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Sensitization to morphine withdrawal in guinea-pigs

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Abstract

The aim of this study was to determine whether sensitization occurred to morphine withdrawal. Guinea-pigs were treated twice daily with increasing doses of morphine (10–100 mg/kg s.c.) for 3 days followed by injection of morphine 100 mg/kg on the fourth day. Sixty min after the last morphine injection, animals were withdrawn from morphine with naltrexone, 15 mg/kg s.c., and locomotor activity and all other behaviours scored over 90 min. Animals were then rested for 3 days. This procedure was repeated twice over the next 2 weeks. Control animals were treated with saline for the first two treatment cycles. Guinea-pigs subjected to three cycles of morphine withdrawal showed a significant increase in the total number of withdrawal behaviour counts over the 90-min observation period following the third cycle of withdrawal compared with the first and second withdrawal cycles. However, locomotor activity, a major sign of morphine withdrawal in guinea-pigs, was not significantly increased. Fos-LI was markedly increased in the repeatedly withdrawn animals in several brain regions, including amygdala, dorsal striatum, thalamus, ventral tegmental area, and ventrolateral periaqueductal gray area. It is concluded that sensitization to morphine withdrawal occurs in guinea-pigs.

Keywords: Morphine withdrawal; Opioid; Naltrexone; Sensitization; Fos; Guinea-pig

1. Introduction

It is now well established that repeated administration of morphine to rats induces sensitization to its motor stimulant effects (e.g., Kalivas and Duffy, 1987). It is also established that sensitization to opioids directly affects rewarding pathways in the brain, which play a role in drug seeking behaviour (Koob and Le Moal, 1997). However, despite extensive investigation of the morphine withdrawal response, no studies appear to have investigated whether sensitization occurs to morphine withdrawal, although it has recently been shown that sensitization occurs to ethanol withdrawal in mice (Veatch and Becker, 2002). Investigation of the sensitizing effect of repeated withdrawal from drugs of abuse is important since many human addicts undergo repeated episodes of withdrawal between drug administrations.

Morphine withdrawal produces behavioural activation and wide-spread expression of the inducible transcription factor, *c-fos*, and Fos-related proteins in the brain of both rats (Hayward et al., 1990; Erdtmann-Vourliotis et al., 1998; Georges et al., 2000) and guinea-pigs (Chahl et al., 1996). In the present study the behaviours of animals and the distribution of Fos-like immunoreactivity (Fos-LI) in the central nervous system following a single episode of morphine withdrawal were compared with those following three cycles of withdrawal. Guinea-pigs were used in these experiments, because like humans and unlike rats, they do not exhibit a motor stimulant response to morphine (Bot et al., 1992). They also have a distribution of brain opioid receptors that is more similar to human than rat (Mansour et al., 1988), and exhibit a marked and readily quantifiable antagonist-induced withdrawal from morphine (Chahl et al., 1996).

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2. Materials and methods

2.1. Treatment of animals

Ethics approval for the following experiments was obtained from the Animal Care and Ethics Committee of the University of Newcastle, an approved research establishment.

Eight male guinea-pigs, weighing between 400 and 750 g at the beginning of the experiment, were housed in individual cages, maintained at room temperature on a 12/12 h light/dark cycle, and allowed free access to food and water containing a vitamin C supplement. Animals were weighed daily throughout the experiment. The guinea-pigs were divided randomly into two groups: those that were subjected to one cycle of morphine withdrawal and those that were subjected to three cycles of morphine withdrawal. Guinea-pigs subjected to three cycles of morphine withdrawal were given one s.c. injection of morphine twice daily in the morning and afternoon during 3 days and one injection on the fourth day. To induce dependence and maintain effectiveness the doses of morphine were increased over the 3 days. For each cycle of morphine treatment the following doses of morphine were given: 10 mg/kg and 20 mg/kg on day 1, 40 mg/kg and 60 mg/kg on day 2, 80 mg/kg and 100 mg/kg on day 3, and 100 mg/kg on day 4. One hour after the last morphine injection on day 4, animals were given 15 mg/kg of the opioid antagonist, naltrexone hydrochloride s.c., to induce morphine withdrawal. This high dose of naltrexone was chosen because earlier unpublished experiments (Chahl, unpublished) showed that this dose produced maximum withdrawal in guinea-pigs following the high doses of morphine used in these experiments. It was considered important to obtain maximum withdrawal following the morphine treatment so that changing sensitivity to naltrexone itself would be less likely to be a variable. Following naltrexone injection the behaviour of the animals was observed for 90 min. The animals were then rested for 3 days, after which the above procedure was repeated twice (total of three cycles). Guinea-pigs subjected to one cycle of withdrawal were given equivalent volumes of saline instead of morphine for the first two cycles followed by naltrexone hydrochloride, 15 mg/kg, at the end of each cycle of saline treatment. During the third cycle these animals were given morphine instead of saline and withdrawn with naltrexone following the same procedure as for the animals subjected to three cycles of withdrawal.

Following the last 90-min observation period the animals were given an intraperitoneal injection of a lethal dose of sodium pentobarbitone (100 mg/kg). Ninety minutes was chosen as this is the time of peak Fos expression following neuronal activation (Morgan and Curran, 1991). The animals were then perfuse-fixed with 0.1 M phosphate-buffered saline (PBS, pH 7.4, 37 °C) containing 5000U/l heparin, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, post-fixed for 24 h at 4 °C and placed in 30% sucrose solution in 0.5% paraformaldehyde at 4 °C for cryoprotection until the brains sank.

2.2. Measurement of behaviour

Locomotor and other behavioural activities of guinea-pigs during withdrawal were measured in a modified animal cage equipped with a single infrared photocell and detector on the longitudinal axis (Bot et al., 1992). A digital counter recorded every crossing of the infrared beam at least 1.5 s apart and a single pulse record was made simultaneously on a chart recorder. Activity scores were obtained from the total number of counts over successive 10-min intervals throughout the experiment. The frequency of other behaviours characteristic of morphine withdrawal in guinea-pigs such as digging, face washing, rearing, head/body shake, scratching, vocalizing, sneezing, chewing, and stretching were recorded by a trained observer for each 10-min interval throughout the 90-min period following naltrexone injection.

2.3. Immunohistochemistry

Coronal sections, 50 μm, were cut on a cryostat at −17 °C and washed in PBS. Free floating sections were incubated in PBS containing 0.3% hydrogen peroxide for 20 min, washed in PBS, and incubated in 10% normal donkey serum (NDS) in PBS, containing 0.1% sodium azide, and 0.075% Triton X-100 for 30 min at room temperature. The sections were then incubated with rabbit Fos antiserum (c-Fos (K-25), Santa Cruz Biotechnology), diluted 1:2000 in 1% NDS in PBS containing 0.075% Triton X-100 and 0.1% sodium azide, for 48 h at 4 °C. The sections were washed with PBS three times for 15 min each, and then incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) diluted 1:1000 in 0.1% NDS in PBS containing 0.075% Triton X-100 and 0.1% sodium azide, for 1 h at room temperature. The sections were then washed with PBS three times, incubated with avidin–biotin–peroxidase complex (ABC, Vectastain Elite ABC kit, Vector Laboratories) in PBS for 1 h at room temperature, and washed three times with 0.05 M Tris-buffered saline (TBS, pH 7.4). To visualize bound antibodies, trays were cooled on ice and cold 0.05% 3,3′-diaminobenzidine (DAB, Sigma) in TBS containing 0.033% hydrogen peroxide, 0.004% nickel ammonium sulphate and 0.004% cobalt chloride was added to each well. After a brown colour had developed (2–4 min), the reaction was stopped by removing the DAB solution and adding deionized water to the wells. Sections were washed three times in deionized water, mounted on gelatin chrom alum-coated slides, air dried, dehydrated in ascending 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 stain were considered to have Fos-like immunoreactivity (Fos-LI).

Microscope slides were examined using a light microscope and computer-assisted camera lucida using the programme Magellan v 5.1 (Paul Halacz, University of New South Wales). The nomenclature and boundaries of brain regions used were extrapolated from those described for rat brain in the atlas of Paxinos and Watson (1998) and with reference to a set of thionine-stained slides of guinea-pig brain. The densities of Fos-LI cells in each brain region were determined by an unbiased observer from slides carefully matched for rostro-caudal level and using a standardized counting box of appropriate size for the region. Mean densities and standard errors (cells per 0.1 mm^2) were computed for each treatment group.

2.4. Analysis of results

Significance of differences in weight changes during treatment with morphine within and between the groups was tested by two-way analysis of variance (ANOVA) (GraphPad Prism 4). Two-way ANOVA for repeated measures was used to analyse significance of differences in locomotor scores for each 10-min interval over the 90-min observation period within the same group over the three withdrawal cycles, and for differences between groups in the third cycle. Behaviour scores were summed over the 90-min observation periods and analysis of differences between the means within the same group of animals was undertaken using repeated measures one-way ANOVA and Bonferroni post-tests, and between the two groups using Students *t* tests. The significance of differences between treatment groups in the mean densities of Fos-LI cells for each brain region was tested using Students *t* tests. In all statistical tests a *P* value of less than 0.05 was considered significant.

3. Results

3.1. Guinea-pig weights

In the one-cycle withdrawal group, the body weight remained stable during the saline treatment periods, and decreased during the morphine treatment in the third week. In the three-cycle withdrawal group, the body weights of guinea-pigs decreased significantly compared with controls after commencement of each series of morphine injections (two-way ANOVA Week 1, $F_{1,24}$=81.66, P<0.0001; Week 2, $F_{1,24}$=37.99, P<0.0001), and returned to the original levels by the beginning of the next cycle of morphine treatment. There was no significant difference in the change in body weight between the two groups in the third week (Week 3, $F_{1,24}$=3.90) (Fig. 1). In the three-cycle withdrawal group the body weight changes during the three treatment periods were not significantly different (Week 1 vs. 2, $F_{1,24}$=1.33; Week 1 vs. 3, $F_{1,24}$=0.20; Week 2 vs. 3, $F_{1,24}$=1.38).

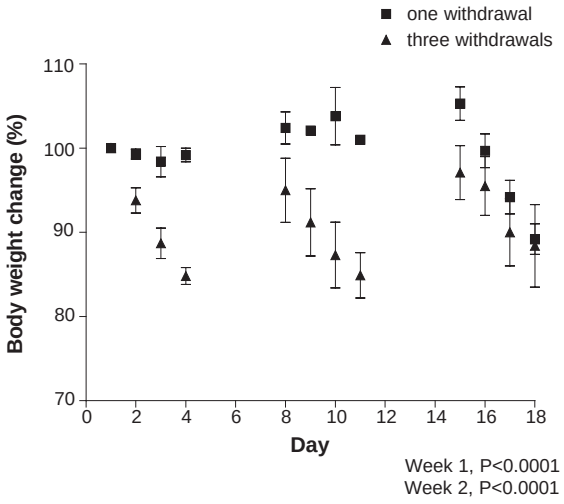

Fig. 1. Mean percentage changes in body weight from weight at commencement of the experiment of guinea-pigs subjected to two cycles of saline injections followed by naltrexone on the fourth day and then one episode of morphine withdrawal (■) and of guinea-pigs subjected to three episodes of morphine withdrawal (▲). Bars are standard errors of the means obtained from 4 animals. The weight change in morphine treated animals was significantly greater compared with saline treated animals in the first 2 weeks (two-way ANOVA, both P<0.0001). Note similar weight losses induced by morphine in the third period of treatment for both groups of animals. See text for doses of morphine and naltrexone.

3.2. Withdrawal behaviour

The behaviours exhibited during morphine withdrawal in guinea-pigs differed from those in rats. Morphine withdrawal in guinea-pigs was characterized by marked increase in locomotor activity commencing immediately after naltrexone injection. Other behaviours included vocalization, digging, face washing, rearing, sneezing, head/body shaking, and scratching. The latter two behaviours occurred rarely in most animals and thus were not analysed separately in this study. However, they were used in the calculation of the "total behaviour counts," which were the counts obtained when all behaviours, other than locomotor activity, that an animal exhibited in each 10-min period were added together. A small increase in the number of faecal pellets compared with controls was observed in some guinea-pigs towards the end of the withdrawal observation period but diarrhoea did not occur and weight loss was therefore minimal in this species during the 90-min period of observation. Thus weight loss following withdrawal was not quantified.

3.2.1. Locomotor activity

Following saline treatment in the first and second weeks, the one-cycle withdrawal group did not show any signs of withdrawal following naltrexone injection, whereas following morphine treatment in the third week they showed

marked locomotor activity on injection of naltrexone that was significantly greater than following saline treatment (two-way ANOVA for repeated measures: Week 1 vs. 2: $F_{1,48}$=4.09; Week 1 vs. 3: $F_{1,48}$=16.00, P=0.0071; Week 2 vs. 3: $F_{1,48}$=23.92, P=0.0027) (Fig. 2). In the three-cycle withdrawal group, marked locomotor activity and withdrawal behaviours were observed following naltrexone injection in each cycle (Fig. 2). There was no significant difference between the withdrawal locomotor activity scores over 90 min in each of the three cycles of the three-cycle withdrawal group (Week 1 v 2: $F_{1,48}$=0.14; Week 1 v 3: $F_{1,48}$=0.31; Week 2 v 3: $F_{1,48}$=0.03), or between the scores obtained in the third week for the three-cycle withdrawal group and the single-cycle withdrawal group ($F_{1,48}$=0.30).

3.2.2. Other behaviours

The mean numbers of counts for particular behaviours over 90 min±standard errors of the means and for the sum of all withdrawal behaviours except locomotor activity (total behaviour count) for guinea-pigs subjected to one and three withdrawal cycles are shown in Table 1. The one-cycle withdrawal animals exhibited virtually no naltrexone-induced changes in behaviour following saline treatment but in the third week following treatment with morphine these animals exhibited withdrawal behaviours, with significant increases in digging, rearing, sneezing, and total behaviour scores compared with responses in the first 2 weeks (Bonferroni t tests following repeated measures one-way ANOVA: digging, Weeks 1 and 2 vs. Week 3, P<0.01; rearing, Weeks 1 and 2 vs. Week 3, P<0.001; sneezing, Week 2 vs. Week 3, P<0.05; total behaviours, Weeks 1 and 2 vs. Week 3, P<0.001). Although the three-cycle withdrawal group did not show significant changes in individual behaviours over the three withdrawal cycles, they showed a significantly greater total behaviour count in the third cycle compared with the first and second cycles (Bonferroni post-tests following repeated measures one-way ANOVA: P<0.01 and P<0.05 respectively).

3.3. Fos-like immunoreactivity

Table 2 shows the brain regions that expressed Fos-LI following withdrawal in the two groups of animals. In the telencephalon, Fos-LI neurons were seen within the entire cortex, as well as in the hippocampus and amygdala. The density of Fos-LI cells in the three-cycle withdrawal group was higher than that in the one-cycle withdrawal group in the medial, central, basomedial, and basolateral amygdaloid nuclei (Students t tests: all P<0.05). Only the three-cycle withdrawal group showed Fos-LI expression in the caudate–putamen (P<0.05), where Fos-LI appeared in the dorsomedial region (Fig. 3). Significantly greater densities of Fos-LI cells in the three-cycle withdrawal group were also seen in the bed n. stria terminalis (P<0.01), in the lateral dorsal and medial septal nuclei (P<0.05), in the median preoptic nucleus (P<0.05), paraventricular nucleus magnocellular

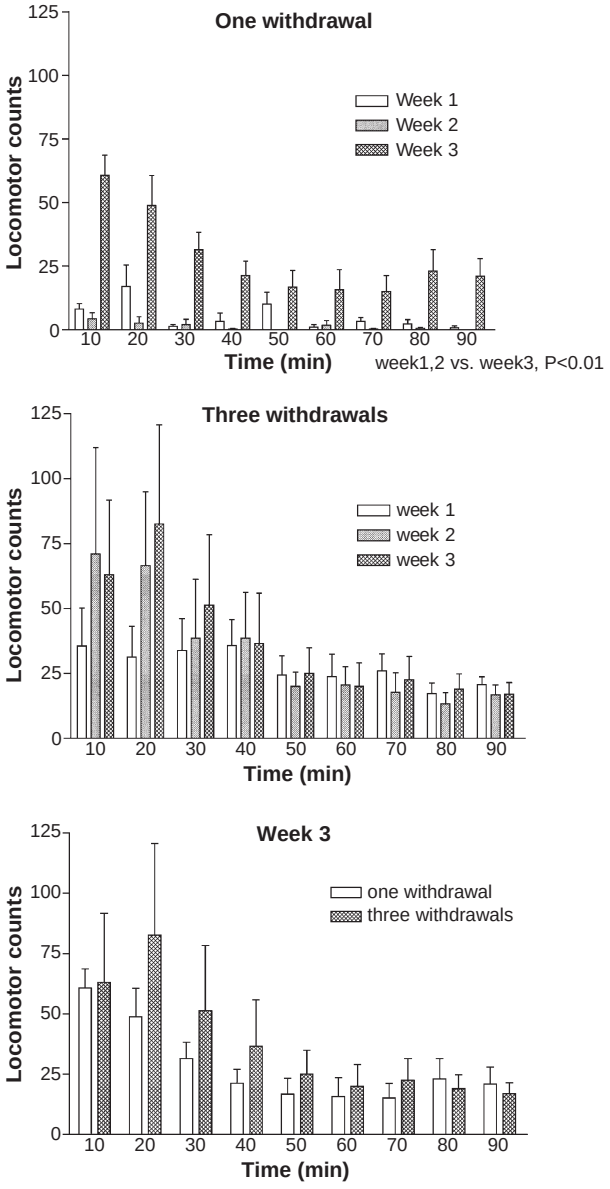

Fig. 2. Locomotor activity over the 90-min period following injection of naltrexone of guinea-pigs subjected to two cycles of saline (Weeks 1 and 2) and one cycle of morphine injections (Week 3) (top graph), and of guinea-pigs subjected to three cycles of morphine injections (middle graph). Note that animals given saline did not give a response to naltrexone. To allow comparison of the locomotor withdrawal responses between animals subjected to one and three cycles of morphine injections, the results for the third week only are shown in the lower graph. Histograms are mean locomotor responses (counts) over intervals of 10 min and bars are standard errors obtained from 4 animals. There was a significant increase in locomotor activity following morphine treatment compared with saline treatment (two-way ANOVA for repeated measures with Bonferroni post-tests: Weeks 1 and 2 vs. Week 3, both P<0.01) (top graph). However, there was no significant difference between the scores in each of the three episodes of withdrawal for animals subjected to three episodes (middle graph), nor was there a significant difference between the scores in the third week for the two groups of animals (lower graph).

(P<0.0001), and the lateral and dorsal areas of the hypothalamus (P<0.05). Furthermore, only the three-cycle withdrawal group showed Fos-LI expression in the para-

Table 1
Mean behavioural counts±standard errors of the means obtained from 4 guinea-pigs observed over the 90-min period following naltrexone-induced morphine withdrawal in animals subjected to one and three withdrawals

Behaviour	One withdrawal group			Three withdrawal group		
	Week			Week		
	1	2	3	1	2	3
Digging	0.3±0.3	0.0±0.0	96.8±25.3 [b,b]	77.8±22.8	98.0±21.2	61.3±11.8
Face washing	2.5±1.4	0.0±0.0	39.8±18.9	51.8±16.2	45.5±8.8	64.0±13.6
Rearing	5.0±2.2	0.3±0.3	247.8±34.2 [a,a]	269.5±86.1	300.5±79.0	477.0±119.0
Vocalizing	0.0±0.0	0.0±0.0	17.3±8.9	28.3±11.4	20.3±7.5	18.0±4.5
Sneezing	0.5±0.5	0.0±0.0	5.0±1.8 [c (week2)]	7.0±2.5	11.5±5.2	17.0±6.3
Total behaviours#	10.3±3.7	0.0±0.0	315.3±24.8[a,a]	437.3±108.9	480.3±115.7	648.5±140.7[b,c]

#Excludes locomotor activity but includes infrequent behaviours not listed in the table.
Significantly greater than responses in Weeks 1 and 2 respectively (Bonferroni post-tests following repeated measures one-way ANOVA), [a]$P<0.001$; [b]$P<0.01$; [c]$P<0.05$.

ventricular nucleus magnocellular and dorsal area. In the thalamus, almost all areas that expressed Fos-LI showed a significantly greater density in the three-cycle withdrawal group. These included the mediodorsal ($P<0.01$), paraventricular ($P<0.0001$), central medial ($P<0.001$), reunien ($P<0.05$), and medial habenular ($P<0.001$) nuclei. Furthermore, only the three-cycle withdrawal group showed Fos-LI expression in the paraventricular nucleus anterior part, central medial, and reunien nuclei. The density of Fos-LI cells was also higher in the three-cycle withdrawal group in the precommissural nucleus ($P<0.0001$), lateral mamillary nucleus ($P<0.05$), ventral tegmental area ($P<0.05$), substantia nigra ($P<0.05$), nucleus of the solitary tract ($P<0.01$), prepositus nucleus ($P<0.05$), medial vestibular nucleus ($P<0.01$), and the locus coeruleus ($P<0.05$).

4. Discussion

The present study has shown that behavioural sensitization occurred in guinea-pigs to the naltrexone-induced morphine withdrawal response, since animals subjected to three episodes of withdrawal showed a greater total behavioural response than those subjected to a single episode. However locomotor activity, one of the prominent signs of morphine withdrawal in guinea-pigs, was not increased.

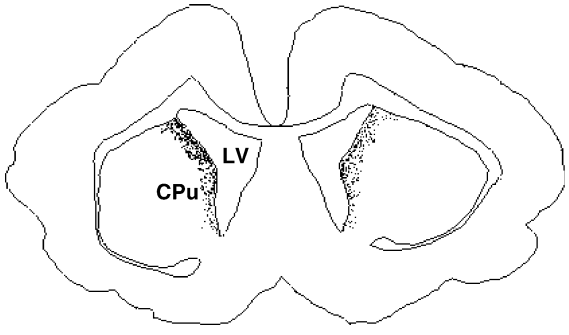

Fig. 3. Camera lucida drawing of a coronal section from the brain of a guinea-pig subjected to three cycles of naltrexone-induced morphine withdrawal showing a high density of Fos-LI neurons in the dorsal and medial caudate–putamen. Animals subjected to only one cycle of withdrawal did not exhibit any Fos-LI neurons in this region.

In a previous study it has been shown that the morphine withdrawal response in rats was dependent on the dose of opioid antagonist (naloxone, 0.1 to 2 mg/kg) used. In particular, the autonomic signs of opioid withdrawal but not the somatomotor signs were related to the dose of naloxone given (Le Guen et al., 2001). In the present study a much higher dose of the long-acting opioid antagonist, naltrexone, 15 mg/kg, was used to induce morphine withdrawal and both control animals that experienced one withdrawal episode, as well as those that experienced three withdrawal episodes, were subjected to the same number of doses of naltrexone. Also both groups of animals exhibited a similar degree of weight loss during the final period of morphine treatment. Thus it is unlikely that the results would have been influenced by differences between animals in sensitivity to naltrexone.

The Fos-LI data from the present study supported the conclusion that sensitization to morphine withdrawal occurred since several brain regions exhibited greater densities of Fos-LI neurons following three episodes of withdrawal than following a single episode. In particular, several nuclei of the amygdala, thalamus, hypothalamus, midbrain regions including the ventral tegmental area and substantia nigra, and hind brain regions such as the medial vestibular nucleus, nucleus of the solitary tract, and locus coeruleus exhibited significantly greater densities of Fos-LI neurons following repeated withdrawal than following a single withdrawal episode. Some regions including the caudate–putamen, paraventricular magnocellular nucleus of the hypothalamus, and anterior paraventricular nucleus of the thalamus exhibited no Fos-LI neurons following one withdrawal but exhibited Fos-LI neurons after three withdrawals. Thus repeated withdrawal resulted in the recruitment of new areas of activation. In contrast to the increased density of Fos-LI neurons exhibited by several subcortical regions following repeated withdrawal, there was no increase in cortical regions. The importance of this observation to addiction research remains to be explored. Nevertheless, it is interesting to note that studies on

Table 2
Mean densities per 0.1 mm^2 of Fos-like immunoreactive neurons in brain regions of guinea-pigs subjected to one and three episodes of naltrexone-precipitated morphine withdrawal

	Three withdrawals from morphine	One withdrawal from morphine
Telencephalon		
Neocortex		
Frontal	95.9±10.4	99.2±10.3
Parietal	103.3±24.3	118.0±35.8
Temporal	199.8±55.1	147.8±39.8
Occipital	121.5±28.4	143.3±42.9
Allocortex		
Piriform	132.5±10.4	107.0±11.6
Olfactory tubercle	237.3±31.5	199.2±15.6
Orbital	191.2±19.4	197.3±22.3
Insular	185.8±28.9	185.0±22.2
Cingulate1	118.9±12.6	88.7±28.2
Cingulate2	127.3±12.6	135.6±20.7
Infralimbic	343.4±37.6	282.4±32.9
Prelimbic	393.0±56.4	267.0±55.1
Hippocampus		
CA1	164.6±17.9	117.0±7.8
CA2	207.5±45.5	124.3±15.3
CA3	68.4±16.6	30.7±25.5
Dentate	312.0±38.2	254.3±35.2
Subiculum	122.8±4.6	102.2±11.8
Amygdala		
Medial	151.5±26.9[c]	77.1±10.3
Central	108.3±19.2[c]	34.3±15.9
Anteriocortical	64.6±9.2	46.4±7.0
Basomedial	102.9±17.6[c]	44.9±12.1
Basolateral	58.3±6.9[c]	27.8±9.7
Striatum		
Caudate–putamen	36.2±13.3[c]	0.0±0.0
Bed n. stria terminalis	60.9±5.2[b]	12.6±9.4
Accumbens n. shell	60.2±21.2	23.2±17.2
Accumbens n. core	29.2±10.1	6.3±5.7
Septum		
Lateral septal n. D.	44.8±5.0[c]	16.8±9.4
Lateral septal n. L.	51.4±11.8	19.4±8.8
Medial septal n.	42.8±2.9[c]	16.5±8.2
Diencephalon		
Hypothalamus		
Median preopt. n.	225.8±52.3[c]	55.8±29.8
Preoptic area	167.2±21.8	76.1±31.8
Supraoptic n.	159.3±53.5	127.8±46.4
Paraventricular n. (parvocellular)	158.9±28.1	71.0±22.7
Paraventricular n. (magnocellular)	157.8±13.5[a]	0.0±0.0
Periventricular n.	108.2±20.2	63.4±16.8
Lateral area	85.8±9.8 [c]	48.8±11.5
Anterior area	104.5±13.6	89.2±16.8
Dorsal area	36.8±14.2[c]	0.0±0.0
Posterior area	83.6±18.4	69.2±11.3
Dorsomedial n.	135.5±35.7	61.8±13.2
Ventromedial n.	130.4±19.5	84.2±20.1
Arcuate n.	422.5±38.1	313.4±46.7
Thalamus		
Mediodorsal thal. n.	152.2±25.1[b]	32.5±18.7
Paraventricular n. A.	139.1±15.9[a]	0.0±0.0
Paraventricular n. P.	89.0±3.9[a]	7.4±4.2
Paratenial n.	85.9±22.6	26.2±13.5
Central medial n.	83.6±11.1[a]	0.0±0.0
Reunien n.	72.2±24.9[c]	0.0±0.0
Lateral hebenular n.	102.9±15.7	56.7±11.7
Medial habenular n.	123.5±13.2[b]	34.7±7.7
Mesencephalon		
Precommissural n.	108.8±8.8[a]	20.9±3.6
Supramammillary n.	141.8±17.8	101.4±14.1
Lateral mammillary n	88.8±10.4[c]	52.3±10.5
Medial mammillary n.(med)	178.8±24.9	147.9±19.1
Medial mammillary n.(lat)	218.3±20.2	235.4±41.6
Dorsomedial periaqueductal gray	145.7±35.8	72.0±14.1
Dorsolateral periaqueductal gray	119.2±35.8	66.6±11.3
Lateral periaqueductal gray	139.5±36.2	85.9±16.6
Ventolateral periaqueductal gray	151.7±40.9	51.2±11.2
Ventral tegmental area	54.5±8.9[c]	31.4±2.4
Substantia nigra	22.7±2.7[c]	12.1±1.3
Rhombencephalon		
Interpeduncular n.	114.8±28.8	67.0±22.8
Doral raphe n. dorsal part	174.9±57.9	64.8±22.8
Dorsal raphe n. ventral part	140.0±42.8	43.9±42.9
Pontine n. (medial)	100.4±7.4	62.5±27.9
Pontine n. (lateral)	73.9±33.7	72.2±7.4
Lateral parabrachial n.	99.6±23.2	56.7±9.8
Superior paraolivary n.	71.5±23.9	41.2±5.9
Raphe magnus n.	38.2±11.4	18.4±10.4
Solitary tract n.	12.4±1.3[b]	5.8±1.2
Prepositus n.	107.3±15.5[c]	41.2±15.6
Medial vestibular n.	145.3±22.1[b]	38.4±10.3
Locus coeruleus	86.3±14.0[c]	39.0±8.3

Values shown are mean densities per 0. 1 mm^2 and standard errors of the means obtained from 4 guinea-pigs. Significant differences obtained in Students *t* tests between the means of the two groups of animals: [a]$P<0.001$; [b]$P<0.01$; [c]$P<0.05$.

sensitization to heroin have also shown that the sensory–motor cortex does not exhibit sensitization (D'Este et al., 2002). Thus behavioural sensitization in general may be a phenomenon restricted to subcortical regions.

The two groups of guinea-pigs used in the present study differed in the amount of morphine exposure. However, it was not considered appropriate to include a control group that received the same amount of morphine but did not receive naltrexone since these animals would undergo spontaneous withdrawal between treatments which would confound the results. Thus it was not possible to determine whether repeated treatment with morphine itself affected the results. However, Mizutani and Chahl (unpublished) found that longer term morphine treatment (10 days) produced a reduction in Fos-LI in many brain regions compared with shorter term (3 days) treatment, and the only area showing significantly increased Fos-LI following longer term morphine treatment was the lateral septal nucleus. Thus it is

concluded that the increased density of Fos-LI neurons found in the present study most likely reflected sensitization to morphine withdrawal rather than to morphine itself.

Schulteis et al. (2003) showed that repeated experience with naloxone following morphine treatment facilitated withdrawal-induced suppression of operant responding for food reward in rats. They proposed that there was an important role of conditioning in opioid withdrawal. The present study has confirmed and extended previous studies in that it has shown sensitization to behaviour and increased neuronal activation in several brain regions, as reflected in Fos-LI, following repeated opioid withdrawal. These findings may reflect 'conditioning' to the withdrawal response.

Withdrawal from opioids is highly aversive and is a major negative reinforcer of continued opioid abuse. Earlier studies by Stinus et al. (1990) identified the nucleus accumbens and amygdala as critical regions for the aversive stimulus of opioid withdrawal. More recent studies have shown that the central nucleus of the amygdala (Watanabe et al., 2003), the extended amygdala, viz the bed nucleus stria terminalis (Delfs et al., 2000) and the nucleus accumbens shell (Gracy et al., 2001), play major roles in the aversive affective component of morphine withdrawal. Indeed, in a dose-response study of naloxone on conditioned place aversion in morphine-dependent rats the nucleus accumbens shell and the central nucleus of the amygdala were found to be the most sensitive regions (Gracy et al., 2001). In the present study the bed nucleus stria terminalis and the central nucleus of the amygdala exhibited significantly higher densities of Fos-LI neurons following repeated withdrawal than following a single withdrawal. There was also an increase in the mean density in the accumbens shell with repeated withdrawal but this did not reach significance. Thus it is likely that repeated withdrawal induced sensitization to its aversive effect.

The induction of Fos-LI in neurons of the caudate–putamen in guinea-pigs following three episodes of withdrawal occurred in the dorsomedial region. Like the nucleus accumbens this region receives inputs from the cingulate cortex (Brown et al., 1998) and other limbic areas (Finch, 1996) considered to be involved in the acquisition of spatial and conditioned reward information (Devan et al., 1999). Therefore it is likely to be involved in limbic as well as motor functions. It is tempting to speculate that activation of the dorsomedial caudate–putamen in the present study reflected sensitization or conditioning to the aversive stimulus of withdrawal.

Several regions of the medial dorsal thalamus including the mediodorsal nucleus and paraventricular nucleus exhibited marked increases in Fos-LI neurons following three episodes of withdrawal. The mediodorsal thalamus receives projections from the ventral striatum and pallidum and basolateral amygdala, and has been found in conditioned place preference paradigms to be involved in reward processes (see McAlonan et al., 1993). In light of the results in the present study that repeated withdrawal increased Fos-LI in other regions associated with aversion, it is suggested that the increased activation in the mediodorsal thalamus was also involved in sensitization to aversion.

The opioid withdrawal response is associated with stress and activation of the hypothalamic–pituitary–adrenal (HPA) axis (McNally and Akil, 2002; Milanes et al., 2002). Following opioid withdrawal it has been shown that Fos is expressed in corticotropin-releasing hormone (CRH) neurons in the parvocellular division of the hypothalamic paraventricular nucleus (Hamlin et al., 2004). In the present study Fos-LI neurons were found in the parvocellular division of the paraventricular nucleus following one withdrawal episode but not in the magnocellular region. Following three episodes, more Fos-LI neurons were present in the parvocellular division, although this did not quite reach significance, but Fos-LI neurons were also found in the magnocellular division. This might reflect a differential sensitivity of CRH and vasopressin neurons to opioid withdrawal as suggested by the results of Milanes et al. (2002).

The opioid withdrawal response is complex and comprises somatic motor, autonomic and sensory responses as well as affective components. Two regions that have been implicated in the behavioural and physical signs of opioid withdrawal are the periaqueductal grey (Kimes and London, 1989) and the locus coeruleus (Rasmussen et al., 1990). Electrophysiological studies have shown that activity in neurons in the ventrolateral periaqueductal gray is enhanced during morphine withdrawal (Chieng and Christie, 1996). Evidence has recently been obtained for gamma-amino butyric acid (GABA)-ergic projections from the ventral tegmental area and substantia nigra to the ventrolateral periaqueductal gray and dorsal raphe (Kirouac et al., 2004). Both the periaqueductal gray and dorsal raphe, together with the ventral tegmental area and substantia nigra have been implicated in cardiovascular depressor responses and pain modulation (see Bandler et al., 2000; Wang and Nakai, 1994). In the present study increased density of Fos-LI neurons following three withdrawals was found in the ventral tegmental area, substantia nigra, and locus coeruleus, and a trend toward an increase was also seen in the periaqueductal gray and dorsal raphe, suggesting that sensitization occurred to the cardiovascular and nociceptive modulatory responses to opioid withdrawal. Sensitization in these neuronal circuits might reflect increased coping of physiological systems to the stress and pain of opioid withdrawal.

The response of central neurons during opioid withdrawal is the resultant of intrinsic central responses as well as responses arising from the peripheral organs that also undergo withdrawal and send sensory inputs to the thalamus and nucleus of the solitary tract (Hamlin et al., 2001). In rats it has been shown that several regions considered to be involved in aversion such as the central nucleus of the amygdala and the bed nucleus of the stria terminalis, as well as the medial prefrontal cortex, were activated to varying

extents by peripheral withdrawal (Hamlin et al., 2001). It is noteworthy, however, that systemic withdrawal produced significantly greater activation in the nucleus accumbens shell and central nucleus of the amygdala, regions that have been particularly implicated in aversion (Hamlin et al., 2001). In the present study the contribution of peripheral withdrawal to the observed sensitization to morphine withdrawal cannot be assessed. Nevertheless, the findings of Hamlin et al. (2001), and the observation that peripheral withdrawal is reputed not to produce aversion (Hand et al., 1988) in rats, suggest that a major component of the neuronal activation observed in the present study in regions reputed to be involved in opioid withdrawal-induced aversion resulted from intrinsic central withdrawal.

In conclusion, this study has demonstrated that sensitization to morphine withdrawal occurs in guinea-pigs. The finding that sensitization occurs to ethanol withdrawal in mice (Veatch and Becker, 2002) suggests that sensitization to withdrawal from several drugs of abuse may occur. However, it remains to be determined whether sensitization to drug withdrawal occurs in human subjects.

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