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Ultrasounds during morphine withdrawal in rats

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Abstract. Ultrasounds (US) in rats may communicate affective states, as they occur only in highly significant situations such as maternal care, sex and aggression. Withdrawal from morphine is a manipulation which dramatically alters autonomic, somatic and motor functions; the present experiment demonstrated the production of US in this context and the influence of previous social experience in their production. Sixty male Long-Evans rats with distinct social experiences (social inexperience, defeat or copulation) underwent 72 h of continuous morphine exposure (4 × 75 mg morphine or placebo pellets) and subsequent withdrawal. The rats were observed for 10 min in equally treated pairs and while solitary at 6, 24 and 96 h after pellet removal. US were emitted by all groups and consisted primarily of two distributions of pure tone whistles with little frequency modulation: 1–2 s 21–25 kHz ("low") signals and the more prevalent 0.02–0.1 s 44–52 kHz ("high") signals. Morphine withdrawn rats lost weight, displayed wet dog shakes, were hypoactive and emitted threefold more US vocalizations with a fourfold greater duration than placebo controls. Defeat-experienced morphine withdrawn rats were more hypoactive than either socially inexperienced or copulatory experienced rats while increasing vocalization rates and total duration. This increased duration of ultrasounds included a shift in the distribution of individual US durations from less than 0.3 s to greater than 1.0 s. US are readily emitted at high rates in morphine withdrawn laboratory rats, which may implicate an opioid involvement in their generation. Furthermore, relevant social experiences such as copulation and defeat facilitate the emission of US during morphine withdrawal and may serve as an index of the affective components of withdrawal.

Key words: Ultrasounds – Vocalization – Morphine – Withdrawal – Aggressive behavior – Social behavior – Sexual behavior – Affect – Opiates

Withdrawal from morphine causes profound disturbances in autonomic, somatic and motor functions, as well as major changes in affect (Jaffe 1985). In the rat, the "classic" opiate withdrawal signs include diarrhea, increased motor activity and grooming, jumping, teeth chattering, weight loss, "wet dog" shakes and writhing (e.g., Gmerek 1988). The time course of each sign differs and depends on the opiate dose regimen and the specific agonist that is used to produce dependence (Bläsig et al. 1973). Additionally, opiate withdrawal includes "scream on touch" vocalizations which are audible to the human experimenter and may indicate a hyperreactivity to pain stimuli (Gianutsos et al. 1975).

Animal vocalizations can be emitted in the human auditory range as well as below and above it. These latter sounds are referred to as ultrasonic vocalizations (US) and have been documented in gerbils (Elwood 1979), hamsters (Floody 1979), mice (Whitney and Nyby 1979), rats, including Long-Evans (Peters et al. 1988), Wistar (Sales 1972) and Sprague-Dawley (Insel and Harbaugh 1989), and other species; these signals occur from 20 to 200 kHz. In rats, US occur at 20–30 kHz or at 40–50 kHz and show relatively little frequency modulation. US are particularly interesting as they occur only in significant situations, such as mating (Whitney et al. 1973; Kaltwasser 1990), maternal (Bell et al. 1971), agonistic behavior (Thomas et al. 1983; Kaltwasser 1990) and exposure to painful stimuli (van der Poel et al. 1989). Furthermore, US can be influenced by behavioral experience (Nyby et al. 1976) and can be modulated pharmacologically (Cuomo et al. 1988a, 1987b). It has been proposed that US may serve a communicative purpose (Nyby and Whitney 1978; Smith 1979) and as an index of an affective state (Tonoue et al. 1986; Cuomo et al. 1988b); however, a functional explanation of different kinds of US remains largely elusive (see however, Blumberg et al. 1987) and information on their neurobiological basis is preliminary.

Endogenous opioid peptides and drugs acting on their receptors have been implicated in the mechanisms mediating the production of US. In an investigation of ultrasonic distress calls during unavoidable aversive stimulation (i.e., mild tailshock), morphine dose-dependently decreased the time spent vocalizing in the pre-stimulation period; at 6.0 mg/kg there was almost a complete suppression of ultrasonic vocalizations (van der Poel et al. 1989). Rats continued to vocalize in the post-

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stimulation period, implying that the suppression of prestimulus US was not due to a complete suppression in pain perception. Opiate specificity was evidenced as naloxone (1.0 mg/kg) reversed the rate attenuating effects on US. Morphine and ethylketocyclazocine proved to be very potent in suppressing ultrasonic vocalizations in rats exposed to the threat of attack at doses even lower than those for antinociceptive responses to threat stimuli (Vivian and Miczek, in preparation). The opiate peptides beta-endorphin, dynorphin, met- and leu-enkephalin strongly suppressed US in reaction to electric foot shock; this effect was not due to analgesia as the audible, or pain-related, squeak was not altered and the dose used was one-tenth that needed for pain suppression (Tonoue et al. 1986). Furthermore, these researchers suggested that ultrasonic vocalizations might serve as a measure of affect, particularly fear, and proposed that the suppression of US by opiates reflects an attenuation of the fear reaction.

Consistent with the proposal that opiates modulate US vocalizations, electrical stimulation of brain sites has revealed that the most sensitive areas for US production are the central gray, posterior hypothalamus and ventral reticular formation (Yajima et al. 1980, 1981). These areas are rich in opiate receptors and agree with the proposed distress vocalization areas of Panksepp et al. (1978a), who included also the dorsomedial hypothalamus and central amygdala. Panksepp et al. (1978a) proposed that opiates mediate social behavior and bonding and that separation-induced audible and ultrasonic distress vocalizations are particularly sensitive to the opiate manipulations; these vocalizations may be indicative of the social development of the neonate, and later, the adult (Panksepp et al. 1978a, b, 1982; Herman and Panksepp 1981). Panksepp has found that opiate agonists, including morphine, the endorphins and enkephalins, reduce audible distress vocalizations while opiate antagonists increase them. Finally, central mediation was evidenced as intracerebral administration of naloxone antagonized morphine's suppression of distress vocalizations (Panksepp et al. 1978a).

It was an objective of the present experiment to address several questions: (1) Are US emitted during opiate withdrawal? (2) Are these US indicators of affective state? (3) Because the neurobiological mechanisms of US vocalizations have yet to be determined, one further objective was to investigate possible experiential and pharmacological factors in their production; specifically, do significant behavioral experiences such as defeat or copulation influence the production of US during morphine withdrawal? (4) Finally, a more detailed analysis of the frequency and temporal characteristics of US vocalizations during withdrawal may differentiate these sounds from other US during sexual and aggressive encounters.

Materials and methods

Subjects

Sixty experimentally naive male hooded Long-Evans rats (Charles River Labs, Wilmington, MA) weighing 300–400 g were housed individually in standard hanging stainless steel cages in an environmentally controlled animal colony (12/12 h light/dark cycle, $21 \pm 1°$ C, 30–40% humidity).

Experimental design and procedures

Subjects were evenly assigned into three groups ($n = 20$ each) according to their behavioral experience prior to pellet implantation: (1) socially inexperienced, (2) defeat experienced or (3) copulatory experienced. Within 72 h of the behavioral experience, subjects were implanted with morphine base or placebo pellets for 72 h. Subsequently, the pellets were removed and equally treated subjects were paired for 10 min dyad tests followed by 5 min solitary tests in $30 \times 30 \times 40$ cm^3 clear Plexiglass test cages at 6, 24 and 96 h after pellet removal (see Fig. 1 for a time line of the events).

Behavioral experience. In the socially inexperienced group, subjects remained in the home cage until pellet implantation. Defeat experienced subjects served as intruders in two agonistic encounters with an experienced resident (Miczek 1979). These encounters were terminated at 5 min or ten bites, whichever came first. Subjects which displayed submissive postures (supine, upright and crouch) and emitted ultrasonic vocalizations were subsequently implanted with pellets. Copulatory experienced subjects were exposed to an estrous female and allowed to copulate. Subjects which ejaculated and emitted ultrasonic vocalizations during the postejaculatory phase (Barfield and Geyer 1975) were subsequently implanted. All implantations occurred within 72 h of the defeat or copulatory experience.

For each group of 20 subjects, half were randomly assigned to the morphine and the other half assigned to the placebo condition. Under ether anesthesia (Fisher Scientific), subjects were subcutaneously implanted with four 75 mg morphine base or placebo pellets (National Institute on Drug Abuse). Each pellet was wrapped in nylon (Cochin et al. 1979) to facilitate pellet removal. The pellets were removed 72 h later under ether anesthesia.

Body weight was measured immediately prior to pellet implantation, at pellet removal, and at 6, 24 and 96 h after removal. At 6, 24 and 96 h after pellet removal, two equally treated subjects were placed in the testing cage for video and audio recordings. These observations were comprised of 10 min dyad tests immediately followed by 5 min solitary tests, in which one subject was removed from the current test cage and placed in a separate test cage.

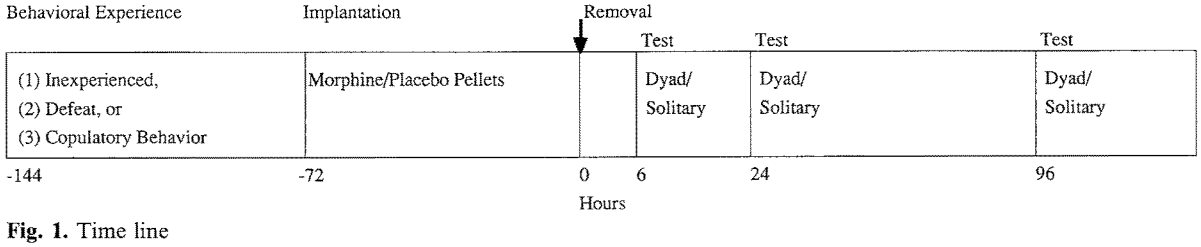

Fig. 1. Time line

Audio recordings. Ultrasonic vocalizations were recorded with a condensor microphone (Bruel & Kjaer Type 4135) suspended 35 cm from the floor of the cage, preamplifier (Bruel & Kjaer Type 2633) and amplifier (Bruel & Kjaer Type 2610) in conjunction with a filter (Krohn-Hite Model 3550R; nominal settings: 20–100 kHz, bandpass). Sounds were recorded onto tapes (Maxell UD 25–120N) using an eight channel instrumentation recorder (Hewlett-Packard Model 3968A). This equipment provided a flat frequency response in the 20–65 kHz range.

Analog signals from the instrumentation recorder were analyzed with two systems: (1) Initial analysis of rate and duration of vocalizations were obtained by playback of the audio tape at quarter speed through an amplifier into headphones (Radio Shack Model 33–2002). Trained listeners depressed keys in response to traces on an oscilloscope (Telequipment Model DM64) and concurrently heard through the headphones. The data were summarized with customized software (Princeton Economics, Princeton, MA). (2) A more detailed analysis of the frequency characteristics was performed with hardware/software developed for the Apple MacIntosh computer. This system (MacSpeech Lab II; GW Instruments, Cambridge, MA) digitizes the output from the instrumentation recorder and displays the vocalizations in five ways: time waveforms, amplitude/energy envelopes, fundamental frequency plots, fast Fourier transforms (FFT) and sound spectrograms. Furthermore, any segment of a vocalization can be expanded for more detailed analysis and these vocalizations can be stored in spreadsheets or graphical form. For the current experiment, the first 20 "low" (20–32 kHz) and "high" (32–65 kHz) ultrasonic vocalizations for every pair of subjects were digitized with the GWI system to determine beginning, end and modal frequencies.

Video recordings. The experimental setting was illuminated with an infrared light source. Behavior was recorded with an infrared-sensitive camera (Canon Model Ci20R) in conjunction with a VHS video cassette recorder (Zenith Model VR 3300) and time code generator (Skotel Model TCG–80N).

Frequency and duration of each behavioral event were observed on a color monitor (NEC Model PM1971A) by trained observers and summarized with customized software. The behavioral events included allogroom, ano-genital contact, autogroom, inactivity, jump, penile lick, rear, stereotyped sniff, teeth chatter, walk, wet dog shake and writhe following the operational definitions of Bläsig et al. (1973), Gianutsos et al. (1975), Gmerek (1988) and Yoburn (1988).

Data analysis

Audio and video measurements were analyzed with a three-factor (treatment × experience × time) ANOVA with repeated measures. When significant effects were present, post-hoc Newman-Keuls analyses were performed on between-group comparisons; for within-group analyses, a difference was considered significant when greater than 2 SD. In the analysis of individual US durations, the z test for proportions was used (Kachigan 1986). Significance was $P<0.05$, two-tailed.

Results

Dyad condition

US vocalizations were detected from all groups and consisted primarily of two distributions of pure tone whistles with little frequency modulation: 1–2 s 21–25 kHz (low) signals and the more prevalent 0.02–0.1 s 44–52 kHz (high) signals. A characteristic spectrogram of low and high frequency US emitted 24 h after pellet removal in defeat experienced subjects is given in Fig. 2. Most US were less than 1.0 s in duration, although some extended up to 5.0 s. The beginning (Mean ± SD: 24 537 ± 798 Hz), modal (23 137 ± 1121 Hz) and end (23 293 ± 1186 Hz) frequencies of low US vocalizations from morphine withdrawn subjects did not differ from the beginning (24 732 ± 1609 Hz), modal (23 293 ± 1548 Hz) and end (23 272 ± 1622 Hz) vocalizations of control subjects. Likewise, there was no evidence of frequency modulation or increased variance in the high frequency vocalizations or signals resulting from different behavioral experience.

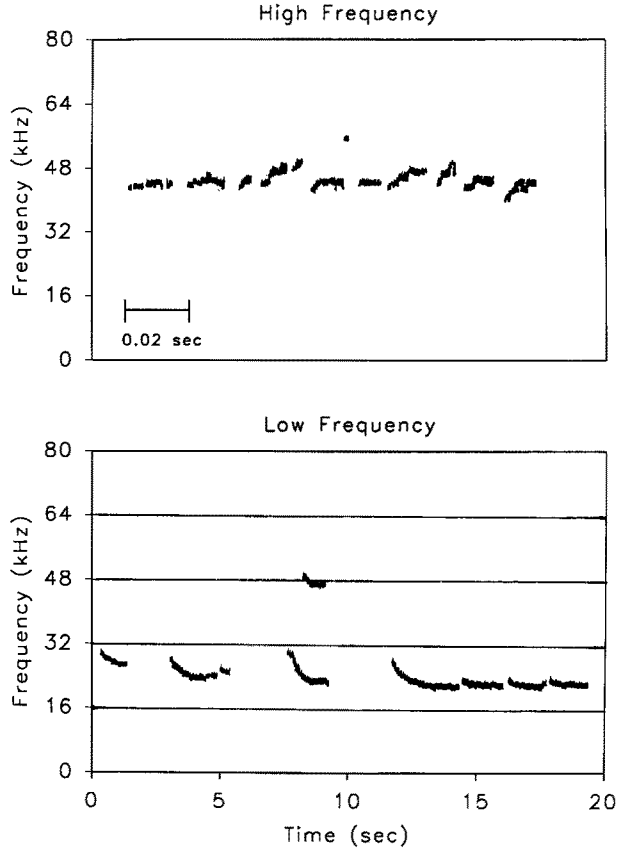

Fig. 2. Spectrograms of high (32–65 kHz) and low (20–32 kHz) frequency vocalizations for a defeat experienced morphine withdrawn rat. Note that the high frequency calls are representative samples and the time base between calls varies; low frequency calls are depicted in real time

As shown in Fig. 3, morphine withdrawal increased the rate and total duration of ultrasounds [$F(1,22)=15.06$, $P<0.01$; $F(1,22)=13.05$, $P<0.01$]. Withdrawal from morphine produced a threefold increase in the rate with a fourfold increase in the total duration of US vocalizations; this potentiation of US was most prominent at 6 h after pellet removal. Prior social experience also influenced the probability of ultrasonic vocalizations. Defeat experienced animals vocalized at a similar rate but with a threefold increase in total duration as compared to sexually experienced animals; sexually experienced animals emitted threefold more frequent vocalizations with an increased duration

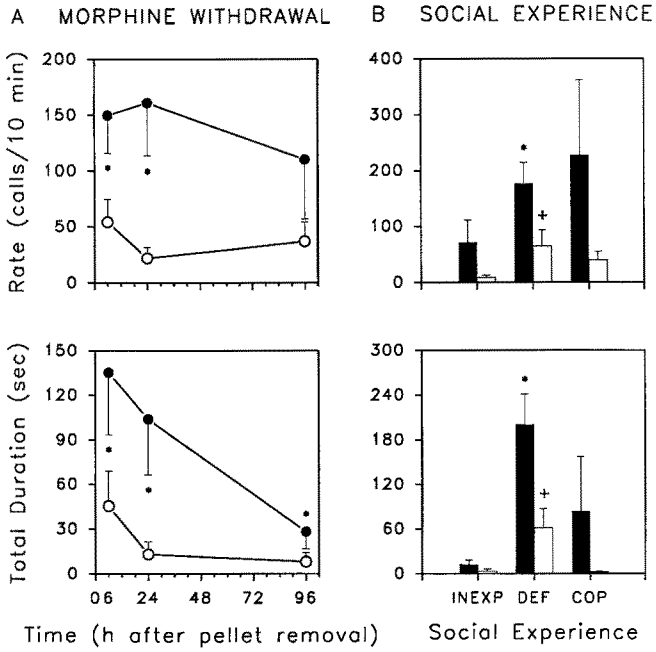

Fig. 3. **A** Mean rate (calls/10 min) and total duration (s) of ultrasonic vocalizations throughout 96 h withdrawal for morphine (●) and placebo (○) implanted rats. Error bars denote SEM, *asterisks* denote significant differences ($P<0.05$) from placebo controls. Each point is the combined vocalizations of socially inexperienced, defeat and copulatory experienced rats. **B** Mean rate (calls/10 min) and total duration (s) of ultrasonic vocalizations for socially inexperienced, defeat and copulatory experienced rats. Error bars denote SEM, *asterisks* and *crosses* denote significant differences ($P<0.05$) from socially inexperienced comparison group. Each bar is the combined vocalizations across the 96 h withdrawal period

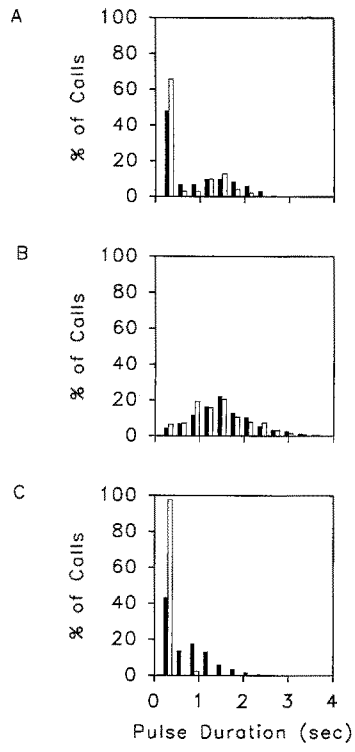

Fig. 4A–C. Frequency histogram of percentage of individual calls of varying durations for (**A**) socially inexperienced, (**B**) defeat and (**C**) copulatory experienced rats after 24 h of withdrawal. ■ Morphine WD; □ placebo

as compared to socially inexperienced animals [$F(2,22)=4.72$, $P<0.05$; $F(2,22)=8.80$, $P<0.01$]. The duration of calls was significantly attenuated over the withdrawal period [$F(2,44)=6.50$, $P<0.01$]; at 96 h after pellet removal, the duration of US vocalization in morphine withdrawn subjects approached control values.

Analysis of the ultrasound pulse durations revealed that morphine treatment consistently decreased the proportion of short 0.3 s pulses (socially inexperienced: $z=4.92$, defeat experienced: $z=7.30$ and sexually experienced: $z=28.74$, $P<0.05$). In contrast, defeat experience influenced not only the 0.3 s vocalizations but also shifted the overall distribution to longer 0.6–2.1 s vocalizations (0.6: $z=10.39$, 0.9: $z=16.98$, 1.2: $z=15.53$, $z=16.77$, 1.8: $z=12.00$, 2.1: $z=9.81$, $P<0.05$). Figure 4 depicts the shift in US pulse duration 6 h after pellet removal.

Morphine pellet exposure did not significantly alter body weights. At pellet implantation, mean body weight of morphine pelleted subjects was 332.0 ± 8.1 g, and at pellet removal mean weight was 330 ± 8.1 g (Mean ± SEM). Similarly, implantation and exposure to placebo pellets did not produce weight changes (implant: 300.5 ± 6.6 g, removal: 308.3 ± 6.8 g). Withdrawal from morphine produced a decrease in body weight which peaked (ca. 10%) at 24 h [$F(6,159)=4.27$, $P<0.01$]; this

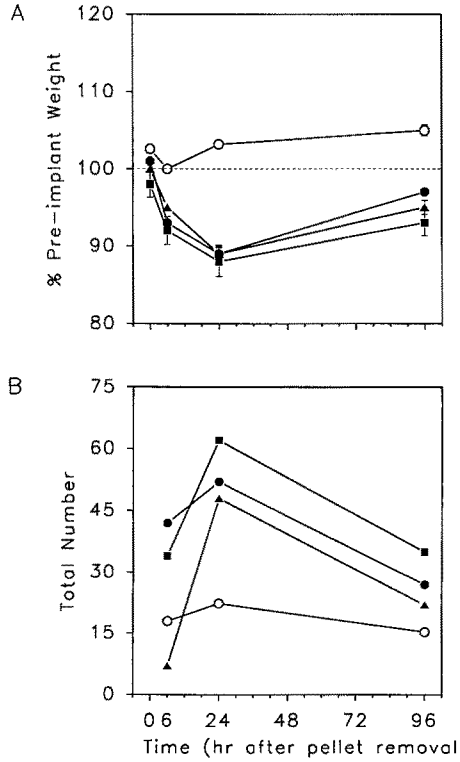

Fig. 5. **A** Percentage pre-implant body weight and **B** total wet dog shakes during 96 h morphine withdrawal. Error bars denote SEM, *asterisks* denote significant differences ($P<0.05$) from placebo control. ● Morphine WD; ▲ MWD + defeat; ■ MWD + copulation; ○ placebo

effect partially recovered by 96 h. Body weight loss was not affected by previous behavioral experience. Similarly, wet dog shakes were pronounced in the morphine withdrawn groups [$F(1,54)=12.35$, $P<0.01$]; particularly at 24 h after pellet removal. Wet dog shakes also were not affected by previous behavioral experience (Fig. 5). Withdrawal jumps were observed 24 and 96 h after withdrawal of morphine pellets.

Motor behavior did not differ between withdrawn and placebo groups; in general, control animals exhibited decreased walking, rearing, allo- and auto-grooming, and prolonged inactivity across all phases of withdrawal. Defeated animals were less active and less likely to initiate behaviors at 6 and 24 h after pellet removal. Inactivity duration was increased in the defeat experienced group, not differing from the socially inexperienced and copulatory experienced subjects at 96 h after pellet removal [$F(4,108)=3.94$, $P<0.01$]. All motor behaviors returned to control values at 96 h after pellet removal.

Solitary condition

Rats in the solitary condition, regardless of treatment or group, were predominantly inactive (83% of the total duration). Ultrasounds were very infrequent during this condition.

Discussion

This experiment describes a feature of opiate withdrawal, namely ultrasonic vocalizations, that has not been documented before. Their rate and duration are increased at 6 and 24 h into the withdrawal period and begin to decline by 96 h. Ultrasonic vocalizations are readily emitted during withdrawal from morphine in rats with previous defeat or copulatory experience. While prior experience did not consistently alter the "classic" behavioral signs of withdrawal, such as wet dog shake, jump and hypoactivity, the large potentiation of temporal and intensity parameters of ultrasonic vocalizations in defeated morphine withdrawn animals differed from those animals with copulatory experience. US resulting from an aversive agonistic situation differ from those in a copulatory context: both low and high frequency US are evidenced in agonistic encounters while only the low frequency US appear relevant in the current copulatory context; this may reflect "distress" or similar affective expressions which can be dissociated from motoric and autonomic signs of withdrawal. In the present experiment, animals receiving prior social experience emitted a greater number of low frequency US than inexperienced subjects, yet frequency modulation did not appear to be relevant in the social interaction tests as neither pellet implantation nor previous behavioral experience altered the beginning, end or modal frequencies.

As decreased body weight provides a reliable index of morphine dependence and withdrawal (Wei and Way 1975), it is clear that the current 4×75 mg morphine pellet regimen produced dependence. The current 10% body weight loss in all morphine implanted groups confirms previous evidence (Wei and Way 1975; Yoburn et al. 1985). The current observations also confirm wet dog shakes as another index of morphine withdrawal (Bläsig et al. 1973; Wei and Way 1975). The frequency of this behavior over the withdrawal period paralleled weight loss (peaking at 24 h after pellet removal) and provided a concurrent assessment of the degree of morphine dependence.

Although morphine treatment had no effect on many of the measures of motor behavior during withdrawal, defeated subjects were consistently less active. Defeated animals are characterized by decreased activity as demonstrated through decreased exploration and a restricted behavioral repertoire (Miczek et al. 1991). Following defeat from an aggressive conspecific, intruder C57 mice displayed an increased duration of immobility, crouch and defensive upright postures in the presence of nonaggressive conspecifics (Siegfried et al. 1982, 1986). Similarly, the attenuation in motor behaviors evidenced in the current defeated subjects may be an "adaptive" conditioned response based on prior social history.

The pharmacological mechanisms of US have only recently begun to be explored. An opiate influence on US has been proposed (Yajima et al. 1980, 1981; Cuomo et al. 1988b; van der Poel et al. 1989). Vocalizations are attenuated by opioid administration which can be reversed with naloxone; furthermore, central sites which are rich in opiate receptors (e.g., central gray, posterior hypothalamus and ventral reticular formation) are correlated with the most sensitive areas for electrical stimulation of US and isolation calls of chicks (Panksepp et al. 1978a).

Additionally, catecholaminergic systems have been implicated in the production of US: apomorphine, haloperidol and clonidine have been found to affect US in gerbils (Thiessen and Upchurch 1981). SCH 23390 (D1 antagonist) increased the duration, sulpiride (D2 antagonist) decreased the rate of US in rats (Cuomo et al. 1987a) and *d*-amphetamine dose-dependently suppressed the rate of US in submissive rats (Miczek 1979). Prolonged postnatal exposure to the D2 antagonist haloperidol reduced the rate but increased the duration of US through day 14 of age (Cagiano et al. 1986). As dopaminergic and opiate neurons may be collocalized, particularly in limbic structures (Attila and Ahtee 1984), US may be ideally suited for questions concerning affective components that are mediated by opioid-dopaminergic mechanisms.

While much of the literature on US contains largely descriptive accounts (rate, duration, intensity, frequency), the present experiment includes an analysis of the temporal structure of vocalizations which will be important for further research. Specifically, defeat experience substantially shifted the proportion of short and long vocalizations, indicating that this type of analysis is sensitive to behavioral manipulations. Interestingly, the respiratory depressant effects of opiates have been dissociated from their ability to reduce US by analyzing the temporal structure of US (van der Poel et al. 1989). This type of analysis will increase our knowledge of behavior-

al and pharmacological effects on US and may provide an insight as to the communicative or functional aspects of US (see also Pontet et al. 1989).

In conclusion, US are readily emitted from rats during morphine withdrawal and their production can be influenced by the animal's previous experience with defeat and copulatory behavior. Unlike the "classic" signs of withdrawal, the analysis of US vocalizations is sensitive to the subject's social behavioral history. The defeat experience provides a strong behavioral manipulation which potentiates US production and shifts the temporal distribution of individual US from short (less than 0.1 s) to long (more than 1.0 s) durations. Frequency modulation of US is of little relevance during morphine withdrawal. US vocalizations during withdrawal from morphine have very similar frequency and temporal characteristics to US vocalizations emitted by rats exposed to the threat of attack (Vivian and Miczek, in preparation) and may communicate a similar affective expression.

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