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Autoradiographical and behavioural effects of a chronic infusion of antisense to the α_{2D}-adrenoceptor in the rat

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1 The aims of this study were, firstly to use receptor autoradiography to investigate the effect of antisense oligonucleotides to the α_{2D}-adrenoceptor on receptor binding and, secondly to measure behavioural and physiological parameters to determine whether the chronic antisense infusion had any effect on α_2-adrenoceptor function *in vivo*.

2 A 3 day infusion of antisense to the α_{2D}-adrenoceptor significantly reduced specific [^{3}H]-RX821002 binding in the septum (20–30%) and anterior hypothalamic area (20–30%). β-Adrenoceptor expression was unaffected in those brain areas examined, indicating the antisense knockdown was specific to the α_2-adrenoceptors.

3 On the second day of the infusion, the hypothermic response to UK 14,304 was significantly attenuated in the antisense-treated group compared with both vehicle and mismatch controls. The effect was fully reversible and a similar decrease in body temperature was observed in all the treatment groups 4 days after the end of infusion.

4 During the second day of the infusion, the effects of UK 14,304 on behaviour were reduced in the antisense-treated rats, but were not significantly lower than those of the vehicle and mismatch, UK 14,304 controls. These trends were not observed 4 days after the end of the infusion.

5 In conclusion, antisense has been shown to selectively knockdown α_2-adrenoceptor expression in specific brain areas. The consequence of this knockdown is a significant attenuation of UK 14,304-induced hypothermia and a reduction in its sedative actions. These changes were fully reversed 4 days after the end of the infusion.

Keywords: Antisense oligonucleotides; α_2-adrenoceptors; [^{3}H]-RX821002; receptor autoradiography; UK 14,304; behavioural profile; temperature; mydriasis

Abbreviations: BST, bed nucleus stria terminalis; CP, caudate putamen; DSP-4, N-(2-chloroethyl)-N-ethyl-2 bromo-benzylamine; FC, frontal cortex; LSN, lateral septal nucleus; SHy, septohypothalamic nucleus

Introduction

α_2-Adrenoceptors are known to mediate many physiological and pharmacological effects in the central nervous system (Szabadi & Bradshaw, 1996; Nutt & Pinder, 1996). The functional role of α_2-adrenoceptors has been investigated using selective α_2-agonists which produce bradycardia, hypotension, hypothermia, sedation, inhibition of motor function, mydriasis and antinociception in animals (Maze & Tranquilli, 1991; French, 1995; MacDonald *et al*., 1997). α_2-Adrenoceptors have also been shown to play an important role in the regulation of neurotransmitter release, inhibiting both neuronal firing and the release of noradrenaline and other neurotransmitters (Correa-Sales *et al*., 1992; Dennis *et al*., 1987). These functions are known to be mediated through both pre- and post-synaptically located α_2-adrenoceptors (French, 1995).

Pharmacological and molecular biological studies have shown that four distinct subtypes of α_2-adrenoceptors exist. In the rat three subtypes of α_2-adrenoceptor have been identified and cloned (Bylund, 1988; MacKinnon *et al*., 1994). These are the α_{2B}, α_{2C} and α_{2D} subtypes (Zeng & Lynch, 1991). Gene sequencing suggests that the rat α_{2D}-adrenoceptor is a species homologue of the human α_{2A}-adrenoceptor although they are pharmacologically distinct (Renouard *et al*., 1994; Simonneaux *et al*., 1991). The lack of highly selective ligands for the different subtypes has limited studies of their individual functions. However, correlations between the affinities of a range of agonists and antagonists for α_2-adrenoceptors in cortical membranes prepared from DSP-4 (N-(2-chloroethyl)-N-ethyl-2 bromo-benzylamine) lesioned rats and α_2-adrenoceptor subtypes in prototypical tissues suggests that post-synaptic α_2-adrenoceptors in the rat cortex are predominantly of the α_{2D}-subtype (Heal *et al*., 1995a; Bylund, 1995). Furthermore, correlations between functional effects of α_2-agonists and antagonists and their affinities for the different α_2-adrenoceptor subtypes suggests that pre-synaptic α_{2D}-adrenoceptors mediate the effects on noradrenaline release and motor function whereas post-synaptically located α_{2D}-adrenoceptors mediate their effects on pupil diameter (Trendelenburgh *et al*., 1993; Millan *et al*., 1994; Limberger *et al*., 1995; Heal *et al*., 1995a).

The roles of the different α_2-adrenoceptor subtypes can be further investigated using antisense technology and transgenic animals. Antisense oligonucleotides target a specific mRNA and inhibit expression of a specific protein through sequence specific hybridization. The technique is therefore a valuable pharmacological tool for investigating the function of a receptor protein, particularly when highly selective ligands are not yet available (Robinson *et al*., 1997).

In the present study we have used receptor autoradiography to investigate the effect of a 3 day i.c.v. infusion of an antisense sequence specific to α_{2D}-adrenoceptors on α_2-adrenoceptor expression in rat brain. This sequence has previously been

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shown to elevate systolic blood pressure in rats (Nunes, 1995). In order, to explore the effects of the antisense treatment on α_2-adrenoceptor function *in vivo*, we have investigated the effects of the α_2-adrenoceptor agonist UK 14,304 on general behaviour patterns, body temperature and pupil diameter in animals given i.c.v. infusion of either vehicle, antisense or the mismatch control. These particular parameters were monitored as α_2-agonists have been shown to produce hypoactivity (Heal *et al*., 1989; Correa-Sales *et al*., 1992), hypothermia (Lin *et al*., 1981; Bill *et al*., 1989) and mydriasis (Heal *et al*., 1995a) in laboratory animals. The challenge with UK 14,304 was given on the second day of the infusion and also 4 days after the end of infusion to explore whether any functional changes were reversible and not simply a result of non-specific toxicity.

Methods

Animals and surgical procedures

Male Wistar rats (Bantin and Kingman, U.K., University of Bristol; Charles River, U.K., Knoll Pharmaceuticals) weighing 270–310 g were housed individually on a 12 h light/dark cycle (lights on at 07.00 h) at 21 ± 1°C and 55% humidity. Rats were allowed free access to food, standard rat diet, and water. At the start of the experiment rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) for receptor autoradiography or isoflurane (5% for induction, 2–3% maintenance) for behavioural testing. Animals were stereotaxically implanted with an i.c.v. cannulae to the left lateral ventricle (0.92 mm caudal to bregma, 1.4 mm lateral and 3.5 mm below the surface of the dura, Paxinos & Watson, 1986). Phosphorothioate oligonucleotides were administered using osmotic mini-pumps, combined with a brain infusion kit (Alzet, Charles River, U.K., 1 μl h^{-1}) which were primed overnight with vehicle, antisense or mismatch oligonucleotide, included as a toxicity control (8 μg μl^{-1} h^{-1}, i.c.v.). The mini-pump was located in the midscapular region and the cannulae fixed to the skull using a bone screw and dental cement. The whole region was then closed using surgical staples. Correct placement of the cannulae was confirmed when the brains were sectioned or using a dye injection at the end of the behavioural experiments.

Receptor autoradiography

Receptor autoradiography was performed on brain sections taken from animals 4 days after the start of the infusion. Rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and perfused, intra-cardiac, with ice-cold phosphate buffered, physiological saline (250 ml, 10 mM, pH 7.4). Brains were removed rapidly over ice and immediately frozen in isopentane at -40°C. Sections (12 μm) were cut on a cryostat at -15°C and thaw-mounted onto gelatin subbed glass slides and stored at -70°C until use.

Prior to binding, sections were thawed and pre-washed in assay buffer (Tris HCl 50 mM, $MgCl_2$ 1 mM, pH 7.4) for 30 min at 24°C. Sections were then dried and incubated with 1 nM [^{3}H]-RX821002 (53 Ci mmol^{-1}), to label α_2-adrenoceptors, in fresh assay buffer in triplicate for 30 min at 24°C. Corresponding triplicate sections were incubated in identical conditions with the addition of 10 μM rauwolscine to determine the non-specific binding. Assays were terminated by two 20 s rinses in ice-cold assay buffer followed by a dip wash in distilled water and rapid drying in a stream of cool air (Hudson *et al*., 1992). For [^{3}H]-dihydroalprenolol binding, to label β-adrenoceptors, sections were thawed then incubated with 1 nM [^{3}H]- dihydroalprenolol (95 Ci mmol^{-1}), using 1 μM (−)propranolol to define the non-specific binding, for 30 min in assay buffer (Tris-HCl 0.17 M, $MgCl_2$ 10 mM, pH 7.7). The assay was terminated by two 10 min washes in fresh assay buffer then dipped in distilled water followed by rapid drying (Biegon & Israeli, 1986).

Bound radioactivity was determined by liquid scintillation counting and the remaining sections were apposed to tritium sensitive film (Hyperfilm, Amersham) with known tritium standards (Amersham) for 4 weeks before photographic development and analysed using computer assisted densitometry (Quantimet 970, Cambridge Instruments, U.K.). Binding densities were determined for each brain region by quantitative analysis of triplicate experiments with a minimum of five animals. Brains were sectioned and analysed using a Paxinos & Watson (1986) stereotaxic atlas to facilitate structural recognition.

Behavioural and physiological measurements

All behavioural and physiological measurements were made in the same animals between 09.00 h and 16.00 h on day 2 of the infusion and 4 days after the end of the infusion, by an experimenter who was unaware of the treatment each animal received. All experiments were carried out under low light conditions to enable pupil diameters to be measured. The treatment groups were as follows: control (vehicle i.c.v., saline i.p.), vehicle (vehicle i.c.v., UK 14,304 i.p.), antisense (antisense i.c.v., UK 14,304 i.p.) and mismatch (mismatch i.c.v., UK 14,304).

Behavioural observations

On each experimental day animals were acclimatized to the test room in their home cages for 1 h prior to testing. Rats were injected with saline or UK 14,304 (1 mg kg^{-1}, i.p., 1 ml kg^{-1}) and immediately placed individually into eight spatially-adjacent cages identical to the home cage (dimensions $33 \times 20 \times 19$ cm high) containing sawdust bedding. Behavioural observations began 5 min after placing the rats in the cage using a time sampling technique. The behaviour of each rat was recorded for 5 s every 60 s over the test period for a total of 60 min, divided into 15 min periods (adapted from Reinstein & Isaacson, 1977). Two rats from each group were tested concurrently on each day and the results were pooled over 3 days so that each treatment group contained six rats. The presence of a behavioural component was counted only once during the 5 s to give a maximum response of 15 for each 15 min time period and the scores accumulated to give total scores for 0–60 min.

Body temperature

Body temperature was measured using a rat rectal probe (inserted 2 cm) and digital thermometer (Model Bat-12, Sensortek; both obtained from CP Instruments, Bishop's Stortford, U.K.). Ambient temperature was maintained at 21 ± 1°C and 55% humidity. Rectal temperature measurements were made 30 min before the start of the experiment, immediately prior to dosing (0 reading) and 60 and 120 min following administration of vehicle or UK 14,304.

Pupil diameter

Pupil diameter was measured using a Wild M1 binocular microscope containing a graticule scale in one eyepiece. The

microscope was illuminated by a Swift light box with the voltage set at 6 V (light intensity 450 lux) The procedure was carried out in an artificially lit room of light intensity of 20 lux. Animals were first acclimatized to the lighting conditions in the test room for at least 30 min before the first reading. Each rat was then gently restrained and held under the light source and its pupil diameter was read off the graticule scale in eyepiece units. This value was then converted into millimetres as described by Heal *et al.* (1995b). Pupil diameter readings were taken 30 min before the start of the experiment immediately prior to dosing (0 reading) and 30, 60 and 120 min following administration of vehicle or UK 14,304.

Drugs

The oligonucleotide sequence was that previously described by Nunes (1995), to the RG20 gene mRNA (antisense 5′-ATCCGGCTGCAGGGAGCC-3′, mismatch 5′-ATC-CAGCGGCTGGGAGCC-3′). The oligonucleotides were fully modified phosphorothioate oligonucleotides synthesised by Dr L. Hall, Biochemistry Department, University of Bristol, U.K. The following drugs were purchased from: [^{3}H]-RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) and [^{3}H]-dihydroalprenolol (Amersham International), rauwolscine hydrochloride (Sigma), (−)propranolol hydrochloride (Sigma) and UK 14,304 (5-bromo-N-(4,5-

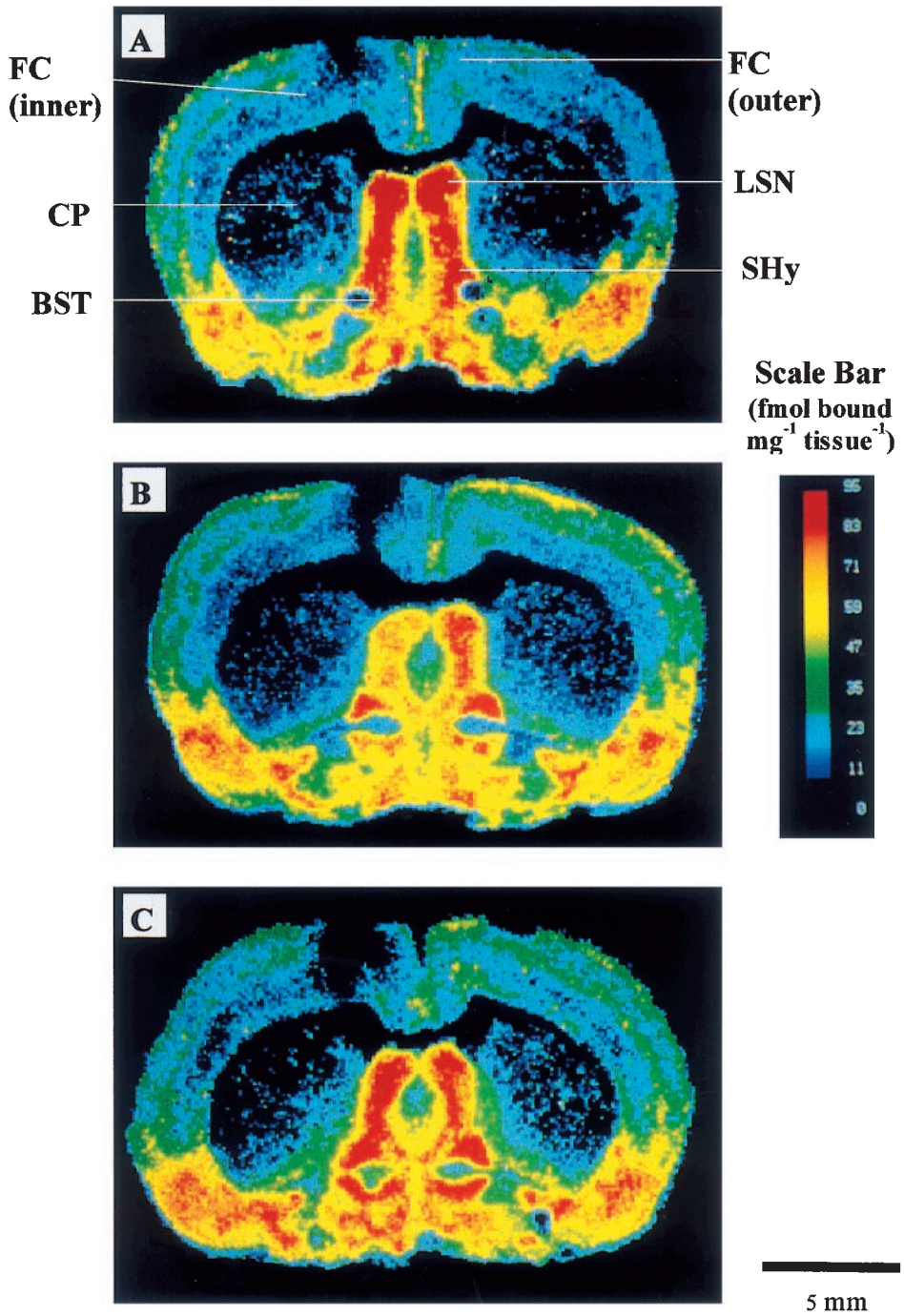

Figure 1 Representative pseudo-colour coded autoradiograms from animals treated with vehicle (A), antisense (B) and mismatch (C). The reduction in [^{3}H]-RX821002 binding in the septum is particularly clear on the left side in the antisense-treated rat. Sections shown were taken approximately −0.4 mm from bregma.

dihydro-1H-imidazol-2-yl)-6-quinoxalinamine, Research Biochemicals Inc.).

Statistics

Receptor autoradiography data are expressed as mean values for each brain region ± s.e.mean and compared statistically using Student's unpaired, two-tailed *t*-test. Temperature and pupil diameter data are shown as mean values at each time point separately and were analysed using a one-way analysis of variance with treatment as factor. *Post hoc* comparisons were then made using the Dunnett's *t*-test. Behavioural profile data were analysed using a non-parametric approach, results are expressed as group median scores ± range and the Cochran–Manzel–Haenszel test was used to test the association between behaviour and treatment. In all statistical analysis a value of $P<0.05$ was considered significant.

Results

Receptor autoradiography

Compared with both the vehicle and the mismatch controls a significant reduction in [^{3}H]-RX821002 binding was seen in the antisense-treated rats in specific brain regions (Figures 1 and 2, and Table 1). In the lateral septal nucleus a reduction of 20–30% was observed, this was significantly different on the left side of the brain i.e. the same side as the infusion. A significant reduction was also seen in the anterior hypothalamic area on both the left and right sides. In addition, other brain areas such as the anterior amygdaloid area and paraventricular nucleus, also showed a reduction in binding of approximately 15% but this failed to reach a level of significance compared to both the vehicle and mismatch treated animals (Table 1). No significant reduction in [^{3}H]-RX821002 binding was seen in other brain structures measured including the frontal cortex, caudate putamen and globus pallidus. Histological examination using cresyl violet staining revealed no difference between the treatment groups and no signs of neuronal loss. [^{3}H]-Dihydroalprenolol binding was not significantly different in any of the brain areas examined for any of the treatment groups on the left or the right side of the brain. In particular, there was no reduction in binding to those brain regions where [^{3}H]-RX821002 was significantly reduced i.e. the lateral septal nucleus and anterior hypothalamic area (data not shown).

Behavioural observations

During the second day of the infusion, UK 14,304 (1 mg kg^{-1}, i.p.) significantly inhibited general activity, locomotion and

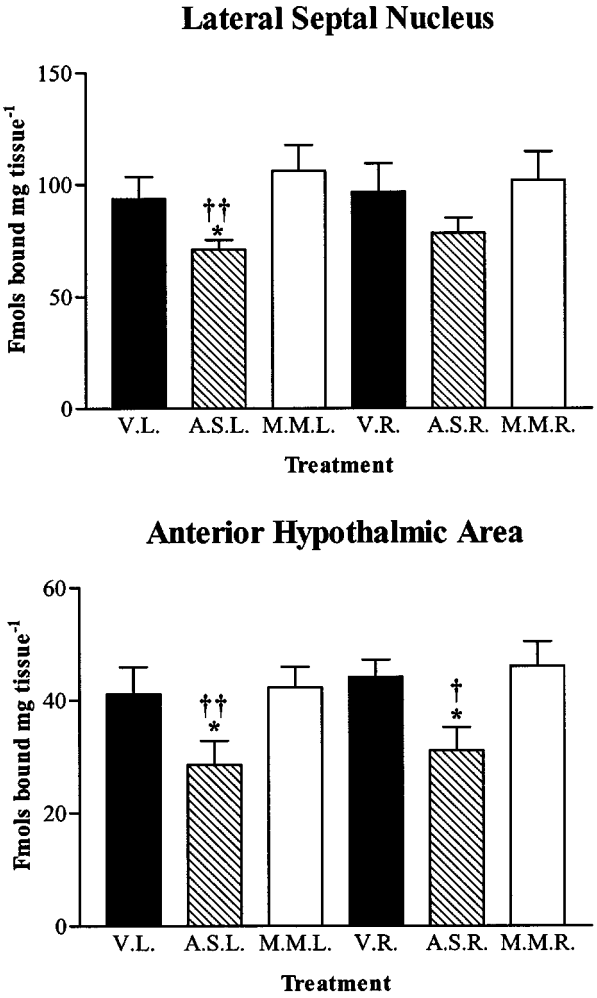

Figure 2 Graphs representing brain areas quantified showing a significant reduction in [^{3}H]-RX821002 binding of 20–30% magnitude. Results shown are specific binding for vehicle left (V.L.), vehicle right (V.R.), antisense left (A.S.L.), antisense right (A.S.R.), mismatch left (M.M.L.) and mismatch right (M.M.R.). *$P<0.05$, **$P<0.01$ compared with vehicle, †$P<0.05$, ††$P<0.01$ antisense compared with mismatch, $n=5-6$ animals per group.

Table 1 Quantified data for [^{3}H]-RX821002 autoradiography from rat brain following a 3 day infusion of vehicle, antisense or mismatch (sequence described by Nunes, 1995)

Region	*Left*			*Right*		
	Vehicle	*Antisense*	*Mismatch*	*Vehicle*	*Antisense*	*Mismatch*
Frontal cortex (outer)	21.67 ± 2.74	21.67 ± 2.19	22.51 ± 3.08	21.69 ± 2.39	22.02 ± 2.08	23.63 ± 3.01
Frontal cortex (inner)	14.36 ± 1.80	14.26 ± 1.66	17.50 ± 2.20	14.09 ± 1.21	13.86 ± 1.03	16.42 ± 1.41
Caudate putamen	35.02 ± 4.25	31.61 ± 3.84	37.05 ± 2.73	36.25 ± 4.94	32.70 ± 4.11	40.50 ± 3.93
Globus pallidus	9.66 ± 1.07	11.47 ± 1.13	13.45 ± 1.50	10.18 ± 1.58	10.98 ± 1.57	11.65 ± 0.81
Septohypothalamic nuc.	53.17 ± 4.13	51.03 ± 4.63	58.78 ± 1.62	51.57 ± 5.73	52.45 ± 3.91	57.67 ± 0.89
Lateral septal nuc.	93.75 ± 10.59	71.17 ± 4.10*‡	106.18 ± 12.56	96.65 ± 13.98	78.37 ± 6.68	101.99 ± 13.72
Ant. hypothalamic area	41.15 ± 4.27	28.47 ± 3.84*‡	42.25 ± 3.30	44.12 ± 2.75	31.05 ± 3.66*†	46.02 ± 3.92
Ant. amygdaloid area	62.20 ± 9.72	49.67 ± 6.58	64.15 ± 6.66	57.87 ± 5.20	59.54 ± 6.95	69.89 ± 2.71
Bed. nuc. stria. med	95.74 ± 6.15	85.42 ± 14.19	91.23 ± 14.27	97.88 ± 4.05	81.58 ± 13.67	96.77 ± 11.44
Paraventricular nuc.	34.85 ± 3.00	29.83 ± 2.32	33.26 ± 3.94	ND	ND	ND
Reticular thalamic nuc.	22.68 ± 2.82	19.21 ± 0.99†	33.26 ± 3.94	21.30 ± 1.83	19.10 ± 0.67	25.46 ± 2.08

Results shown as mean value in fmol bound mg wet tissue^{-1} ± s.e.mean. *$P<0.05$, **$P<0.01$ compared to vehicle, †$P<0.05$, ‡$P<0.01$ antisense compared to mismatch, $n=5-6$ animals per group.

Table 2 Observational analysis of the behavioural effects of UK 14,304

Behaviour (frequency score)	*Day*	*Treatment*			
		Control	*Vehicle*	*Antisense*	*Mismatch*
Locomotion	2	4 (0–9)	0* (0–1)	2 (0–6)	0* (0–3)
	7	3 (2–5)	0** (0–2)	0** (0–2)	1** (0–1)
Wall climbing	2	3.5 (0–15)	0* (0–1)	0 (0–8)	0* (0–2)
	7	5.5 (1–21)	0** (0–0)	0.5*† (0–1)	0.5* (0–7)
Rearing	2	0.5 (0–3)	0 (0–0)	0 (0–0)	0 (0–3)
	7	2 (0–6)	0* (0–0)	0* (0–1)	0* (0–1)
Grooming	2	5.5 (1–11)	0.5* (0–3)	1.5* (0–3)	0* (0–2)
	7	5 (2–8)	0** (0–0)	0** (0–3)	0** (0–1)
Scratching	2	0.5 (0–6)	0 (0–0)	0 (0–0)	0 (0–0)
	7	0.5 (0–2)	0 (0–0)	0 (0–1)	0 (0–3)
Eating	2	0.5 (0–1)	0 (0–2)	0 (0–1)	0.5 (0–2)
	7	0.5 (0–4)	0* (0–0)	0 (0–2)	0 (0–2)
Activity	2	15 (2–43)	1* (0–5)	4 (0–17)	1.5* (0–10)
	7	20 (12–31)	0** (0–2)	1** (0–5)	1.5** (0–10)
Stationary awake	2	41.5 (17–58)	59* (55–60)	56* (43–60)	58.5* (50–60)
	7	37 (22–48)	60** (58–60)	59** (55–60)	58.5** (50–60)
Stationary asleep	2	0 (0–7)	0 (0–0)	0 (0–0)	0 (0–0)
	7	0 (0–5)	0 (0–0)	0 (0–0)	0 (0–0)
Low body posture	2	0 (0–0)	35* (0–60)	4 (0–54)	36** (1–60)
	7	0 (0–3)	46.5* (0–57)	29* (0–60)	24.5 (0–60)
Sniffing	2	17 (7–33)	0.5** (0–6)	10.5 (2–25)	2 (0–17)
	7	19.5 (17–42)	0.5** (0–13)	4** (0–15)	5* (5–10)

Results shown are median scores ± range for six animals per group. *$P<0.05$, **$P<0.01$ compared to vehicle, †$P<0.05$ antisense compared to mismatch.

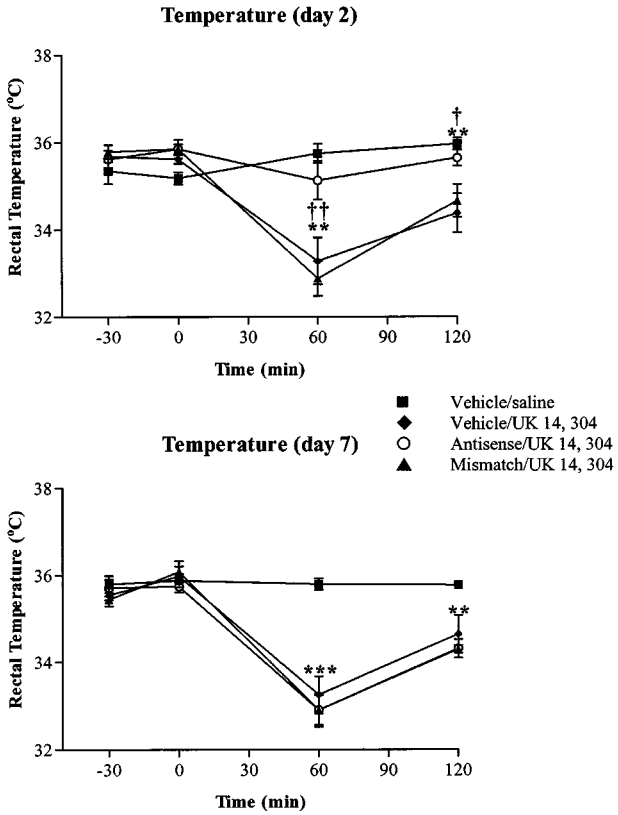

Figure 3 The top graph shows the effect of UK 14,304 (1 mg kg^{-1}, i.p.) on rectal temperature during the second day of infusion. Results shown as mean temperature ± s.e.mean, *$P<0.05$, **$P<0.01$ antisense compared to vehicle infused, UK 14,304 treated animals and †$P<0.05$ antisense compared to mismatch infused, UK 14,304 treated animals ($n=6$ animals per group). This effect was reversed by 4 days after the end of infusion when all the treatment groups were significantly reduced (***$P<0.001$ compared to the vehicle infused, saline controls, $n=6$ animals per group).

increased low body posture in animals given vehicle or mismatch i.c.v. The decrease in activity was predominantly due to decreased locomotion, wall-climbing and grooming. The behavioural effects of UK 14,304 were not significantly attenuated in animals infused with antisense to α_2-adrenoceptors compared to animals given an i.c.v. infusion of vehicle or mismatch and UK 14,304. However, UK 14,304 did not significantly reduce locomotion, wall-climbing, general activity or sniffing, or produce a significant increase in low body posture in the antisense-treated group (compared to the vehicle infused, saline group) suggesting that some reduction in α_2-adrenoceptor function had occurred. These trends were not observed 4 days after the end of the vehicle or oligonucleotide infusions. A summary of the data for the individual behaviours on day 2 and day 7 is shown in Table 2.

Body temperature

UK 14,304 (1 mg kg^{-1}, i.p.) produced a significant reduction in rectal body temperature in vehicle and mismatch i.c.v. infused groups compared to vehicle infused, saline controls (Figure 3). A reduction in body temperature of 2.9°C ($P<0.01$, $n=6$) in the vehicle-treated group and 2.5°C ($P<0.01$, $n=6$) in the mismatch-treated group was observed at 60 min compared to saline control. However, the hypothermic effect of UK 14,304 was significantly attenuated in the antisense-treated rats. The body temperatures of these animals were similar to those in the control group at the 60 and 120 min reading and significantly higher than the vehicle ($P<0.01$, $n=6$) and mismatch-treated ($P<0.01$, $n=6$) groups that received UK 14,304 (Figure 3). Four days after the end of infusion, UK 14,304 produced a similar hypothermic response in the vehicle, mismatch and antisense-treated groups, i.e. the inhibitory effects of the antisense treatment on the decrease in body temperature induced by UK 14,304 was fully reversible (Figure 3).

Pupil diameter

A significant increase in pupil diameter was seen in the antisense-treated animals during base-line readings at time −30 and 0 min on day 2 (0.14 mm vs vehicle $P<0.01$, 0.16 mm vs mismatch $P<0.05$, $n=6$). There was no significant difference in baseline readings between any of the treatment groups 4 days after the end of infusion.

During day 2 of the infusion, UK 14,304 (1 mg kg^{-1}, i.p.) caused a significant increase in pupil diameter at 30 min after injection, in the vehicle (0.7 mm, $P<0.05$, $n=6$), antisense (0.64 mm, $P<0.05$, $n=6$) and mismatch-treated (0.56 mm, $P<0.05$, $n=6$) animals compared to saline-treated, vehicle controls (Figure 4). An increase of magnitude 130–172% was seen 30 min after injection and pupil diameter was still significantly higher in all the groups at 60 min (53–93%) compared to saline. At 120 min pupil diameter was still significantly elevated in the antisense-treated animals (0.23 mm, $P<0.05$ vs control). Subsequently, 4 days after the end of infusion, UK 14,304 produced a significant increase of 0.88 mm in vehicle, 0.70 mm in antisense and 0.52 mm in mismatch rats at 30 min. Pupil diameter in all three treatment groups were still elevated at 60 min but returned to baseline values by 120 min (Figure 4). There was no significant difference between any of the UK 14,304-treated groups at any time point.

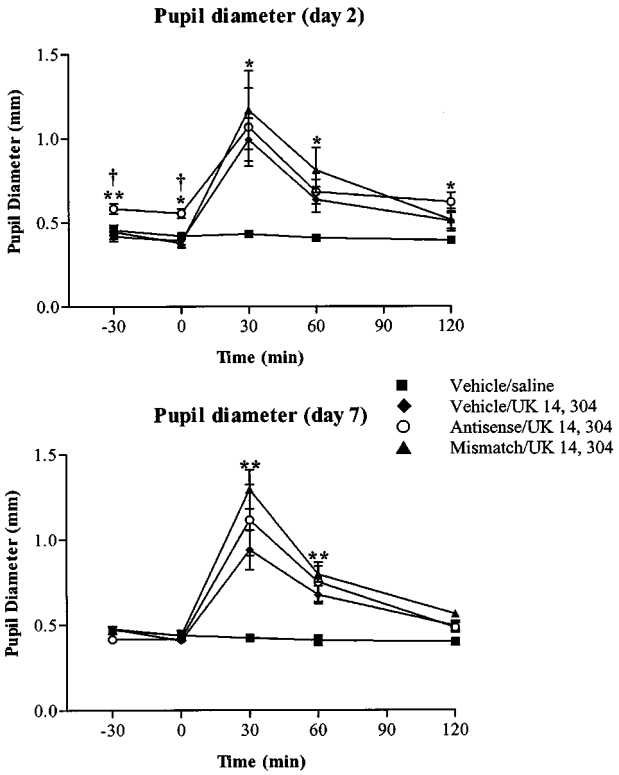

Figure 4 The effect of UK 14,304 (1 mg kg^{-1}, i.p.) on pupil diameter in rats treated with antisense to the α_{2D}-adrenoceptor, mismatch and vehicle compared to vehicle infused saline controls. The top graph shows the results for pupil diameter during the second day of the infusion. Results shown as mean temperature ± s.e.mean, $*P<0.05$, $**P<0.01$ antisense compared to vehicle infused UK 14,304 treated animals and $\dagger P<0.05$ antisense compared to mismatch infused, UK 14,304-treated animals ($n=6$ animals per group). The bottom graph shows the results for 4 days after the end of infusion, $**P<0.01$ antisense compared to vehicle infused saline treated animals ($n=6$ animals per group). No significant difference was seen between the UK 14,304-treated groups.

Discussion

This study has used an antisense sequence described by Nunes (1995) to further investigate the role of α_2-adrenoceptor subtypes in the rat. The major findings of this study are that a 3 day infusion with antisense to α_{2D}-adrenoceptor produced a significant decrease in α_2-adrenoceptor expression in specific brain areas. A reduction of 20–30% was achieved in the lateral septal nucleus and anterior hypothalamic area, with a greater effect seen on the same side as the infusion. In addition, there was also a reduction in [^{3}H]-RX821002 binding in the anterior amygdaloid area and the paraventricular nucleus (10–15%). In the vehicle and mismatch control-treated animals the autoradiographic distribution for α_2-adrenoceptor was similar to that previously shown with this ligand (Hudson *et al.*, 1992). The reduction in binding was specific to the antisense-treated animals since β-adrenoceptor expression, quantified using [^{3}H]-dihydroalprenolol, was not altered by the antisense treatment. This is the first report that antisense to α_{2D}-adrenoceptor reduces α_2-adrenoceptor expression in the CNS. Previous studies using antisense to the α_{2D}-adrenoceptors have shown that it inhibits agonist challenged behaviour in the rat (Mizobe *et al.*, 1996; Hunter *et al.*, 1997a) and increase systolic blood pressure. Data from the present study, with the results from the histological staining, show that this antisense sequence does not cause any neurological toxicity and the receptor knockdown is not as a result of neuronal loss. This antisense sequence can selectively knockdown α_2-adrenoceptor binding and can, therefore, be used to identify the functional consequence of the inhibition of expression of this receptor in specific brain regions.

There are two factors which may have influenced the results of the autoradiography experiments. Firstly, distribution of the oligonucleotide and secondly, the equal affinity of [^{3}H]-RX821002 for all the α_2-adrenoceptor subtypes (Hudson *et al.*, 1992). Distribution studies using a radio-labelled oligonucleotide have shown that following an i.c.v. infusion the oligonucleotide is not distributed to all brain areas and is limited to structures close to the site of infusion (Ogawa *et al.*, 1995; Yaida & Nowak, 1995). Noradrenergic cell bodies of neurones projecting to the frontal cortex are located in the locus coeruleus and an i.c.v. antisense infusion is unlikely to reach this brain structure at sufficient levels to inhibit expression. The brain structures shown in this study to be affected by the antisense were all located close to the ventricle and a greater effect was seen on the same side as the infusion.

The lateral septal nucleus has a high level of α_{2D}-adrenoceptor mRNA and the anterior hypothalamic area also expresses high levels of this receptor subtype mRNA (Zeng & Lynch, 1991). However, the striatum contains predominantly the α_{2C}-adrenoceptor subtype and the caudate putamen and globus pallidus showed no effect with the α_{2D}-antisense treatment, which is further evidence of the specificity of this antisense sequence. Antisense targeting the α_{2C}-adrenoceptor subtype has been shown to locally inhibit the expression of this receptor in the striatum following i.c.v. administration (Lu & Ordway, 1997). Using a non-subtype selective ligand to label receptor density, such as [^{3}H]-RX821002 the antisense-mediated knockdown of receptor protein in some brain areas may have been masked or detection reduced by the presence of additional α_2-subtypes. Further studies using selective ligands to label α_{2D}-adrenoceptors when they become available, will help to clarify this issue.

The level of receptor knockdown achieved using a 3 day infusion varied from 10–30% in this study. Experiments using antisense specific to other receptors, i.e. opioid, dopamine,

neuropeptide Y-Y1 receptor and *N*-methyl-D-aspartate receptor subunit (NMDA-R1), have shown similar levels of receptor knockdown and a reduction of this magnitude was sufficient to inhibit function (Lai *et al*., 1995; Wahlestedt *et al*., 1993a,b; Weiss *et al*., 1997). Although the turnover rate for α_2-adrenoceptor is thought to be approximately 4 days (Adler *et al*., 1985) antisense oligonucleotide have been shown to inhibit function rapidly after the start of treatment (Nunes, 1995; Mizobe *et al*., 1996). Both dopamine (Weiss *et al*., 1993, Zhou *et al*., 1994) and opioid (Standifer *et al*., 1994) antisense experiments have also shown changes in function earlier than expected from receptor binding data and a poor correlation with changes in function. One possible explanation for these results is that the newly synthesized receptors are those functionally active (Qin *et al*., 1995) and it is these that are first affected by the inhibition of receptor synthesis (Weiss *et al*., 1997).

Our study demonstrates a number of important features necessary to *in vivo* antisense experiments, which include; the specific decrease in receptor protein, the inhibition of specific receptor mediated function and the reversibility of any functional inhibition. An agonist challenge study with UK 14,304 was used in the functional tests during and after the infusion to determine the consequence of receptor knockdown on agonist-induced responses and its reversibility. The sedative effects of UK 14,304 are mediated by post-synaptic α_2-adrenoceptors on the locus coeruleus and pre-synaptic inhibition of neurotransmitter release in the frontal cortex (Correa-Sales *et al*., 1992; Dennis *et al*., 1987). In this study only a small non-significant reduction in sedation was observed, suggesting locus coeruleus α_2-adrenoceptor function was not significantly affected. In contrast, Mizobe *et al*. (1996) observed that local administration of antisense to the locus coeruleus significantly and reversibly inhibited the hypnotic response to another α_2-agonist dexmedetomidine. This discrepancy most likely reflects the location of administration of the antisense. Direct infusion of an antisense oligonucleotide over the locus coeruleus is more likely to affect the receptors in this region than administration into the lateral ventricle. Studies using knockout mice lacking the α_{2D}-adrenoceptor subtype also showed the α_{2D}-subtype to be the predominant subtype involved in mediating sedation (Hunter *et al*., 1997b).

The hypothermic response to UK 14,304 was significantly attenuated by the antisense infusion. The significant reduction in α_2-adrenoceptor expression in the anterior hypothalamic area, shown in the autoradiographic studies, and the attenuation in the hypothermic response seen in the antisense-treated rats suggests this response may be mediated by the α_{2D}-adrenoceptor. Studies using the α_{2D}-adrenoceptor transgenic mice have also shown a reduction in the hypothermic response to dexmedetomidine. However, there is still some discrepancy over the subtype responsible for mediating the hypothermic action of α_2-adrenoceptor agonists, as a study using knockout mice and mice overexpressing the α_{2C}-adrenoceptor by Sallinen *et al*. (1997) showed a reduction in the hypothermic response to dexmedetomidine implicating the α_{2C}-adrenoceptor in this response. The reason for this discrepancy is, as yet, unclear, but may be an effect of the genetic alteration and development of knockout mice lacking a specific receptor subtype (Gerlai, 1996).

α_2-Adrenoceptor-induced mydriasis has been shown to be mediated by post-synaptic α_{2D}-adrenoceptors in the Edinger-Westphal nucleus (Koss, 1986, Heal *et al*., 1995a,b) which is located in the mid- to hindbrain region. Previous studies suggest that oligonucleotides given i.c.v. may not penetrate this brain region (Yaida & Nowak, 1995). The spontaneous change in pupil diameter seen in the antisense-treated group on day 2 at baseline readings is still being investigated but may be due to an affect of the antisense distant from the Edinger-Westphal nucleus. The spontaneous increase was not observed 4 days after the end of the infusion, suggesting it is fully reversible and a consequence of the antisense and not as a result of non-specific toxicity.

In conclusion, the present study confirms the use of antisense oligonucleotides as a tool for investigating the function of specific receptor proteins. These results show that antisense to the α_{2D}-adrenoceptor, administered i.c.v., can selectively knockdown receptor expression in specific brain areas. The functional consequence of this knockdown is the attenuation of the hypothermic effect, and a small reduction in the behavioural depression induced by UK 14,304 in the rat. However, UK 14,304-induced mydriasis, a response thought to be mediated by α_{2D}-adrenoceptors was not inhibited by the antisense treatment, suggesting that such studies may still be limited by the restricted distribution of the oligonucleotides in the central nervous system.

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