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Mediation of Mydriasis in Conscious Rats by Central Postsynaptic α_2-Adrenoceptors

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HEAL, D. J., M. R. PROW, S. A. BUTLER AND W. R. BUCKETT. *Mediation of mydriasis in conscious rats by central postsynaptic α_2-adrenoceptors.* PHARMACOL BIOCHEM BEHAV **50**(2) 219-224, 1995.—The α_2-adrenoceptor agonist, clonidine (0.001-1 mg/kg, IP), dose-dependently induced mydriasis in conscious rats (ED_{50} 0.088 mg/kg). This response was maximal when measured 10 min after clonidine injection and was of about 30-min duration. The noradrenaline releasing agent, methamphetamine (0.75 mg/kg, IP), also increased pupil diameter. Clonidine (0.03 mg/kg, IP)-induced mydriasis was inhibited in a dose-related fashion by the α_2-adrenoceptor antagonists, idazoxan (0.03-3 mg/kg, IP) and yohimbine (0.03-3 mg/kg, IP), but was unaltered by the α_1- or β-adrenergic antagonists, prazosin (1 and 3 mg/kg, IP) or pindolol (1 and 3 mg/kg, IP). Methamphetamine (0.75 mg/kg, IP)-induced mydriasis was similarly inhibited by idazoxan (1 mg/kg, IP) and yohimbine (1 mg/kg, IP). These data argued strongly that central α_2-adrenoceptors are involved in the mediation of mydriasis. The synaptic location of these receptors was determined using DSP-4 (50 mg/kg × 2, IP) to lesion noradrenergic neurones: this produced a 64% depletion of noradrenaline in the midbrain (containing the Edinger-Westphal nucleus responsible for mydriasis) and reduced the mydriatic effect of methamphetamine (0.75 mg/kg, IP) to a similar extent (72%), whereas clonidine mydriasis remained unaltered. Therefore, these results show that the mydriasis responses induced by either clonidine or methamphetamine are mediated by central postsynaptic α_2-adrenoceptors.

α_2-Adrenoceptors Postsynaptic α_2-adrenoceptors Mydriasis Central nervous system Clonidine
Functional model Conscious rats

α_2-ADRENOCEPTOR agonists are known to induce mydriasis in a wide variety of animal species including dogs (25), cats (21), rats (4,9), and mice (14). This response is mediated centrally and results from the activation of a population of α_2-adrenoceptors that are postsynaptic to noradrenergic neurones (15,16,18,20). These receptors are probably located in the Edinger-Westphal complex (26) where they act to modulate the parasympathetic tone to the iris (16,20,21). With the exception of the protocol that we have described for measuring mydriasis in conscious mice (14), almost all of the studies have been conducted in anaesthetised animals [e.g., (4,9, 18,21-23)]. There are a number of disadvantages with the use of anaesthetised animals for measuring mydriatic responses, the most obvious being that the anaesthetic directly affects the mydriasis produced by α_2-adrenoceptor agonists, as has been previously suggested by an earlier study using conscious rats (28). Secondly, the use of anaesthesia precludes or prejudices the testing of other biochemical and behavioural parameters in these animals; in a previous study (15), clonidine-induced mydriasis and hypoactivity were both measured in the same conscious mice and, in addition, clonidine's effects on brain 3-methoxy-4-hydroxyphenylglycol (MHPG) concentrations were also determined. Finally, anaesthesia also complicates the repeated use of animals in chronic studies. Recently (6), it was reported that it is also possible to measure α_2-adrenoceptor-mediated mydriasis in conscious rats under conditions of low lighting. We have now conducted experiments in rats to confirm these findings and, in addition, have pharmacologically characterised clonidine-induced mydriasis in conscious rats using various α- and β-adrenoceptor antagonists to determine whether this response could be used as a specific model of central α_2-adrenoceptor function. Furthermore, the synaptic location of the central α_2-adrenoceptors involved with the mydriasis response has been determined using the noradrenergic neurotoxin, *N*-(2-chloroethyl)-*N*-ethyl-bromobenzylamine (DSP-4), which was used because it is transported into noradrenergic neurones via the high-affinity noradrenaline reuptake system (5,11) where it binds covalently to electrophilic centres (7), rendering the neurones inactive.

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METHOD

Drugs and Reagents

Drugs were obtained from the following sources: clonidine HCl, methamphetamine HCl, pindolol, yohimbine HCl (Sigma, Poole); prazosin HCl (Pfizer, Sandwich); idazoxan HCl (Reckitt and Colman, Hull); DSP-4 HCl, zimeldine $(HCl)_2$ (Research Biochemicals International, Natick, MA).

Reagents were obtained from Sigma (Poole), BDH (Poole), Rathburn Chemicals (Walkerburn), and FSA (Loughborough). Reagents for high performance liquid chromatography (HPLC) methods were of the highest purity available and water was distilled and deionised prior to use.

Animals and Drug Administration Procedures

Adult male CD rats (Charles River), weighing 150–200 g, were used. They were housed in groups of three on a 12L : 12D cycle (commencing 0700 h) at a temperature of 21 °C and 55% humidity. The rats were allowed free access to food and water and, in addition, wet mash and 1% glucose (w/v) in the DSP-4 experiments.

All drugs were dissolved in 0.9% (w/v) sodium chloride solution (saline), with the exception of prazosin, which was dissolved in distilled water. Drugs were injected intraperitoneally in weight-related doses in a volume of 2 ml/kg body weight. Control rats were injected with saline, or in the case of prazosin, distilled water intraperitoneally. The doses of antagonists and pretreatment times chosen are those that have been shown to be appropriate from earlier studies (12,14).

DSP-4 treatment involved the following protocol. At approximately 0900 h, male CD rats (180–200 g) were pretreated with zimeldine (10 mg/kg, IP) to protect 5-hydroxytryptamine (5-HT)-containing neurones, with DSP-4 (50 mg/kg, IP) being injected 30 min later. Control animals received zimeldine (10 mg/kg, IP) followed by saline (2 ml/kg, IP). Six hours later, this procedure was repeated. Rats were also administered 1 ml of 0.9% (w/v) saline (SC) and 2 ml of 4% (w/v) glucose (PO) twice daily for the duration of the experiment. Animals were killed by cervical dislocation 72 h after the initial injection of DSP-4 or saline.

Pupil Diameter Measurement

The rats were allowed at least 30 min to adapt to the lighting conditions before pupil diameter measurements were made. The pupil diameter of the right eye was measured using a Wild M1 binocular microscope containing a graticule scale in one eyepiece. This was linked to a Swift light box (setting 1, light intensity 450 lx). The procedure was carried out in an artificially lit room (light intensity 20 lx). The rat was carefully held underneath the light source and its pupil diameter was read off the graticule scale in eyepiece units. This figure was then converted to millimetres.

Measurement of Brain Monoamine Concentrations

Measurement of monoamine (noradrenaline, 5-HT, and dopamine) concentrations in the whole cortex and midbrain [containing the Edinger-Westphal nucleus responsible for mydriasis, see (19,26)] was performed using HPLC with electrochemical detection (HPLC-ECD) to confirm the selectivity and extent of the DSP-4 lesioning procedure. Rats were killed by cervical dislocation; whole cortices and midbrains were rapidly dissected and snap frozen in liquid nitrogen. Tissues were then prepared and HPLC analyses were carried out according to a method previously described (13).

Statistics

The following statistical analyses were carried out:

a. The time course for clonidine-induced mydriasis used a repeated-measures analysis of covariance (ANCOVA) (29) carried out on pupil diameter, with treatment as the factor and baseline measurement as the covariate, followed by Dunnett's multiple comparisons test (8) with Sidak adjustment (27).
b. The dose–response for clonidine-induced mydriasis used Dunnett's multiple comparisons test.
c. The study of the effects of α- and β-adrenoceptor antagonists on pupil diameter used one-way analysis of variance (ANOVA) (1) with treatment as the factor, followed by Dunnett's multiple comparisons test when a saline group had more than one related antagonist group. If a saline group had only one related antagonist group, then this was equivalent to carrying out a Student's unpaired *t*-test (2).
d. The effects of α- and β-adrenoceptor antagonists on clonidine (0.03 mg/kg, IP)-induced mydriasis and the effects of α_2-adrenoceptor antagonists on methamphetamine (0.75 mg/kg, IP)-induced mydriasis were statistically analysed using the same methods as in c. The ID_{50} values for idazoxan and yohimbine were calculated by linear regression of change in pupil diameter plotted against log dose of antagonist. The 95% confidence limits were calculated by Fieller's theorem.
e. The increase in pupil diameter over its own basal value induced by methamphetamine (0.75 mg/kg, IP) was statistically evaluated using Student's paired *t*-test.
f. The effect of DSP-4 lesioning on pupil diameter and on brain monoamine concentrations used Student's unpaired *t*-test.
g. The effects of DSP-4 on the mydriasis induced by clonidine (0.03 mg/kg, IP) or methamphetamine (0.75 mg/kg, IP) were statistically evaluated using the tests carried out in c and d.

RESULTS

Time Course for Clonidine-Induced Mydriasis in Rats

Rats were injected with either clonidine (0.03 mg/kg) or saline, and pupil diameter measurements were taken as described in the Method section. Clonidine (0.03 mg/kg) markedly increased pupil diameter compared with saline-treated controls (Fig. 1) with an increase of 53% 10 min after injection. Pupil diameter was still significantly greater than the control values after 30 min, but had returned to control values by 60 min. In all subsequent experiments, pupil diameter was measured immediately prior to and 10 min after clonidine administration.

The Dose–Response Relationship for Clonidine-Induced Mydriasis in Rats

Injection of clonidine (0.001–1 mg/kg) produced a sigmoidal, dose-dependent increase in rat pupil diameter (Fig. 2). A maximum increase of 235% was observed after administration of 1 mg/kg, and the ED_{50} (dose producing a 50% increase in pupil diameter) for this response was 0.088 mg/kg (95% confidence limits 0.058–0.135 mg/kg) (Fig. 2).

The Effects of α- and β-Adrenoceptor Antagonists on Pupil Diameter and on Clonidine-Induced Mydriasis

Rats were injected with idazoxan (0.03–3 mg/kg, 30 min), yohimbine (0.03–3 mg/kg, 30 min), prazosin (1 or 3 mg/kg,

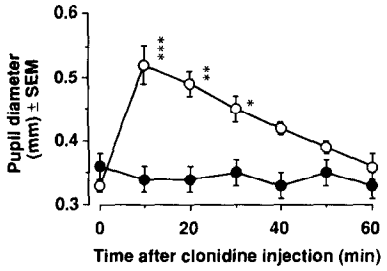

FIG. 1. Time course for clonidine-induced mydriasis in conscious rats. Animals were given an intraperitoneal injection of clonidine (0.03 mg/kg) (○) or saline (2 ml/kg) (●). Pupil diameter was measured immediately before drug injection and at 10-min intervals for up to 60 min thereafter. Values are the mean pupil diameter (mm) ± SEM (for groups of eight rats) plotted against the time after clonidine injection (min). Significantly different compared with saline controls: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ using a repeated-measures ANCOVA followed by Dunnett's multiple comparisons test with Sidak adjustment.

60 min), pindolol (1 or 3 mg/kg, 45 min), or saline prior to injection of clonidine (0.03 mg/kg). Pupil diameter measurements were taken immediately prior to injection of α- or β-adrenoceptor antagonists, immediately before clonidine administration and 10 min after injection of this α_2-adrenoceptor agonist. Pretreatment with the α_2-adrenoceptor antagonists, idazoxan (1 or 3 mg/kg) and yohimbine (1 or 3 mg/kg), caused a dose-related decrease in pupil diameter (Table 1). However, the α_1-adrenoceptor antagonist, prazosin, and the β-adrenoceptor antagonist, pindolol, were without effect (Table 1).

Idazoxan (0.03–3 mg/kg) and yohimbine (0.03–3 mg/kg) evoked a dose-dependent attenuation of the mydriasis produced by clonidine (0.03 mg/kg; Fig. 3). The ID_{50} (dose producing a 50% inhibition of clonidine mydriasis) values for idazoxan and yohimbine were 0.43 mg/kg (95% confidence

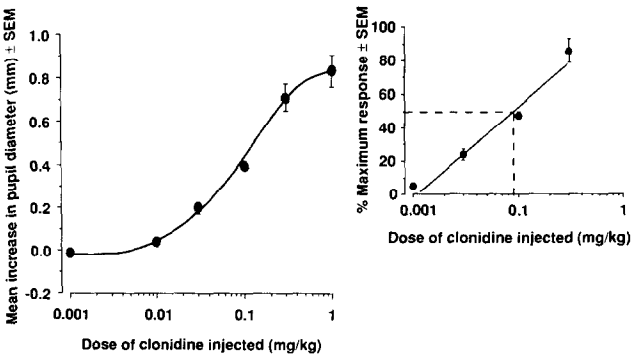

FIG. 2. Dose-response relationship for clonidine-induced mydriasis in conscious rats. Animals were injected intraperitoneally with clonidine (0.001–1 mg/kg). Pupil diameter was measured immediately prior to clonidine injection and 10 min later. The main figure shows the mean increase in pupil diameter (mm) ± SEM 10 min after clonidine injection. Each point represents the mean response of eight rats. Doses of clonidine ≥ 0.03 mg/kg produced a highly significant ($p < 0.01$) increase in pupil diameter compared with saline-treated controls using Dunnett's multiple comparisons test. The inset shows a linear transformation of the data and the ED_{50} for this response was 0.088 mg/kg (95% confidence limits 0.058–0.135 mg/kg).

TABLE 1

EFFECTS OF α- AND β-ADRENOCEPTOR ANTAGONISTS ON PUPIL DIAMETER

Antagonist	Dose (mg/kg)	Pretreatment (min)	Change in Pupil Diameter (mm)
Combined vehicle control	–	30	−0.009 ± 0.010
Idazoxan	1	30	−0.100 ± 0.014*
	3	30	−0.139 ± 0.018*
Yohimbine	1	30	−0.078 ± 0.008*
	3	30	−0.125 ± 0.014*
Combined vehicle control	–	60	−0.006 ± 0.012
Prazosin	1	60	−0.029 ± 0.012
	3	60	−0.015 ± 0.007
Combined vehicle control	–	45	−0.034 ± 0.014
Pindolol	1	45	−0.028 ± 0.013
	3	45	−0.019 ± 0.015

Rats were dosed with adrenergic antagonists for the pretreatment periods stated. Values are mean change in pupil diameter (mm) ± SEM (for groups of eight rats) between injection of antagonist and end of pretreatment period. Control groups ($n = 8$) have been combined for the sake of clarity.

Significantly different from own vehicle control, $*p < 0.01$ using one-way ANOVA followed by Student's unpaired t-test or Dunnett's multiple comparisons test when appropriate.

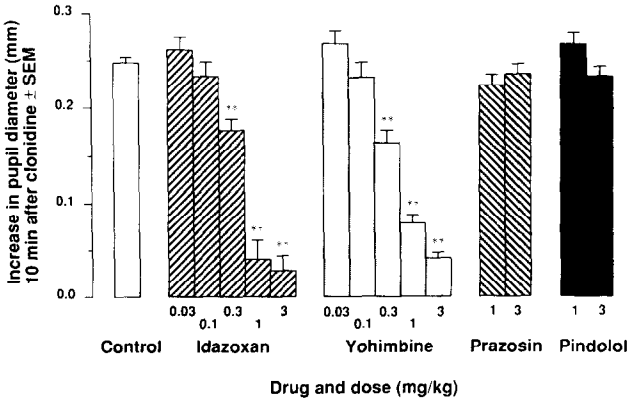

FIG. 3. The effects of α- and β-adrenoceptor antagonists on clonidine (0.03 mg/kg, IP)-induced mydriasis. Rats were injected intraperitoneally with one of the following: idazoxan (0.03–3 mg/kg, 30 min), yohimbine (0.03–3 mg/kg, 30 min), prazosin (1 or 3 mg/kg, 60 min), pindolol (1 or 3 mg/kg, 45 min), or saline. All groups of rats were injected with clonidine (0.03 mg/kg, IP) after the pretreatment period shown in parentheses. Pupil diameter was measured immediately before and 10 min after clonidine administration. Values are the mean increase in pupil diameter induced by clonidine (mm) ± SEM for groups of eight rats. Control groups ($n = 7$–8) have been combined for the sake of clarity. Significantly different from own control $**p < 0.01$ using one-way ANOVA followed by Student's unpaired t-test or Dunnett's multiple comparisons test when appropriate.

limits 0.32–0.56 mg/kg) and 0.53 mg/kg (95% confidence limits 0.41–0.68 mg/kg), respectively.

Methamphetamine-Induced Mydriasis and Its Reversal by α_2-Adrenoceptor Antagonists

When rats were injected with methamphetamine (0.75 mg/kg), this produced a 56% increase in pupil diameter [mean basal pupil diameter (mm) ± SEM = 0.327 ± 0.012, mean pupil diameter (mm) 10 min after methamphetamine ± SEM = 0.511 ± 0.011; $p < 0.001$ Student's paired t-test]. This increase was markedly attenuated by pretreating the rats with either idazoxan (1 mg/kg) or yohimbine (1 mg/kg) 30 min before administration of methamphetamine (0.75 mg/kg) [mean increase in pupil diameter (mm) ± SEM 10 min after methamphetamine: saline pretreated 0.184 ± 0.008; idazoxan pretreated 0.080 ± 0.009 (57% decrease, $p < 0.01$); yohimbine pretreated 0.090 ± 0.017 (52% decrease, $p < 0.01$)].

The Effects of DSP-4 Lesioning on Pupil Diameter and on the Mydriasis Induced by Clonidine or Methamphetamine

Rats were injected with DSP-4 (50 mg/kg) or saline (2 ml/kg) twice over a 6-h period, 30 min after zimeldine (10 mg/kg) pretreatment. Seventy-two hours later, rats were given clonidine (0.03 mg/kg) or methamphetamine (0.75 mg/kg). Pupil diameter measurements were made immediately prior to clonidine or methamphetamine injection and again 10 min later.

DSP-4 treatment produced a 100% depletion of noradrenaline in the whole cortex of rats used in the clonidine and methamphetamine mydriasis experiments, with no effect on 5-HT concentrations and a large, though only marginally significant, increase in dopamine levels (methamphetamine mydriasis experiment only). This lesioning agent also caused a highly significant overall 67% reduction of midbrain (containing Edinger-Westphal nucleus) noradrenaline concentrations (Table 2) with a small, but significant, decrease in dopamine concentrations (clonidine mydriasis experiment only) and no effect on 5-HT levels.

Administration of DSP-4 (50 mg/kg × 2) induced significant decreases in the pupil diameter of rats used in clonidine and methamphetamine mydriasis experiments (22% and 31%, respectively) (Table 3).

DSP-4 lesioning did not affect the increase in pupil diameter produced by clonidine (Table 3). However, pretreatment with this noradrenergic neurotoxin evoked a marked 72% reduction in the mydriatic response to methamphetamine (Table 3).

TABLE 3

EFFECTS OF DSP-4 TREATMENT ON THE MYDRIASIS INDUCED BY CLONIDINE OR METHAMPHETAMINE

Treatment	Basal Diameter (mm)	Treated diameter (mm)	Increase in Diameter (mm)
Clonidine mydriasis			
Saline	0.439 ± 0.014	0.652 ± 0.015	0.213 ± 0.011
DSP-4	0.344 ± 0.021*	0.562 ± 0.030	0.218 ± 0.017
Methamphetamine mydriasis			
Saline	0.417 ± 0.023	0.624 ± 0.018	0.207 ± 0.012
DSP-4	0.289 ± 0.023*	0.346 ± 0.036	0.058 ± 0.015†

Rats were given DSP-4 or saline using the protocol outlined in the Method section. Seventy-two hours after initial DSP-4 injection, clonidine (0.03 mg/kg, IP) or methamphetamine (0.75 mg/kg, IP) was administered and mydriasis experiments were performed. Values are expressed as pupil diameter (mm) ± SEM for groups of 8–12 rats. Basal diameter = pupil diameter measured immediately before clonidine or methamphetamine injection. Treated diameter = pupil diameter measured 10 min after clonidine or methamphetamine injection. Increase in diameter = treated diameter − Basal diameter.

Significantly different from saline control basal diameter, $*p < 0.01$ using Student's unpaired t-test.

Significantly different from saline control, $\dagger p < 0.01$ using one-way ANOVA followed by Student's unpaired t-test.

DISCUSSION

The majority of studies of the mydriatic responses induced by α_2-adrenoceptor agonists such as clonidine have been carried out in anaesthetised animals (4,9,18,21–23). Although these experiments have been invaluable in determining the role of central α_2-adrenoceptors in mediating mydriasis, there have been relatively few considerations (6,28) of the potential interference caused by general anaesthesia. It has previously been found (6) that the mydriatic responses of conscious rats to clonidine were attenuated at higher levels of ocular illumina-

TABLE 2

CONCENTRATIONS OF NORADRENALINE, DOPAMINE, AND 5-HT IN RAT WHOLE CORTEX AND MIDBRAIN AFTER DSP-4 TREATMENT

	Noradrenaline			Dopamine			5-HT		
	Control	DSP-4	Depletion (%)	Control	DSP-4	Depletion (%)	Control	DSP-4	Depletion (%)
Clonidine mydriasis									
Whole cortex	96 ± 7	0 ± 0†	100	57 ± 6	72 ± 10	+26	324 ± 8	343 ± 9	+6
Midbrain	424 ± 18	131 ± 6†	69	174 ± 5	152 ± 8*	13	807 ± 19	802 ± 18	1
Methamphetamine mydriasis									
Whole cortex	146 ± 8	0 ± 0†	100	61 ± 9	112 ± 17*	+84	294 ± 13	271 ± 15	8
Midbrain	441 ± 12	160 ± 17†	64	151 ± 7	143 ± 5	5	739 ± 45	709 ± 48	4

Rats were given DSP-4 or saline using the protocol outlined in the Method section. Seventy-two hours later clonidine or methamphetamine mydriasis experiments were performed and then whole cortex and mid-brain samples prepared for HPLC analysis of monoamines. Values are mean monoamine concentrations expressed as ng/g tissue wet weight ± SEM for groups of 8–12 rats.

Significantly different from saline control, $*p < 0.05$, $\dagger p < 0.001$ using Student's unpaired t-test.

tion that the authors concluded to be a clear example of "physiological antagonism" (i.e., the light reflex is more pronounced in unanaesthetised than in anaesthetised rats). These findings were supported in other studies (17,28) that again found agents inducing mydriasis were far less efficacious in conscious animals under conditions of "moderate to normal" laboratory illumination. Clearly, although anaesthetised animals are more sensitive to the mydriatic effects of clonidine than their conscious counterparts, as we have demonstrated here, provided the ambient lighting used is of sufficiently low intensity, then the measurement of mydriasis in conscious rats is quite achievable. In this regard, we have confirmed the feasibility of measuring mydriasis in conscious rats under low light conditions as previously reported (6). The ED_{50} for clonidine-induced mydriasis in conscious rats of 0.088 mg/kg is similar to the value of 0.054 mg/kg that we obtained in conscious mice (14). The duration of clonidine-induced mydriasis in the rat was approximately 30 min in our experiments, which is very similar to that found previously in conscious rats under similar lighting conditions (6) and again is almost identical to the result obtained using conscious mice (14).

Pharmacological characterization of the clonidine-induced mydriasis with α- and β-adrenoceptor antagonists revealed that this response was potently and dose-dependently reversed by idazoxan and yohimbine, but unaltered by pretreatment with prazosin or pindolol. These findings are consistent with specific mediation by α_2-adrenoceptors and are in agreement with results previously obtained in both anaesthetised (4,16) and conscious rats (6). Earlier studies conducted in anaesthetised rats have shown that clonidine-induced mydriasis is mediated exclusively by α_2-adrenoceptors located in the CNS (16), and it has also been suggested from experiments in anaesthetised cats and rats that these receptors are located in the Edinger-Westphal complex, where they control parasympathetic tone to the iris (16,26). It has also been postulated that the α_2-adrenoceptors responsible for the induction of mydriasis in several species, including the rat, are not inhibitory prejunctional autoreceptors controlling noradrenaline efflux, but are part of the population that is postsynaptic to noradrenergic neurones (15,16,18,20). Consistent with this hypothesis, we have demonstrated that the response can also be evoked in conscious rats by the administration of methamphetamine, which increases noradrenaline outflow by inhibiting reuptake and possibly by enhancing release (3,10,24). The resulting mydriasis is also specifically mediated by α_2-adrenoceptors because it is potently reversed by idazoxan and yohimbine. A postsynaptic location for the α_2-adrenoceptors mediating mydriasis was confirmed using DSP-4 to selectively lesion noradrenergic neurones. DSP-4 treatment totally depleted noradrenaline in the whole cortex and markedly decreased the concentration of this monoamine (by 64%) in the midbrain (containing the Edinger-Westphal nucleus responsible for the mydriasis response). In the same rats, this neurotoxin also reduced the mydriasis induced by the noradrenaline releasing agent, methamphetamine (by 72%). The response to the α_2-adrenoceptor agonist clonidine, however, remained unaltered. This confirms that in conscious rats mydriasis is also mediated by postsynaptic α_2-adrenoceptors. Methamphetamine acts indirectly on these receptors by increasing noradrenaline outflow; conversely, clonidine, by acting directly on postsynaptic α_2-adrenoceptors, is unaffected by the neurotoxin. This conclusion is consistent with our findings in conscious mice (15).

Overall, the results presented show that pupil dilatation can be measured in conscious rats under low light conditions. Pharmacological characterization revealed that mydriasis is specifically mediated by postsynaptic α_2-adrenoceptors in the CNS and it can be initiated either directly using specific agonists or indirectly with noradrenaline releasing agents. This physiological response, therefore, provides a simple and rapid model for assessing postsynaptic α_2-adrenoceptor function in rat brain that avoids any potential interference by general anaesthesia and could be used to simultaneously study other biochemical and/or behavioural parameters in either acute or chronic studies.

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