranolol and by destroying adrenergic neurones with 6-hydroxydopamine (6-OHDA (Woolfe *et al.*, 1982). The 6-OHDA-induced up-regulation was shown to be restricted to the $\beta_1$-adrenoceptor subtype using the selective agonist zinterol (Minneman *et al.*, 1979a). Administration of prenalterol had no significant effect on the $K_D$ values of IPIN binding to total, $\beta_1$- or $\beta_2$-adrenoceptors in rat cortex or cerebellum.

*Effect of corwin treatment*

As can be seen in Figures 3a and 3b, the $\beta_1$-selective partial agonist corwin (which has a 10 fold selectivity for $\beta_1$-adrenoceptors in the CNS, Beer *et al.*, 1987), given subcutaneously at either 10 or 20 mg kg$^{-1}$ daily had no significant effect on total number of $\beta_1$- or $\beta_2$-adrenoceptors or on the $K_D$ value for IPIN binding in rat cortex or cerebellum. This is possibly due to an inability to penetrate the blood brain barrier.

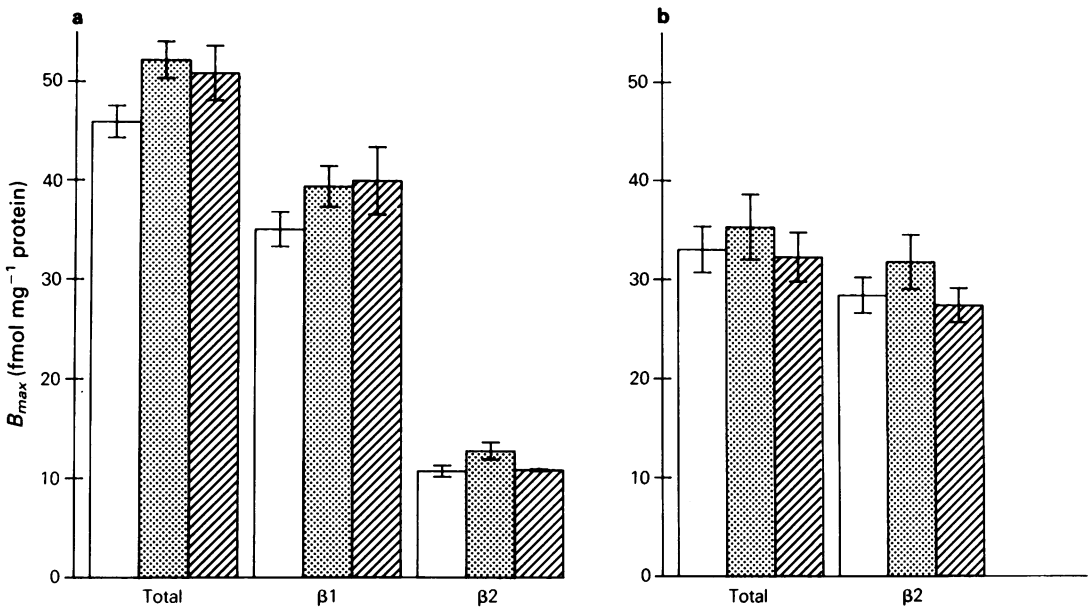


**Figure 3** Effect of eight days treatment with vehicle (open columns, $n = 9$), 10 mg kg$^{-1}$day$^{-1}$ corwin (stippled columns, $n = 5$), 20 mg kg$^{-1}$day$^{-1}$ corwin (hatched columns, $n = 5$) on the maximal binding capacity of (−)-[$^{125}$I]-pindolol to total, $\beta_1$- and $\beta_2$-adrenoceptors in rat cerebral cortex (a) and cerebellum (b). Each column represents the mean value and the vertical lines show s.e.means.

## Discussion

Our results demonstrate that chronic administration of DMI down-regulates total β-adrenoceptors in cortex, to 68% of the control value, but has no significant effect in cerebellum. This effect of DMI is now well documented (Minneman *et al.*, 1979a; Nimgaonkar *et al.*, 1985) and is known to be correlated with a desensitization of adenylate cyclase activity (Motulsky & Insel, 1982; Stiles *et al.*, 1984). However, a closer examination of the resolved $\beta_1$- and $\beta_2$-adrenoceptor subtypes in our study revealed that the effect of DMI in rat cortex is due entirely to a 40% reduction in the $\beta_1$-adrenoceptor population, the $\beta_2$-adrenoceptor subtype, as in cerebellum being essentially unaffected.

This selective down-regulation by DMI has previously been suggested by Minneman *et al.* (1979a) employing the $\beta_1$-selective agonist zinterol in binding studies, by Dooley *et al.* (1983) using various tissue types and from the behavioural studies of Kitada *et al.* (1986). Hence, we can conclude from our as well as others' data that DMI preferentially down-regulates $\beta_1$-adrenoceptors. Any potential fast acting 'β-agonist type antidepressant' would have to mimic this action if this is the primary antidepressant action of DMI.

The first β-agonist tested in our study was clenbuterol, an aminohalogen substituted phenyl-ethanolamine. Despite its apparent lack of $\beta_1$:$\beta_2$ selectivity in CNS binding studies (Frazer *et al.*, 1986), this lipophilic partial agonist (Cohen *et al.*, 1982; Waldeck &

Widmark, 1984) was chosen because of its ability to penetrate the blood brain barrier (Ross, 1980; Brunswick & Conway, 1984) and also because of its suggested antidepressant like properties in clinical evaluations (Simon *et al.*, 1984).

Chronic treatment with clenbuterol had no significant effect on total β-adrenoceptor population in rat cortex but significantly reduced the total β-adrenoceptor population in cerebellum. This is in agreement with the findings of Ordway *et al.* (1985) and O'Donnell *et al.* (1985) although Hall *et al.* (1980) and Nimgaonkar *et al.* (1985) report down-regulation of total β-adrenoceptors in cortex after chronic clenbuterol treatment. We believe that the apparent lack of effect by clenbuterol seen in our study is due to the preferential down-regulation (40%) of the $\beta_2$-adrenoceptor subtype. Cortex is composed of 70–80% $\beta_1$-adrenoceptors (Minneman *et al.*, 1979b; Rainbow *et al.*, 1983), and measurements of total β-adrenoceptors may mask the selective down-regulation of $\beta_2$-adrenoceptors in this particular tissue. This selective action by clenbuterol is confirmed by the results obtained in cerebellum, which contains mainly (90–95%) $\beta_2$-adrenoceptors (Minneman *et al.*, 1979b; Rainbow *et al.*, 1983), and where the total and $\beta_2$-adrenoceptor populations are reduced to 33% and 24% of the control values respectively. Ordway *et al.* (1985) and Dooley & Hauser (1983) also report 80% and 65% reductions in total β-adrenoceptor density respectively in cerebellum following clenbuterol treatment.

Clenbuterol treatment caused a small increase in the $K_D$ value for IPIN binding in cortex. O'Donnell *et al.* (1985) consider similar findings as evidence for the uncoupling of the receptor from its GTP binding protein. An alternative explanation, which also accounts for the unaltered $K_D$ in cerebellum, is the 3 fold selectivity IPIN has for $\beta_2$-adrenoceptors (McGonigle *et al.*, 1986). As the $\beta_2$-adrenoceptors are down-regulated the $K_D$ value for IPIN to the remaining receptor is correspondingly increased. This is not observed in cerebellum which is virtually devoid of $\beta_1$-adrenoceptors.

There are numerous reports in the literature of antidepressant drugs failing to down-regulate β-adrenoceptors. A number of groups, namely Mishra *et al.* (1980), Dooley *et al.* (1983) and O'Donnell & Frazer (1985) claim this to be the case with clenbuterol, despite being able to demonstrate a reduced responsiveness of the noradrenaline (NA)-stimulated adenylate cyclase. Such reports have led to the theory that such drugs promote their effects by 'uncoupling' the receptor from its N-protein without necessarily causing its internalization (O'Donnell *et al.*, 1985, Okada *et al.*, 1986). Stadel *et al.* (1983) have suggested that this 'uncoupling' action by antidepressants could be due to phosphorylation of the receptor site. However, Hancock & Marsh (1985) have demonstrated that antidepressants cause a reduced adenylate cyclase responsiveness without altering the affinity of the β-adrenoceptor for agonists which would tend to argue against this "uncoupling" theory.

This study offers an alternative explanation for these findings. The possibility remains that in measuring total β-adrenoceptor populations any subtype specific down-regulation could be masked, especially if that subtype was the minor component in the particular tissue being studied, as was found to be the case with clenbuterol in cerebral cortex. In view of the reports that certain antidepressants i.e. mianserin (Sellinger-Barnette *et al.*, 1980), zimeldine and bupropion (Suranyi-Cadotte *et al.*, 1985) and iprindole (Hancock & Marsh, 1985) do not down-regulate β-adrenoceptors, Sugrue (1983) dismisses alterations in β-adrenoceptor density as a marker of antidepressant efficacy. However, this may prove not be be the case if the separate subtype populations were monitored.

Methodological considerations may also explain these discrepant findings between different groups. For example some lipid-soluble radioligands used in binding studies may not discriminate between available and internalized receptors.

The possibility also remains that certain antidepressants do, in fact, mediate their effects in a manner unrelated to β-adrenoceptor density. Hirata & Axelrod (1978) have suggested that a β-adrenoceptor-mediated phospholipid methylation could result in changes in membrane fluidity and hence adenylate cyclase sensitivity with or without concomitant β-adrenoceptor down-regulation. In the extreme case, the pivotal site of action of some drugs may be post-receptor and totally divorced from receptor density.

A further question raised by this study is why does (±)-clenbuterol, which we have previously shown to be non-selective for β-adrenoceptors in CNS binding studies (Beer *et al.*, 1987), preferentially act on $\beta_2$-adrenoceptors? Frazer *et al.* (1986) also failed to find any evidence that clenbuterol acts at $\beta_1$-adrenoceptors in cerebral cortex or cerebellum from autoradiographic studies and also after investigating the reduced adenylate cyclase responsiveness to (−)-isoprenaline and clenbuterol in cortex and cerebellum (Ordway *et al.*, 1985, Frazer *et al.*, 1986).

Minneman *et al.* (1979a) has suggested that $\beta_2$-adrenoceptors in brain are associated with non-nerve cells i.e. glia and blood vessels, their endogenous input being adrenaline released from the adrenals. It is therefore conceivable that clenbuterol acts only on $\beta_2$-adrenoceptors because of an inability to penetrate the blood brain barrier, thus acting only on the glia and blood vessels of the blood brain barrier itself. This would seem unlikely in view of the behavioural findings of Ross (1980), the *in vivo* studies of Brunswick & Conway (1984) and also the $\beta_2$-selective action

of clenbuterol in the periphery.

Secondly, Honegger *et al.* (1986) have suggested that $\beta_2$-adrenoceptors are more tightly coupled to the cyclic AMP secondary messenger system which would preferentially increase the rate of their internalization.

Finally, Nerme *et al.* (1985) have suggested that endogenous NA is tightly bound to β-adrenoceptors in rat heart. As NA has a higher affinity for $\beta_1$-adrenoceptors, these are more likely to be masked from the action of clenbuterol.

The conclusions to be drawn from this study are that none of the chosen β-adrenoceptor agonists were able to mimic the biochemical action of DMI on adrenoceptors. This may be a result of the choice of agonists used but the possibility remains, especially in view of the clenbuterol results, that CNS $\beta_1$-adrenoceptors cannot be down-regulated by β-receptor agonists. Perhaps the mechanism by which conventional antidepressants bring about this action does not involve the direct action of NA on the receptor, a view that is supported by the recently emerging evidence for the involvement of the phospholipid matrix in receptor turnover (Takamura *et al.*, 1985; Honegger *et al.*, 1986).

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