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Ethopharmacological analysis of naloxone-precipitated morphine withdrawal syndrome in rats: a newly-developed "etho-score"

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Abstract The intensity of opiate withdrawal syndrome in rats is usually quantified on the basis of selected physical signs or global scores. However, the selection criteria of signs and scores have not been subjected to an ethological discussion, hence they appear to be somewhat arbitrary. The objectives of this study were thus: i) to analyse the rat's behaviour during the naloxone-precipitated morphine withdrawal syndrome, ii) to evaluate the validity of classic methods, and iii) to design a new "etho-score". Ten rats were implanted with morphine pellets (75 mg × 2, SC), all receiving different naloxone doses following a within-subject design (0, 0.01, 0.05, 0.1, 0.5, 1 mg/kg SC). Twenty unexperienced rats and 20 with placebo pellets were injected with either saline or naloxone. Behaviour was videotaped and later analysed by computer-based ethological techniques. The ethogram encompassed 16 patterns displayed by rats during morphine withdrawal. Frequency, duration and latency of each pattern was measured, and a cluster analysis allowed discerning the structure of behaviour. Several physical signs and the Gellert-Holtzman score were also evaluated. The data revealed that writhing responses linearly changed in a dose-related fashion, and mastication was also enhanced after naloxone. Wet-dog shakes and jumping changed following an U-shaped curve. Significant changes in weight loss were found to be dose-dependent, and highly correlated to diarrhea. Learning effects were found to reliably affect exploration, writhing responses and some physical signs. The Gellert-Holtzman score was gradually enhanced after naloxone, being affected by learning as well. Naloxone affected lying and self-care responses in placebo rats. To sum up, the data indicated that: i) classic signs are useful, although most of them are disrupted by high naloxone or affected by learning effects, ii) the Gellert-Holtzman score was validated in this study, and iii) mastication and weight loss are good indicators of naloxone-precipitated morphine withdrawal, representing the basis of an "etho-score" which is herein proposed.

Key words Behaviour · Morphine · Naloxone · Withdrawal syndrome · Ethopharmacology · Rat

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Introduction

Chronic administration of morphine is known to induce physical dependence (Wei et al. 1972, 1973; Bläsig et al. 1973; Koob et al. 1989). Morphine withdrawal is considered as the physical manifestation of dependence, emerging after morphine administration is stopped or after administration of an opiate antagonist such as naloxone. The acute phase of the morphine withdrawal is characterized by specific behavioural and vegetative signs, the intensity of which reflects the degree of dependence. Most laboratories use their own individualized procedures for measuring the degree of physical dependence on opioid drugs in rats. These procedures range from quantifying some selected physical signs to using global rating scales in which withdrawal signs are weighted (Bläsig et al. 1973; Frederickson et al. 1976; Bläsig and Herz 1997; Gellert and Holtzman 1978; Gmerek 1988; Malin et al. 1990; Spanagel et al. 1994).

The rat's behaviour during the opiate withdrawal syndrome is complex, comprising different responses such as exploration, writhing posture, wet-dog shakes and escape attempts (Kerr and Pozuelo 1971; Wei et al. 1972, 1973; Gellert and Holtzman 1978; Calvino et al. 1979; Tremblay and Charton 1981; Espósito

et al. 1987; Stinus et al. 1990, 1992; Maldonado et al. 1992; Spanagel et al. 1994). However, analysis of all of the responses of a rat has not yet been performed. In this context, the selection criterion of physical signs has not been subjected to an ethological discussion, and hence appears to be somewhat arbitrary. An appropriate ethological study would allow elucidation of the structure of behaviour as well as establishment of the behavioural "weight" of each physical sign. Ethological techniques have been shown to be very appropriate for study of changes in the structure of behaviour (Silverman 1965; Colgan 1978), being applied to assess drug effects in various behavioural situations in rats for 3 decades (Silverman 1965; Miczek and Grossman 1972; Mackintosh et al. 1977; Olivier 1981; Poshivalov 1981; Miczek 1982; McAllister et al. 1985; Mos et al. 1987; Espejo et al.1994).

The objectives of this study were: i) to analyse, from an ethological point of view, the rats' withdrawal syndrome to morphine, ii) to evaluate the validity of classic methods, and iii) to determine the behavioural "weight" of each physical sign in order to propose a new "etho-score" for measuring the precipitated morphine withdrawal. Morphine dependence was induced by subcutaneous implantation of morphine pellets and morphine withdrawal precipitated by administering the opiate antagonist naloxone.

Materials and methods

Animals and apparatus

Fifty male Wistar rats (250–300 g) from IFFA Credo (France) were housed in the vivarium in groups of five. Laboratory temperature was kept at 22 ± 1°C, and a 12-h light-dark cycle (lights on at 0800 hours) was maintained throughout the experiment. Food (lab chow) and water were available ad lib. Animals were tested in a transparent cylindrical cage (31 × 40 cm) which was covered to prevent the rat from escaping. Each test lasted 20 min, a duration of exposure which allows the animal to display most of its behavioural responses during the withdrawal syndrome. Every test was carried out during the light period (1000–1400 hours) in a separate room where rats were placed at the start of the session day. Behaviour was videotaped under white light illumination. Video tapes were later played and behaviour analysed automatically after direct keyboard entry to a computer programmed to perform statistical and ethological analyses. Tape speed could be modified (x1, x1/5) for scoring fast behavioural patterns more accurately.

Procedure

Rats were randomly allocated in three groups: i) repeatedly tested (NalR group, $n = 10$), ii) singly tested (NalS group, $n = 20$), and iii) placebo pelleted group (PL group, $n = 20$). One week after housing, rats were subcutaneously implanted in the back with either two placebo pellets or two morphine pellets (75 mg each, National Institute of Drug Abuse, Baltimore, Md., USA), under halothane anesthesia. The incision was sealed with surgical clips and antiseptic applied to the wound area. Two 75-mg morphine pellets maintain a constant level of morphine dependence during at least 13 days (Gold et al. 1994). Naloxone hydrochloride was dissolved in saline (0.9% NaCl) and used to precipitate morphine withdrawal, being supplied by Research Biochemicals International (ref. O-002, Natick, Mass., USA). In the NalR group, naloxone was subcutaneously injected at the nape of the neck at 2, 4, 6, 8, 10, and 12 days after morphine pellet implantation. Every rat received saline control, 0.01, 0.05, 0.1, 0.5 and 1 mg/kg naloxone (NalR-0, NalR-0.01, NalR-0.05, NalR-0.1, NalR-0.5 and NalR-1 groups, respectively), following a within-subject design and progressively changing the initial dose, in order to minimize learning effects of repeated tests (Maldonado et al. 1992; Gold et al. 1994). The singly tested group encompassed rats which were subcutaneously injected with either 1 mg/kg naloxone (NalS-1, $n = 10$) or saline (NalS-0, $n = 10$), 12 days after morphine pellet implantation. The singly tested group was created in order to better discern learning effects. Placebo animals were similarly treated with 1 mg/kg naloxone (PL-1, $n = 10$) or saline (PL-0, $n = 10$), 12 days after placebo pellet implantation. The placebo groups allowed determining behavioural changes induced by naloxone per se. Every solution was injected in a dose volume of 1 ml/kg body weight. Experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) as well as the French Directive concerning the employment of laboratory animals (décret n° 87-848, 19 October 1987).

Ethogram

Behaviour was recorded by using the complete sampling method (Slater 1978). A 16-pattern ethogram, which was made to this end, was employed. Table 1 shows pattern denominations, abbreviations and brief descriptions, which were taken partially from several authors (Grant and Mackintosh 1963; Barnett 1975; Gmerek 1988; Espejo et al. 1994). Table 2 shows behavioural categories or group of patterns with similar ethological "meaning", derived from cluster analysis.

Data analysis

A multivariate cluster analysis based on similarity values between patterns was employed. Individual transition matrices of observed occurrences between elements were obtained, which showed the frequency with which a pattern followed the previous one (predecessors in rows and successors in columns). Total transition matrices were calculated for each group of rats by summing individual transition matrices. Each total transition matrix was transformed into a half similarity matrix by applying a formula (Mos et al. 1987):

$$\text{S.V. AB} = \left(\frac{\alpha + \beta}{\text{A}} + \frac{\alpha + \beta}{\text{B}} \right) * 50$$

S.V. AB similarity value between patterns A and B; *A* absolute frequency of pattern A; *B* absolute frequency of pattern B; α observed occurrences between A and B; β observed occurrences between B and A; * 50 to obtain more manageable values.

Similarity matrices were represented by dendrograms, composed of solid lines connecting those patterns which are linked by the highest similarity values. Matrices and dendrograms only included the most frequently displayed elements, because similarity values of the other elements were comparatively low. Moreover, since small sample sizes were used, the effectiveness of cluster treatment was improved by considering few variables (Short and Horn 1984). The behavioural categories and the ethological "meaning" of each pattern could thus be more readily discerned from cluster analysis (Colgan 1978).

Table 1 Ethogram of the rat's behaviour during morphine withdrawal

Pattern	Abbr.	Description
Immobile-sniff	is	the rat explores the environment, standing on the surface. Movements of head and vibrissae are clearly present
Walk-sniff	ws	the rat moves around the enclosure to explore it
Rearing	re	investigatory upright posture. The rat does not lean against the wall of the enclosure
Leaning-posture	le	erect posture with forelegs leaning on the wall of the cage
Freezing	fr	the rat firmly stands in a quiet and alert posture
Self-grooming	gr	the rat licks or scratchs its fur or hands
Face-washing	fw	cleaning the face with forelegs
Genital grooming	gg	the rat licks its genitalia. It usually follows ejaculation
Body-shake	bs	the rat shakes its body
Hand-shake	hs	the rat moves hands with quick sideway movements
Writhing-posture	wp	the rat lies on the floor, while the belly is firmly pressing the surface. Abdominal contraction are usually present. rat can also elongate the body, yawn or masticate
Attenuated-gait	ag	the rat moves around the surface with short steps, while displaying the writhing posture
Mastication	ms	the act of masticating and/or swallowing
Jumping	ju	leaping off the surface of the cage
Teeth-chattering	tt	the rat rapidly clicks teeth together
Lying	ly	the rat quietly lies or sits on the surface

Table 2 Behavioural categories and most frequent patterns during morphine withdrawal

Category	Abbr.	Patterns
Exploration	EX	Immobile-sniff Walk-sniff Rearing Leaning posture[a]
Self-care	SC	Self-grooming Face-washing Genital-grooming
Writhing behaviour	WR	Writhing posture Attenuated gait
Escape	ES	Jumping Leaning posture[a]
Wet-dog shakes	WS	Body-shake Hand-shake

[a] Escape/exploratory pattern

Several descriptive parameters were quantified: mean frequency, duration and latency for the first occurrence of each pattern. They were calculated by summing every individual absolute measure divided by the total number of animals. A latency of 1200 s was recorded if the animal failed to display the element within this time. For measuring mastication frequency, each "burst" of mastication activity was quantified as 1, regardless of its duration. Writhing reactions and lying were considered as temporary patterns only (duration and latency were measured). Duration of wet-dog shakes and jumping, fast reactions, were not quantified.

Statistical significances were evaluated, on either basic data or logarithmically (log) transformed data if variance was not homogeneous, by one-way ANOVA (dose and pattern, dependent factors). Post-hoc treatment was based on either Dunnett's or Newman-Keuls tests. Comparisons between two groups were performed with the Student's *t*-test. Correlations were carried out using the Pearson's product moment. Printouts comprising descriptive and multivariate data are available from the authors on request.

Physical signs and the Gellert-Holtzman score.

Several single physical signs were quantified: weight loss, number of defecations and micturitions as well as number of animals suffering from diarrhea. Defecation refers to the act of defecating, regardless of whether or not the boli were diarrheic. Weight loss was evaluated before (matching group) and 1 hour after each test. Each 1% loss above the matching weight was quantified as 1. Morphine withdrawal syndrome was also rated according to the Gellert-Holtzman rating score. This scale consists of graded and checked signs, as shown in Table 3. For the Gellert-Holtzman scale, weight loss was evaluated before and 2.5 h after the test.

Results

Cluster analysis

The basic criterion to select categories was a similarity value (S) higher than 80. Dendrograms of the control groups (NalR-0, NalS-0 groups) were quite similar, indicating that only four patterns could be classified into two clear-cut categories: "exploration" (EX) comprising immobile-sniff and walk-sniff (NalR-0, S = 140), and "self-care" (SC) made up of face-washing and self-grooming (NalR-0, S = 155). Leaning posture, another frequently displayed pattern in the control group, was highly associated with exploratory elements (NalR-0, S = 75). Figure 1 shows the dendrogram of the NalR-0 group.

In repeatedly tested rats, naloxone treatment maintained above categories, but two new categories were observed with doses equal or higher than 0.05 mg/kg: "writhing behaviour" (WR) comprising writhing posture and attenuated gait (NalR-0.05, S = 95; NalR-1, S = 118), and "escape" (ES) including jumping and leaning posture (NalR-0.05, S = 83; NalR-1, S = 84).

Table 3 The Gellert-Holtzman rating score

Sign	Weighting factor
Graded signs	
Weight loss in 2.5 h (each 1% above the weight lost by control rats)	1
No. of escape attempts	
2–4	1
5–9	2
10 or more	3
No. of abdominal contractions (each one)	2
No. of wet-dog shakes	
1–2	2
3 or more	4
Checked signs	
Diarrhea	2
Facial fasciculations or teeth chattering	2
Swallowing	2
Profuse salivation	7
Chromodacryorrhea	5
Ptosis	2
Abnormal posture	3
Erection, ejaculation or genital grooming	3
Irritability	3

Body-shake and hand-shake could not be included in a category. Nevertheless, body-shake and hand-shake were usually linked to each other in the naloxone-treated groups, mainly for high doses (NalR-0.5, S = 27; NalR-1, S = 26). Hence, a "wet-dog shakes" category emerged (Fig. 1). Mastication, another independent pattern, was highly linked to writhing responses (NalR-0.05, S = 67; NalR-1, S = 55). Genital grooming, a pattern which was elicited in rats only after naloxone treatment, was highly linked to self-care patterns (NalR-1, S = 33). Dendrograms of the NalS-0 and NalS-1 groups were similar to those of the NalR-0 and NalR-1 groups, respectively. All the behavioural categories and comprising patterns are shown in Table 2.

Descriptive statistics

Behavioural patterns

Significant changes in frequency, duration and latency of the main patterns are shown in Table 4 in repeatedly tested animals. ANOVA revealed a significant dose effect on frequency measures in NalR groups for immobile-sniff, walk-sniff, self-grooming, face-washing, body-shake,z mastication, and jumping. Post-hoc treatment revealed that exploratory elements, and self-care patterns were dose-dependently decreased. As shown in Fig. 2, mastication frequency was gradually enhanced, body-shake frequency was

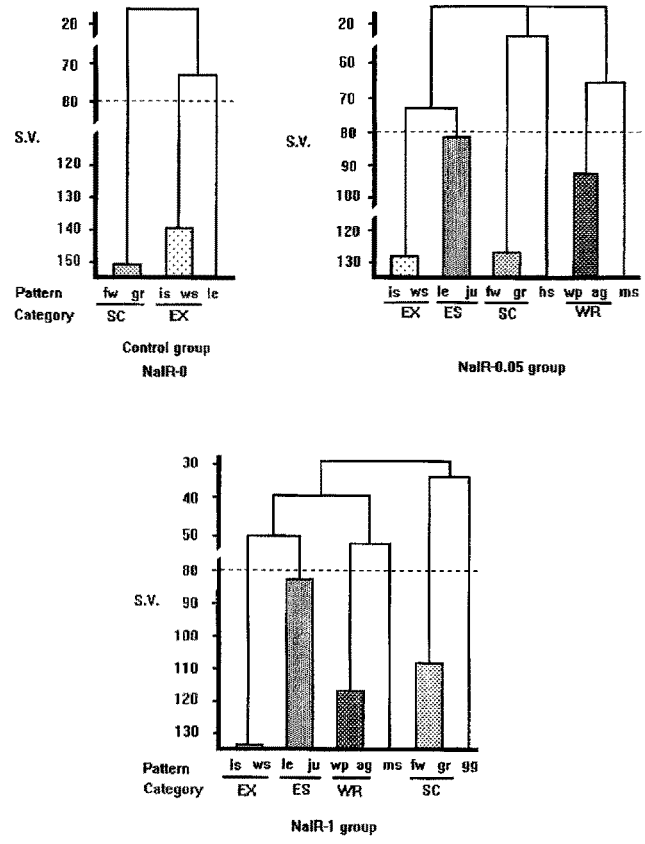


Fig. 1 Representative dendrograms of control (*NalR-0*) and naloxone-treated rats (*NalR-0.05* and *NalR-1* groups). Dendrograms are composed of *solid lines* connecting those patterns which are linked by the highest similarity values. The basic criterion to select categories was a similarity value higher than 80 (*dashed line*). Patterns comprising the same category are *underlined*, and the *filled areas* correspond to each behavioural category. For abbreviations of the patterns and categories, see Tables 1 and 2. *S.V.* similarity value between patterns

Fig. 2 Frequency of mastication, body-shake and escape attempt (jumping). Repeatedly and singly tested groups are separatedly represented along the horizontal axis. Mean + SEM; $*P < 0.05$, $**P < 0.01$ vs. control group (Dunnett)

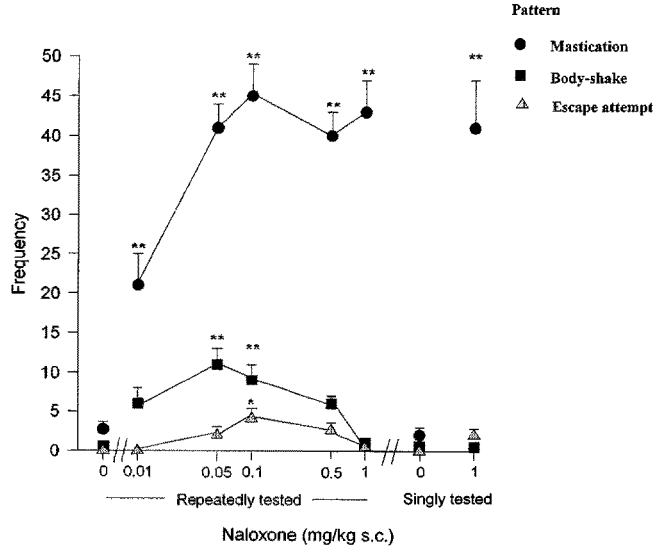


Table 4 Frequency, duration and latency of the main patterns during morphine withdrawal in repeatedly tested rats

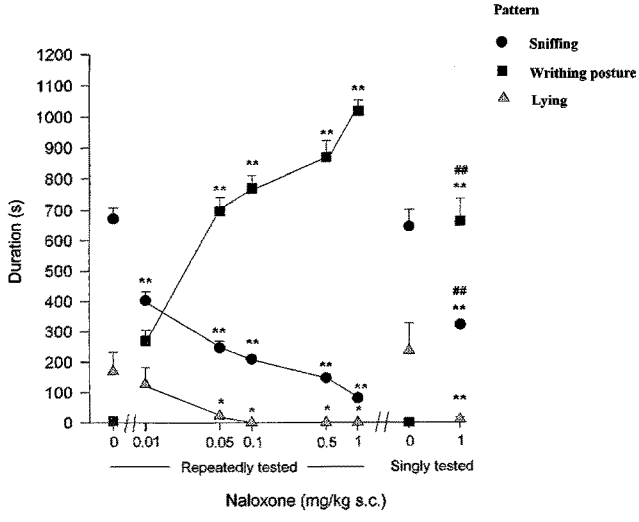
Pattern	NalR-0	NalR-0.01	NalR-0.05	NalR-0.1	NalR-0.5	NalR-1	F
Frequency							
Immobile-sniff	132.4 ± 16.9	98 ± 9.1#	70.3 ± 6.3##	56.1 ± 6.6##	39.3 ± 5.7##	21.6 ± 4.6##	19.5 ****
Walk-sniff	101.2 ± 15.7	73 ± 8.1#	48.3 ± 4.6##	42.5 ± 6.2##	27.2 ± 3.5##	16.5 ± 4.5##	14.4 ****
Self-groom	14.5 ± 12.3	12.6 ± 2.4	11.7 ± 2.8	7.1 ± 2.6	4.1 ± 1.2#	4.7 ± 1.9#	3.8 **
Face-washing	14.5 ± 2.2	13.7 ± 2.4	13.4 ± 2.7	9.8 ± 2.6	4.5 ± 1.8#	4.5 ± 1.5#	4.1 **
Body-shake	0.5 ± 0.3	5.6 ± 1.7	10.9 ± 2.3##	9.1 ± 2.3##	5.9 ± 1.2	0.7 ± 0.4	6.9 ****
Mastication	2.7 ± 1.2	21.2 ± 3.6##	40.9 ± 2.9##	44.8 ± 4.8##	40 ± 2.8##	43 ± 3.5##	24.9 ****
Jumping	0	0	2.1 ± 1.1	4.1 ± 1.3#	2.6 ± 0.9	0.5 ± 0.3	2.5 *
Duration (s)							
Immobile-sniff	539 ± 42	317 ± 31##	191.9 ± 28.1##	152.5 ± 18.5##	116.7 ± 16.7##	62.7 ± 10.2##	41.6 ****
Walk-sniff	134.4 ± 19	86.2 ± 8##	54.3 ± 8.2##	55.9 ± 10.7##	30.2 ± 4.9##	17.2 ± 4.1##	15.6 ****
Self-groom	51.1 ± 8.6	39.2 ± 9.8#	23.6 ± 6.9##	11.6 ± 2.2##	23.6 ± 7.6##	11.5 ± 3.8##	5.3 ***
Face-washing	41.1 ± 7	35.5 ± 7.1	25.4 ± 5.1	17.1 ± 4.6#	7.1 ± 3.1##	8.1 ± 2.2##	7.4 ****
Writhing post.	4.9 ± 2.2	268 ± 36##	696.1 ± 44.3##	769.1 ± 42.2##	869 ± 52.6##	1018.2 ± 34.5##	39.8 ****
Attenuated gait	0	5.9 ± 2.4	17.9 ± 4.1	31.1 ± 8.4#	22.8 ± 5.9#	35.4 ± 7.5##	6.2 ***
Lying	169 ± 64	126 ± 54	2.3#	0#	0#	0#	5.1 ***
Latency (s)							
Writhing post.	910 ± 570	505 ± 142##	230.8 ± 94.8##	167.1 ± 22.5##	113.5 ± 16.5##	73.5 ± 8.8##	19.6 ****
Atten. gait	1200	812 ± 154##	413.3 ± 65.9##	276.8 ± 50.5##	306.8 ± 83.4##	123.8 ± 20.4##	29.5 ****
Body-shake	1046 ± 551	462.8 ± 246	209.5 ± 94.8##	165.1 ± 48.8##	196.6 ± 88.9##	860.1 ± 570.5	6.2 ***
Mastication	874 ± 410	358.2 ± 181	240.8 ± 90.1##	210.1 ± 81##	125.4 ± 32.5##	84.2 ± 29.1##	38.2 ****
Jumping	1200	1200	926.1 ± 541.1	477.8 ± 184##	560.6 ± 302##	1067.6 ± 735	6.1 ***

Mean ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ (one-way ANOVA); #$P < 0.05$, ##$P < 0.01$ vs. NalR-0 group (Dunnett)

significantly enhanced in the NalR-0.05 and NalR-0.1 groups ($P < 0.01$), and jumping frequency was significantly enhanced at 0.1 mg/kg naloxone ($P < 0.05$).

A significant dose effect on duration measures was found for immobile-sniff, walk-sniff, self-grooming, face-washing, writhing posture, attenuated gait, and lying. Post-hoc treatment revealed that sniffing and self-care reactions were reliably decreased in a dose-related fashion (Table 4). Naloxone treatment also induced a significantly dose-dependent increase in duration of writhing posture and attenuated gait ($P < 0.01$). Lying duration was found to be decreased in NalR-0.01 and NalR-0.05 groups with respect to control group and this pattern was not performed following higher naloxone doses. Figure 3 shows duration of sniffing responses, writhing posture and lying.

Fig. 3 Duration of sniffing elements, writhing posture and lying. Sniffing responses comprise immobile-sniff, walk-sniff and rearing. Repeatedly and singly tested groups are separately represented along the horizontal axis. Mean + SEM; * $P < 0.05$, ** $P < 0.01$ vs. control group (Dunnett); ## $P < 0.01$ vs. 1 mg/kg naloxone in the repeatedly tested group (Student's t-test)



ANOVA indicated a significant dose effect on latency measures for writhing posture, attenuated gait, body-shake, mastication and jumping (Table 4). Post hoc treatment revealed that body-shake and jumping latency was reliably decreased after 0.05, 0.1 and 0.5 mg/kg naloxone ($P < 0.01$). Latency to writhing posture and mastication was dose-dependently decreased ($P < 0.01$).

The data of singly tested groups are shown in Table 5. After comparing the NalR-1 versus NalS-1 groups, frequency and duration of exploratory patterns (immobile-sniff, sniff and rearing) were significantly higher in the NalS-1 group. Duration of writhing posture was found to be significantly higher in the NalR-1 group ($t = 2.8$, $P < 0.005$). Besides, writhing posture latency was found to be significantly lower in the NalR-1 group than in the NalS-1 group ($t = 1.9$, $P < 0.05$).

Considering placebo groups, the structure of behaviour was similar in both groups except for duration of lying and self-care patterns. Lying was found to be significantly higher in the PL-1 (522 ± 76.6 s) than in the PL-0 group (216.7 ± 88 s; $t = 2.9$, $P < 0.05$). Self- grooming duration was found to be significantly lower in the PL-1 (90.6 ± 20 s) versus the PL-0

Table 5 Frequency, duration and latency of the main patterns during morphine withdrawal in singly tested rats

Pattern	Frequency		Duration (s)		Latency (s)	
	NalS-0	NalS-1	NalS-0	NalS-1	NalS-0	NalS-1
Immobile-sniff	78.2 ± 15.1	77.3 ± 9.8###	541 ± 65.2	258.4 ± 4.1**##	2.9 ± 10.5	2.6 ± 0.5
Walk-sniff	77.8 ± 17.5	72.3 ± 8.8###	105 ± 21.1	63.3 ± 8.3*###	2.2 ± 9.9	2.5 ± 1.4
Self-grooming	16.6 ± 3.3	3.9 ± 0.5**	121 ± 27.2	4.7 ± 0.5**#	211 ± 110	273 ± 121
Face-washing	10.2 ± 1.3	3.5 ± 0.7**	29.3 ± 4.1	3.4 ± 0.9**	201 ± 115	272 ± 122
Body-shake	0.6 ± 0.4	0.4 ± 0.2	–	–	893 ± 671	809 ± 757
Mastication	1.9 ± 1.2	41.6 ± 6.3**	–	–	975 ± 322	372 ± 212*
Writhing post.	–	–	0	664.1 ± 72.2**##	1200	148 ± 47.3**#
Attenuated gait	–	–	0	36.4 ± 17.5**	1200	302 ± 91**#
Jumping	0	1.8 ± 0.8	–	–	1200	692 ± 318
Lying	–	–	235 ± 102	10.3 ± 7.5**	516 ± 265	860 ± 537

Mean ± SEM; *$P < 0.05$, **$P < 0.01$ vs. NalS-0 group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. NalR-1 group (see Table 4; Student's t-test). – not quantified

group (171.1 ± 47 s; $t = 2.6$, $P < 0.05$). Face-washing duration was also significantly lower in the PL-1 (10.8 ± 3.4 s) versus the PL-0 group (29.3 ± 4; $t = 2.4$, $P < 0.05$).

Physical signs

Physical signs were significantly enhanced in the repeatedly tested groups in a dose-related fashion (5, 54 *df*), as shown in Table 6: weight loss ($F = 35.5$, $P < 0.0001$), number of defecations ($F = 12.2$, $P < 0.0001$), and number of micturitions ($F = 7.6$, $P < 0.0001$). The number of animals suffering from diarrhea ranged from 0 (NalR-0 group) to 10 (NalR-1 group). Pearson's correlation revealed significantly high correlations between weight loss and number of micturitions ($r = 0.93$, $P = 0.005$), as well as weight loss and number of animals with diarrhea ($r = 0.98$, $P = 0.003$). A moderately positive correlation was found between weight loss and number of defecations ($r = 0.78$). As shown in Table 6, the number of singly tested animals suffering from diarrhea was 0 (NalS-0) and 7 (NalS-1). Frequency of micturitions was found to be significantly lower in the NalS-1 than in the NalR-1 group ($t = 4.6$, $P < 0.005$).

Etho-score

The ethological analysis allowed the conclusion that mastication and weight loss are good indicators of the intensity of morphine withdrawal over a wide range of naloxone doses. Both indicators are easy to score and were little affected by learning (see Discussion). An "etho-score" is proposed as follows:

Etho-score = (MF/10) + WL
MF: mastication frequency; WL: weight loss

The first component of the formula is a" behavioural" variable, based on mastication frequency divided by ten to obtain more manageable values. As explained, each "burst" of mastication activity was quantified as 1, regardless of its duration. The second component is a "physical" factor, based on weight loss (WL). As explained, weight loss was evaluated before (matching) and 1 h after the test, and each 1% loss above the matching weight was quantified as 1.

Global scores and comparisons

In the repeatedly tested group, the Gellert-Holtzman score was significantly enhanced after naloxone

Table 6 Physical signs and scores during morphine withdrawal

Parameter	Repeatedly tested					Singly tested	
	NalR-0	NalR-0.01	NalR-0.05	NalR-0.1	NalR-0.5	NalR-1	NalS-0	NalS-1
Weight loss	0	0.5 ± 0.2	0.4 ± 0.2	1.4 ± 0.4*	3.3 ± 0.3**	4.3 ± 0.3**	0	5.2 ± 0.4**
Number of defecations	5.6 ± 1.3	5.9 ± 1.1	13.1 ± 0.9**	14.9 ± 1.2**	16.0 ± 1.3**	14.7 ± 1.7**	2.1 ± 0.2	14.8 ± 0.8**
Number of micturitions	1.8 ± 0.1	2.7 ± 0.6	4.1 ± 0.3**	5.5 ± 0.7**	6.4 ± 1**	7.9 ± 0.3**	0.8 ± 0.2	2.1 ± 0.1**##
Number of animals with diarrhea	0	1	3	4	7	10	0	7
Gellert-Holtzman score	1.3 ± 0.5	13.2 ± 1.2**	20.4 ± 1.1**	23.1 ± 1.2**	31 ± 1.7**	30 ± 1.6**	1.5 ± 0.6	25 ± 1.5**#
Etho-score	0.2 ± 0.1	2.6 ± 0.4**	4.5 ± 0.4**	5.9 ± 0.4**	7.2 ± 0.3**	8.7 ± 0.4**	0.3 ± 0.1	9.4 ± 0.8**

Mean ± SEM; *$P < 0.05$, **$P < 0.01$ vs. control group (Dunnett repeatedly tested; Student's *t*-test, singly tested); #$P < 0.05$, ##$P < 0.01$ vs. NalR-1 group (Student's *t*-test)

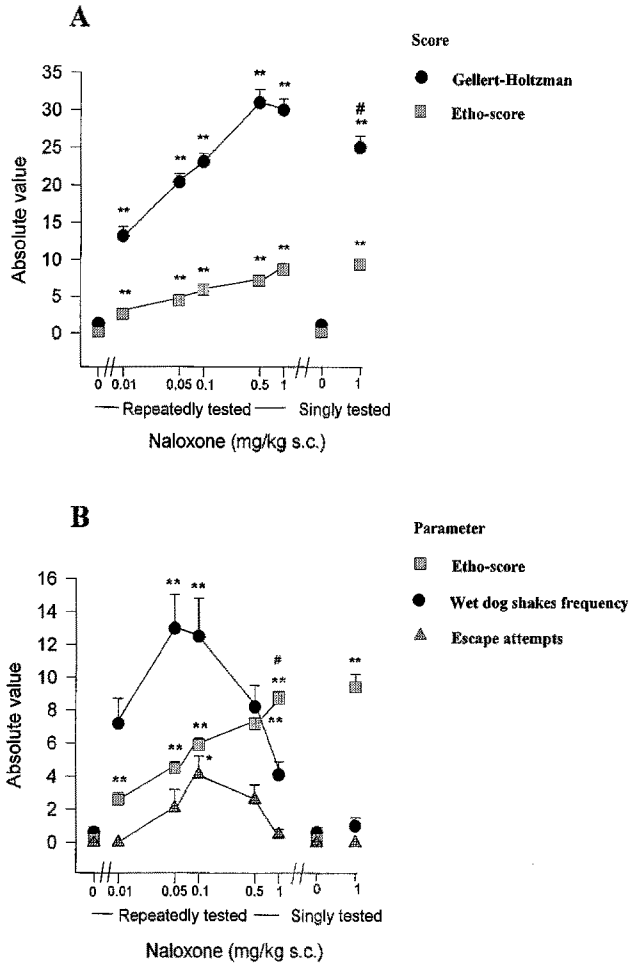


Fig. 4 **A** Values of the Gellert-Holtzman score and the etho-score. **B**: Values of the etho-score, wet-dog shakes frequency and escape attempts. This figure represents a comparison among classic indexes for measuring opiate withdrawal and the etho-score. Wet-dog shakes category is composed of both body-shake and hand-shake. Repeatedly and singly tested groups are separatedly represented along the horizontal axis. Mean + SEM; * $P < 0.05$, ** $P < 0.01$ vs. control group (Dunnett); # $P < 0.05$ vs. 1 mg/kg naloxone in the repeatedly tested group (Student's t-test)

[$F(5, 54) = 76.8$, $P < 0.0001$], as shown in Fig. 4A. After comparing both the NalR-1 and NalS-1 groups, the Gellert-Holtzman score was significantly lower in the NalS-1 than in the NalR-1 group ($t = 2.3$, $P < 0.05$). Etho-score values were also significantly enhanced after naloxone [$F(5, 54) = 60.1$, $P < 0.0001$], as observed in Fig. 4. After comparing both the NalR-1 and NalS-1 groups, etho-score values were found to be quite similar in both groups (NalR-1, 8.7 ± 0.4; NalS-1, 9.4 ± 0.8). In repeatedly tested groups, a significant correlation was found between the Gellert-Holtzman score and the etho-score ($r = 0.97$, $P < 0.001$). Non-significant correlations were found between the etho-score and wet-dog shakes frequency ($r = 0.34$) and escape attempts ($r = 0.52$; Fig. 4B).

Discussion

The ethological analysis revealed that the rat's behaviour during morphine withdrawal is made up of exploratory, self-care, wet-dog shakes, escape, mastication and writhing responses. The first four categories were strongly diminished after high naloxone doses, hence writhing posture and mastication provide the best behavioural indexes for measuring the naloxone-precipitated morphine withdrawal. Writhing posture was, however, highly influenced by learning. Weight loss, the principal physical sign, was also found to be closely related to the morphine withdrawal degree. An "etho-score", based on mastication and weight loss, is herein proposed.

With respect to cluster analysis results, control rats' behaviour was mainly composed of two clear-cut categories: "exploration" and "self-care". Exploration encompassed quiet (immobile-sniff) and mobile (walk-sniff) patterns (Espejo and Mir 1993). Self-care was made up of self-grooming and face-washing. Naloxone treatment maintained these categories, and two new categories were induced: "writhing behaviour" and "escape". Writhing behaviour was composed of writhing posture and attenuated gait. Escape was made up of jumping, an intense escape reaction, and leaning posture. Leaning posture was also linked to exploratory reactions, hence it seems to be an escape/exploratory pattern (Espejo et al. 1994). Genital grooming emerged as a self-care pattern after naloxone. Although the basic criterion to select categories was a similarity value higher than 80, the information derived from similarity matrices enhanced category boundaries and helped elucidate the ethological "meaning" of other patterns. Body-shake and hand-shake were usually linked to each other, hence the classic "wet-dog shakes" category emerged (Wei et al. 1972, 1973; Calvino et al. 1979; Malin et al. 1990). Mastication can be considered an "abnormal" pattern highly linked to writhing responses. Finally, freezing is classically considered as an alert reaction (Barnett 1975).

The ethological data of repeatedly tested rats revealed that writhing behaviour was enhanced in a dose-dependent manner. Mastication was also enhanced, although a "ceiling" effect was apparent after 0.1 mg/kg naloxone. Exploration, self-care and lying responses decreased in a parallel fashion. These behaviours are strongly sensitive signs of abstinence in rats implanted with two standard pellets of morphine, and most of them are already used as indicators of withdrawal degree (Kerr and Pozuelo 1971; Bläsig et al. 1973; Bläsig and Herz, 1977; Espósito et al. 1987; Stinus et al. 1990, 1992; Maldonado et al. 1992; Spanagel et al. 1994). In this context, it is worth noting that very low doses of naloxone precipitated signs of abstinence in rats implanted with two standard pellets of morphine. Higgins and Sellers (1994) reported that no physical signs were noted with naloxone doses

lower than 0.05 mg/kg. Nevertheless, they employed a single morphine 75 mg pellet to render rats tolerant to morphine. With respect to wet-dog shakes and escape attempts, behaviours which are usually selected to evaluate opiate withdrawal degree, the data revealed that they are very sensitive for measuring abstinence degree at moderate naloxone doses. However, in contrast to previous statements (Gmerek 1988), changes in wet-dog shakes and escape attempts were not well correlated to every naloxone dose, as revealed by the U-shaped curve. These responses appear not to be very sensitive for doses lower than 0.05 mg/kg or higher than 0.5 mg/kg, being affected by the strong increase in mastication and writhing posture. Taken together, our findings indicated that wet-dog shakes and jumping are useful for evaluating moderate morphine withdrawal, and mastication and writhing posture for evaluating the effect of a wider range of naloxone doses. In this context, the writhing posture has already been highlighted as a very useful indicator by Von Voigtlander and Lewis (1983). These authors quantified withdrawal from morphine agonists by the increased incidence of writhing responses to intraperitoneal hypertonic saline injections.

Learning reliably affected some behavioural indicators such as exploration and writhing behaviour. Naive or unexperienced rats displayed more exploratory behaviour and less writhing responses than experienced animals after naloxone. Other indicators such as mastication, wet-dog shakes and escape attempts were not reliably modified. The experimental protocol which was used for test repetition minimized learning effects on rats' behaviour except for exploration and writhing responses.

With respect to physical signs, decreases in body weight were closely related to the intensity of morphine withdrawal in repeatedly tested animals, confirming that loss of body weight is a very sensitive physical sign of abstinence (Bläsig et al. 1973; Gellert and Holtzman 1978; Gmerek 1988; Higgins and Sellers 1994; Spanagel et al. 1994). As expected, weight loss degree was highly correlated to the presence of diarrhea, suggesting that diarrhea is the main factor influencing weight loss. Besides, weight loss and diarrhea were little enhanced by learning. On the other hand, number of micturitions was reliably enhanced by learning.

The Gellert-Holtzman score was validated during this study, being an accurate tool for evaluating morphine withdrawal. Learning reliably modified the Gellert-Holtzman score value, a finding that could be explained because this scale encompasses several physical factors (salivation, ptosis, chromodacryorrhea) which were strongly enhanced by learning.

In rats with placebo pellets, lying was reliably lower and self-care responses were higher in saline than in naloxone-treated rats. Numerous reports indicate that opioid systems are activated by exposure to stress, inducing locomotor hypoactivity and grooming behaviors in the rat (Bläsig et al. 1978; Roth and Katz 1980; Arnsten et al. 1985). Hence, findings would suggest that the experimental test represents a stressful situation, which caused the release of endogenous opiates. Besides, the data indicated that naloxone alone did not cause signs which characterize withdrawal in morphine-dependent rats. Curiously, body-shake was displayed by placebo and singly tested rats without naloxone, indicating that this pattern would have a stress-related component.

Mastication and weight loss represent the basis of a newly developed rating score, namely an "etho-score". They were selected to design the "etho-score" because they are easily quantifiable signs, were little affected by learning, and weight loss was progressively enhanced by increasing naloxone doses. Mastication was affected by a "ceiling" effect as explained, but this fact did not reliably alter the progressive increase in the "etho-score" values. Thus, the "etho-score" was very sensitive to the entire range of naloxone doses used during the study, gave a good picture of the morphine withdrawal degree, and it was not reliably influenced by learning in contrast to the Gellert-Holtzman score. Besides, the "etho-score" possesses other advantages: i) it is simpler than other scales, ii) it does not even require a videotaping system or extensive computer analysis as factors can be easily scored during direct observation, and iii) its variance is very low. In summary, the "etho-score" was proved to be accurate, providing one more alternative to the methodologies that are available for measuring the intensity of naloxone-precipitated morphine withdrawal.

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