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The effect of clonidine on the naltrexone-induced withdrawal response in morphine-treated guinea-pigs

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Abstract

Rapid opioid withdrawal induced by naltrexone is now used as a treatment for heroin addiction. The α_2-adrenoceptor agonist, clonidine, is currently used in clinical practice to reduce opioid withdrawal in humans. However, few studies have been reported on its effectiveness for this purpose. Guinea-pigs were made dependent and tolerant to morphine using a 3-day chronic morphine regimen (total 410 mg kg^{-1} morphine base), and injected with either clonidine (0.1 mg kg^{-1}, s.c.) or saline, 1 h before induction of withdrawal with naltrexone (15 mg kg^{-1}, s.c.). Withdrawal behaviours were measured for 90 min and animals were then euthanased and the brains removed. The presence of the immediate early gene protein product, c-Fos, was detected using immunohistochemical techniques. Clonidine reduced the number of head/body shakes, but had no effect on the total withdrawal behaviour score. In the CNS, clonidine increased the number of Fos-LI neurons in the central amygdala. In conclusion, the modest effect of clonidine in the present experiments suggests that the efficacy of clonidine in humans undergoing naltrexone-induced opioid withdrawal requires further investigation.

Introduction

Clonidine has been reported to suppress opioid withdrawal in humans (Gold et al 1978), rats (Tseng et al 1975) and guinea-pig isolated ileum (Collier et al 1981; Chahl 1985). Clonidine is now used in clinical practice to reduce the symptoms of rapid opioid withdrawal induced by naltrexone in heroin addicts.

In rats, clonidine has been found to reduce immunosuppression, ptosis, diarrhoea and weight change (West et al 1999) induced by opioid withdrawal. However, clonidine does not attenuate all behavioural responses induced by opioid withdrawal. Earlier studies reported that aggression (Gianutsos et al 1976) and jumping (Tseng et al 1975) during opioid withdrawal were increased by clonidine. A more recent study has shown that the effect of clonidine on withdrawal jumping in rats is dose-dependent, with low doses attenuating jumping behaviour and high doses increasing it (Pinelli & Trivulzio 1998).

Clonidine is an α_2-adrenoceptor agonist that has several actions in common with the opioids, including inhibition of adenylyl cyclase activity, inhibition of voltage-gated Ca^{2+} channels, opening of inwardly rectifying K^+ channels and inhibition of neurotransmitter release (for review see Harrison et al 1998). Thus, during withdrawal, clonidine has been proposed to substitute for the activity of opioids at those synapses that have both opioid receptors and α_2-adrenoceptors (Chahl 1985).

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Induction of an opioid withdrawal-like response in rats by electrical stimulation of the locus coeruleus, which contains a dense population of noradrenergic cell bodies and receptors, was effectively blocked by clonidine (Taylor et al 1988). However, localized injection of clonidine into this area did not abolish the opioid withdrawal response, indicating that its site of action was not solely in the locus coeruleus. It is interesting to note that the actions of clonidine to decrease withdrawal signs are not limited to the CNS. ST-91, a non-lipophilic and hence peripherally acting α_2-adrenoceptor agonist, had a similar, albeit reduced, effect on withdrawal to clonidine (Taylor et al 1988). Indeed, clonidine was found to be most effective when given peripherally, in contrast to intracerebral infusion, presumably because it interacted with both the central and peripheral α_2-adrenoceptors. However, some behaviours, such as the wet-dog shake in rats, which are presumably entirely central in origin, were not affected by peripheral administration of α_2-adrenoceptor agonists (Taylor et al 1988). Therefore, it would appear that both central and peripheral mechanisms are involved in the action of clonidine to inhibit opioid withdrawal.

Guinea-pigs are a suitable model for studies on opioid mechanisms since they exhibit a similar response to opioids as humans (Bot & Chahl 1996) and have a similar distribution of central opioid receptors (Mansour et al 1995). Since there have been few studies in species other than rats, the present study was carried out to determine the effect of clonidine on the naltrexone-induced response following chronic morphine treatment in guinea-pigs.

Materials and Methods

Animals

The experiments were authorized by the Animal Care and Ethics Committee of the University of Newcastle (an accredited research establishment) (Animal Research Authority 689 0201). Adult tri-coloured guinea-pigs of either sex, weighing 290–650 g, were used. The guinea-pigs were housed in a room maintained at 23°C and on a 12-h light–dark cycle. Food and water were freely available and a vitamin C supplement was added to the drinking water daily.

Drugs

The drugs used were: morphine sulphate (David Bull Laboratories, Rydalmere, NSW, Australia); morphine tartrate (David Bull Laboratories); clonidine hydrochloride (Catapress; Boehringer Ingelheim Pty Ltd, Artarmon, NSW, Australia); naltrexone hydrochloride (Sigma, Castle Hill, NSW, Australia); sodium pentobarbitone (Lethabarb; Virbac (Australia) Pty Ltd, Peakhurst, NSW, Australia).

Treatment of animals

The guinea-pigs were allowed to acclimatize to their environment for 7 days before the start of treatment. The animals were handled 3 days before treatment to familiarize them with the handler.

All guinea-pigs were made morphine tolerant and dependent by twice daily administration of subcutaneous injections of morphine sulphate (30 mg mL^{-1}) or morphine tartrate (120 mg/1.5 mL) in increasing doses for 3 days (total dose 410 mg kg^{-1} morphine base). On the first day, each animal received doses of 10 and 20 mg kg^{-1}, on the second day, 40 and 60 mg kg^{-1}, and on the third day, 80 and 100 mg kg^{-1}. In the morning and afternoon of the second day and the morning of the third day, the guinea-pigs received 10-mL intraperitoneal injections of warm saline (approx. 37°C) to counter dehydration induced by chronic morphine treatment. On the morning of the fourth day, all animals were given a final dose of morphine (100 mg kg^{-1}) followed immediately by the test drug (clonidine or saline) and placed into activity cages for a 1-h habituation period.

There were three treatment groups with four animals in each group: group 1 received clonidine (0.1 mg kg^{-1}, s.c.) before the 1-h habituation period, followed by naltrexone hydrochloride (15 mg kg^{-1}) after the habituation period; group 2 received saline (0.66 mL kg^{-1}, s.c) instead of clonidine, followed by naltrexone hydrochloride; and group 3 received clonidine as for group 1 animals, but they were not withdrawn and received saline (0.5 mL kg^{-1}, s.c.) instead of naltrexone hydrochloride. All animals were observed for 90 min by a trained observer who quantified withdrawal behaviours (digging, face-washing, head/body shake, rearing, grooming). Locomotor activity of guinea-pigs was measured in a modified animal cage (40 cm × 28 cm × 15 cm) equipped with a single infrared photocell and detector on the long axis, located 5 cm above the floor and 14 cm from either end of the cage. Every crossing of the beam at least 1.5 s apart was recorded on a digital counter. The time delay was chosen to avoid measurements of small body movements.

At 90 min after the naltrexone or saline injections, the animals were anaesthetized with sodium pentobarbitone (100 mg kg^{-1}, i.p.) and perfuse-fixed by transcardial

perfusion with heparinized (5000 U L^{-1}) phosphate-buffered saline (PBS; pH 7.4, 37°C) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 37°C). Brains were removed, post-fixed overnight, and transferred to a cryoprotectant solution of 30% sucrose and 0.5% paraformaldehyde in PBS at 4°C until they sank.

Localization of c-Fos

Coronal sections, 50 μm, were cut on a cryostat at −20°C. Free floating sections were washed twice in PBS, incubated for 20 min in 0.3% hydrogen peroxide, washed three times in PBS and incubated for 1 h at room temperature in a blocking solution (PBS containing 10% normal donkey serum (Sigma) and 0.1% sodium azide (Sigma)). They were then incubated with polyclonal rabbit c-Fos antisera (K-25; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:2000 (in PBS containing 1% normal donkey serum, 0.1% sodium azide and 0.075% Triton X-100 (BDH Chemicals Ltd, Poole, UK)) for 48 h at 4°C. The sections were washed three times in PBS, incubated for 1 h at room temperature in biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:1000 in PBS (0.1% normal donkey serum), washed three times in PBS and incubated for 1 h in avidin–biotin horseradish peroxidase complex (Vectastain Elite kit; Vector Laboratories Inc., Burlingame, CA). The sections were then washed three times in Tris-buffered saline (TBS; pH 7.4, 50 mM Tris-HCl, 150 mM NaCl). Bound antibody was visualized by addition of cold 3,3′-diaminobenzidine (DAB; Sigma) solution (TBS containing 0.033% DAB, 0.03% hydrogen peroxide, 0.004% nickel ammonium sulphate). The reaction was terminated with excess deionized water. Sections were washed three times in TBS, mounted on gelatin chrom-alum-coated slides, air-dried, dehydrated in increasing concentrations of ethanol (70%, 95%, 100%), cleared with Histolene (Fronine, Riverstone, NSW, Australia) and cover-slipped with Ultramount (Fronine). Cells in which the nucleus contained a dark brown/black stain were considered to have Fos-like immunoreactivity (Fos-LI).

Specificity of antibody

The c-Fos antibody (K-25; Santa Cruz) used in the study was raised against a conserved domain of c-Fos p62 of human origin, identical to mouse, rat and chicken sequences. This Fos antibody recognizes a conserved region common to several members of the Fos family, including c-Fos, Fos-B, and Fos-related antigens (Fra-1 and Fra-2). This antibody has been extensively characterized in immunohistochemical studies. Sections incubated without primary antibody developed no staining.

Microscopy

The slides were examined under a Zeiss Axioskop light microscope equipped with a camera lucida and Magellan V5.3 software (Paul Halasz, University of NSW, Australia). The microscope and computer were used to record the location of each cell containing Fos-LI, and to measure the areas of particular brain regions. The cell densities were then calculated as number of positive cells per unit area (cells mm^{-2}). The brain regions were determined by extrapolation from the rat atlas of Paxinos & Watson (1986).

Statistics

The data were analysed statistically using GraphPad Prism3 (GraphPad Software Inc.). Two-way analysis of variance for repeated measures, and Bonferroni post-tests were used to compare the behaviours and densities of Fos-LI cells between animal groups.

Results

Effect on behaviour

Chronic morphine-treated guinea-pigs that were pre-treated with clonidine (0.1 mg kg^{-1}, s.c.) and injected with saline rather than naltrexone showed no behaviour throughout the 90-min observation period. The animals

Table 1 Effect of clonidine on behavioural responses to naltrexone-induced morphine withdrawal in guinea-pigs.

Behaviour	Clonidine–naltrexone	Saline–naltrexone
Total behaviour scores	470±50.9	464±48.7
Locomotor counts	290±44.7	257±33.4
Digging	46.3±10.8	37.0±14.8
Face-washing	10.5±4.13	21.5±5.2
Head/body shakes	0.50±0.50***	4.75±1.0
Rearing	110±5.73	139±31
Grooming	6.00±2.80	5.75±2.25

Values are means±s.e.m. of total number of observations over 90 min from four animals. All animals were treated with morphine over a 3-day period and naltrexone-induced withdrawal was induced on the fourth day. The clonidine-treated group received 0.1 mg kg^{-1} clonidine, 1 h before naltrexone-induced withdrawal. The saline control group received 0.66 mL kg^{-1} saline, 1 h before withdrawal. ***$P < 0.001$.

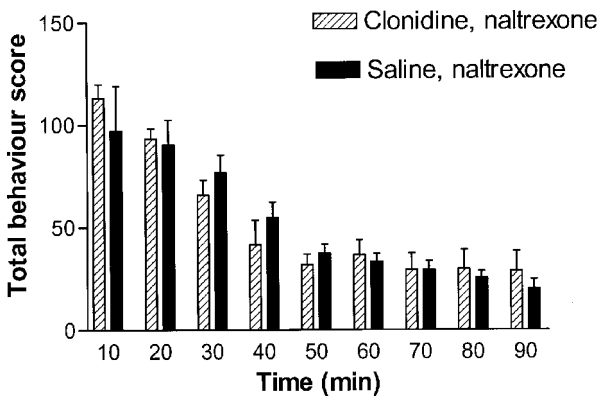

Figure 1 Effect of clonidine on total behaviour scores in guinea-pigs following naltrexone-induced morphine withdrawal. Histograms represent means±s.e.m. from four animals. All animals were injected with increasing doses of morphine over 3 days. Withdrawal was induced with naltrexone (15 mg kg^{-1}) on the fourth day. Responses of clonidine-treated animals (total of 0.1 mg kg^{-1} s.c., 1 h before naltrexone-induced withdrawal) are shown as hatched bars. Control animals, shown as closed bars, received 0.66 mL kg^{-1} saline subcutaneously, 1 h before naltrexone-induced withdrawal. Total behaviour scores were obtained by adding all the behaviour scores over successive 10-min periods.

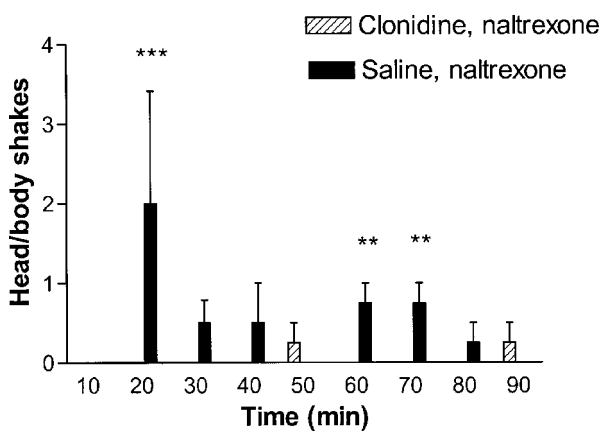

Figure 2 Effect of clonidine on head/body shakes in guinea-pigs following naltrexone-induced morphine withdrawal. Histograms represent means±s.e.m. from four animals. Refer to Figure 1 for treatment of animals. ***$P < 0.001$; **$P < 0.01$.

lay on their side during the observation period, but showed signs of aggression when disturbed. In contrast, guinea-pigs pre-treated with clonidine or saline and given naltrexone exhibited marked withdrawal behaviour. It was noted that clonidine-treated animals displayed "start–stop" morphine withdrawal behaviour, which comprised very intense bouts of activity interrupted by periods of quiescence, presumably because the animals were exhausted.

The total behaviour score of animals pre-treated with clonidine and withdrawn with naltrexone was not significantly different from that of animals pre-treated with saline (Table 1; Figure 1). The only individual behavioural score that was affected by clonidine was the number of head/body shakes, which was markedly reduced by clonidine ($P < 0.0001$; two-way analysis of variance) (Figure 2).

Effect on Fos-LI neuron density

Naltrexone-induced withdrawal increased the Fos-LI neuron density in clonidine pre-treated guinea-pigs compared with animals that did not receive naltrexone ($P < 0.0001$; two-way analysis of variance) (Table 2). The regions where naltrexone-induced withdrawal significantly increased Fos-LI neuron density were the nucleus accumbens core ($P < 0.05$; Bonferroni post-test), lateral septal nucleus ($P < 0.05$), central amygdala ($P < 0.001$), lateral habenula ($P < 0.01$), paraventricular hypothalamic nucleus ($P < 0.05$), interpeduncular nucleus ($P < 0.001$), ventral tegmental area ($P < 0.05$) and the dorsal raphe ($P < 0.01$) (Table 2, Figure 3).

Animals pre-treated with clonidine and withdrawn with naltrexone showed no significant difference in the overall Fos-LI neuron density, compared with animals that were pre-treated with saline and withdrawn with naltrexone ($P = 0.62$; two-way analysis of variance) (Table 2). However, animals pre-treated with clonidine displayed significantly increased Fos-LI neuron density in the central amygdala ($P < 0.001$; Bonferroni post-test) (Table 2; Figure 3).

Discussion

In this study, clonidine (0.1 mg kg^{-1}) significantly reduced the number of head/body shakes that occurred in guinea-pigs during naltrexone-induced withdrawal. The reduced number of head body/shakes is in agreement with the results of Taylor et al (1988) that clonidine infused into the locus coeruleus attenuated withdrawal-induced head/body shakes in the rat. This particular withdrawal sign has been found to be exclusively under the control of the locus coeruleus, an area that has a high density of α_2-adrenoceptors (Taylor et al 1988). Since other withdrawal behaviours were not reduced by clonidine, it is likely that the major effect of clonidine on the opioid withdrawal response in the guinea-pig directly or indirectly involved the locus coeruleus.

It is now widely accepted that Fos-LI is a useful marker of neuronal activation. The only significant effect

Table 2 Effect of clonidine on Fos-LI neuron density in the guinea-pig brain.

Brain region	Clonidine–naltrexone (cells mm^{-2})	Saline–naltrexone (cells mm^{-2})	Clonidine–saline (cells mm^{-2})
Cingulate cortex	283±89.3	210±45.0	124±37.5
Accumbens nucleus, core	446±90.8	356±40.0	153±37.9*
Accumbens nucleus, shell	352±80.0	371±102	263±41.3
Lateral septal nucleus	800±169	864±145	511±59.0*
Central amygdala	1350±126†††	968±223	0.00±0.00***
Paraventricular thalamic nucleus	408±30.0	475±34.6	205±36.5
Lateral habenula	696±87.1	776±51.2	375±99.5**
Supraoptic nucleus	1880±248	2050±385	1750±262
Paraventricular hypothalamic nucleus	1230±267	1300±169	954±71.0*
Interpeduncular nucleus	509±126	398±34.0	35.1±20.3***
Ventral tegmental area	255±60.2	280±60.0	0.00±0.00*
Substantia nigra, pars reticulata	108±25.1	186±44.0	22.4±14.7
Periaqueductal grey, dorsomedial	230±43.5	245±39.4	231±17.1
Periaqueductal grey, lateral	257±23.5	372±35.8	124±35.2
Periaqueductal grey, dorsolateral	222±23.0	311±34.7	207±31.6
Dorsal raphe	464±38.9	514±102	131±65.3**

Comparison of Fos-LI neuron density in brain regions of guinea-pigs treated with clonidine–naltrexone, saline–naltrexone and clonidine–saline. All animals were injected with increasing doses of morphine over 3 days. Withdrawal was induced with naltrexone (15 mg kg^{-1}) on the fourth day, except in animals treated with clonidine–saline, which received the equivalent volume of saline instead of naltrexone. Values represent means±s.e.m. of Fos-LI-positive neurons (cells mm^{-2}) from four animals. †††$P < 0.001$ clonidine–naltrexone vs saline–naltrexone. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ clonidine–naltrexone vs clonidine–saline.

of clonidine treatment on the density of Fos-LI neurons following morphine withdrawal was an increase in the central amygdala. It is unknown whether the Fos-LI neurons in the amygdala were excitatory or inhibitory. Previous reports have demonstrated both excitatory and inhibitory effects of clonidine on cell activity (reviewed by Pinelli & Trivulzio 1998). The increase in Fos-LI neuron density in the amygdala of clonidine-treated guinea-pigs following opioid withdrawal might have resulted from direct activation of α_2-adrenoceptors in the amygdala by clonidine. However, indirect activation from any of the large number of areas of the CNS, including the hypothalamus, hippocampus and cortical areas (McDonald 1992), from which the amygdala receives inputs, might also have occurred. Furthermore, it is possible that the increased density of Fos-LI neurons in the amygdala was a result of peripheral inputs related to hypotension that was presumably induced by clonidine. The central amygdala is involved in autonomic and endocrine regulation and in responses to fear and stress (Davis 1993). Thus, the increased density of Fos-LI neurons in the amygdala might have been a reflection of the increased aggression observed in guinea-pigs following clonidine treatment. It is noteworthy that increased aggression following clonidine has also been observed in the rat (Harrison et al 1998).

The modest effect of clonidine on the withdrawal response in guinea-pigs was unexpected in light of the widespread use of clonidine in treating opioid withdrawal in humans (Pozzi et al 2000). It is unlikely that the dose of clonidine (0.1 mg kg^{-1}) was inadequate since after administration of clonidine, the animals were seen to lie on their sides before the induction of withdrawal indicating that they were hypotensive and that the dose was effective. This effect of clonidine on guinea-pigs precluded the use of higher doses. Furthermore, the marked reduction of head/body shake withdrawal behaviour by the dose of clonidine used indicated that the dose was effective.

It might be argued that the doses and routes of administration of naltrexone and clonidine used in the present experiments were not comparable with those used in humans undergoing opioid withdrawal. Naltrexone (15 mg kg^{-1}) administered subcutaneously following 3-day treatment of guinea-pigs with morphine, has been used extensively in the laboratory as a model of opioid withdrawal since a robust, but submaximal, withdrawal response is reliably induced (L. A. Chahl,

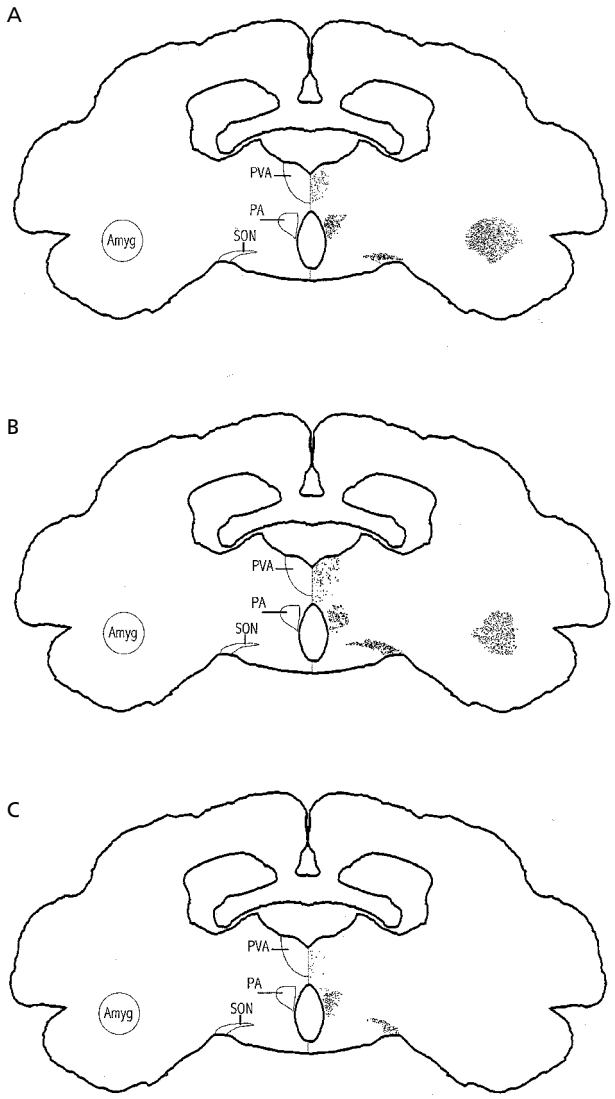

Figure 3 Camera lucida drawings representative of the distribution of Fos-LI neurons in the paraventricular thalamic nucleus (PVA), paraventricular hypothalamic nucleus (PA), supraoptic nucleus (SON) and the central amygdala (Amyg) in sections of guinea-pig brain following naltrexone-induced morphine withdrawal with (A) and without (B) clonidine treatment. Refer to Figure 1 for treatment of animals. Note the greater density of Fos-LI neurons in the central amygdala of the clonidine-treated guinea-pig withdrawn with naltrexone compared with the saline-treated withdrawn animal. No Fos-LI neurons were found in morphine-treated animals given clonidine, but not withdrawn with naltrexone (C).

unpublished observations). Furthermore, the dose of clonidine (0.1 mg kg^{-1}) was in the range used by other investigators using small animals (e.g. Pinelli & Trivulzio 1998). Nevertheless, the possibility of species variation between guinea-pigs and humans in response to clonidine must be considered. It is difficult to translate the effect of clonidine on head/body shakes in guinea-pigs to human withdrawal. Indeed, withdrawal signs observed in small animals might not adequately reflect the subjective emotional component of human withdrawal, which might be affected by clonidine.

In conclusion, the results from the present study suggest that the efficacy of clonidine, alone and in combination with other drugs commonly used in humans undergoing naltrexone-induced opioid withdrawal, requires further investigation.

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