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Profound decrement of mesolimbic dopaminergic neuronal activity during ethanol withdrawal syndrome in rats: Electrophysiological and biochemical evidence

(dopamine neurons/mesolimbic system/craving)

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ABSTRACT **Activity of the mesolimbic dopaminergic system was investigated in rats withdrawn from chronic ethanol administration by single-cell extracellular recordings from dopaminergic neurons of the ventrotegmental area, coupled with antidromic identification from the nucleus accumbens, and by microdialysis-technique experiments in the nucleus accumbens. Spontaneous firing rates, spikes per burst, and absolute burst firing but not the number of spontaneously active neurons were found drastically reduced; whereas absolute and relative refractory periods increased in rats withdrawn from chronic ethanol treatment as compared with chronic saline-treated controls. Consistently, dopamine outflow in the nucleus accumbens and its acid metabolites were reduced after abruptly stopping chronic ethanol administration. All these changes, as well as ethanol-withdrawal behavioral signs, were reversed by ethanol administration. This reversal suggests that the abrupt cessation of chronic ethanol administration plays a causal role in the reduction of mesolimbic dopaminergic activity seen in the ethanol-withdrawal syndrome. Results indicate that during the ethanol-withdrawal syndrome the mesolimbic dopaminergic system is tonically reduced in activity, as indexed by electrophysiological and biochemical criteria. Considering the role of the mesolimbic dopaminergic system in the reinforcing properties of ethanol, the depressed activity of this system during the ethanol-withdrawal syndrome may be relevant to the dysphoric state associated with ethanol withdrawal in humans.**

Alcoholism is a major economic, social, and health problem (1). Indeed, alcohol is the most abused substance after nicotine in the Western world, and alcohol abuse and dependence ranked first of all psychiatric disorders in lifetime prevalence rates (2). As for many other abused drugs, this compulsive behavior seems to be elicited and maintained by the powerful reinforcing properties of the drug.

Dopamine (DA) is one of the major candidates suggested to mediate reinforcement in animals (3): accordingly, rats will self-stimulate when electrodes are placed near DA neurons in the ventrotegmental area (VTA) (4, 5), and many addicting drugs, including ethanol, increase DA release in the nucleus accumbens (6) and increase DA firing in the VTA (7).

The ethanol-withdrawal syndrome begins after cessation of prolonged ethanol administration. A symptom common to withdrawal syndromes, regardless of the substance abused, is the dysphoria associated with absence of the drug. In spite of the evidence linking an increase in dopaminergic transmission to the hedonic properties of ethanol (8), no study has tested *in vivo* whether the ethanol-withdrawal syndrome is associated with a decline in mesolimbic dopaminergic activity. However, a reduction of DA turnover has been reported (9), and a decrease in K^+-stimulated DA release *in vitro* has been seen in slices obtained from rats withdrawn from chronic ethanol administration (10). These findings are consistent with the possibility that dopaminergic tone in the mesolimbic system might be reduced *in vivo* during the ethanol-withdrawal syndrome. On this basis we decided to investigate whether DA release *in vivo*, as measured by microdialysis, is reduced during the ethanol-withdrawal syndrome and whether this release depends on the reduced neuronal activity of mesolimbic dopaminergic neurons.

Preliminary experiments on this issue have supported this hypothesis (11).

MATERIALS AND METHODS

Subjects and Ethanol Treatment. Male Sprague–Dawley rats (Charles River, Como, Italy) weighing 200–225 g were used. Ethanol (20% vol/vol) was administered intragastrically every 6 hr for 6 consecutive days at a dose of 2–5 g/kg, according to the method of Majchrowicz (12). The dose was adjusted to the degree of ethanol intoxication, assessed by the experimenter, to maintain steady intoxicating levels of plasma ethanol (12). Briefly, before each ethanol administration, rats were evaluated, and a score from neutrality to coma was assigned. When neutrality was scored, rats received the highest dose (5 g/kg) of ethanol. When severe signs of intoxication, such as loss of the righting reflex and coma, were scored, no ethanol was administered in that session. At intermediate intoxication levels (sedation, ataxia 1, 2, and 3), an intermediate ethanol dosage was administered (4, 3, 2, and 1 g/kg, respectively).

Microdialysis Experiments. On the morning of the sixth day of ethanol treatment (2 hr after the last ethanol administration), subjects were anesthetized with chloral hydrate (200 mg/kg i.p.) and placed in a stereotaxic frame for fiber implantation, as already described for the striatum (13). One dialysis fiber (AN69, Hospal, France) per rat was inserted horizontally at the level of the nucleus accumbens [at coordinates; anterior: 1.7 mm; ventral: 7.3 mm from bregma, according to the atlas of Paxinos and Watson (14)]. Fiber inlet and outlet were secured to the skull with dental cement, and rats were then returned to their cages to undergo the last day of ethanol treatment. On the seventh day (10–12 hr after the last ethanol administration) rats were connected to the infusion pump, and the dialysis membrane was perfused (2 μl/min) with Ringer solution (147 mM NaCl/3 mM KCl/2.2 mM $CaCl_2$/1.2 mM $MgSO_4$/0.4 mM KH_2PO_4, pH 7.2).

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Abbreviations: DA, dopamine; VTA, ventrotegmental area; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid.

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Twenty-minute perfusates (40 μl) were injected into the HPLC column (5 mm Beckman ultrasphere C-18, 150 × 4 mm) for separation of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). Quantification was done by electrochemical detection (Waters Amperometric detector M 460; electrode voltage was set at 0.65 V). The mobile phase consisted of 50 mM citrate/acetate buffer, pH 4.6, containing octyl sulfate at 30 mg/l, 0.1 mM EDTA, and 7% (vol/vol) methanol.

Electrophysiological Experiments. Single-cell extracellular recordings from antidromically identified mesoaccumbens dopaminergic neurons were done, as already described for nigrostriatal DA cells (15). Briefly, on the seventh day from onset of chronic ethanol treatment (10–12 hr after the last ethanol dose), rats were tested for withdrawal signs and assigned a withdrawal score according to the rating scale of Lal *et al.* (16). Most animals (74%) chronically treated with ethanol displayed a robust withdrawal syndrome characterized by severe (head, body, and tail) tremors, vocalization on handling, bracing posture, rigidity, etc. Each item was scored separately, and the sum of the individual scores was used to measure the severity of the withdrawal syndrome. Subjects were temporarily anesthetized with halothane/room air inhalation anesthesia, and the femoral vein was cannulated for i.v. administration of pharmacological agents. The trachea was then exposed and incised to allow tracheal intubation with a Teflon catheter for artificial respiration. The paralyzing agent (d)-tubocurarine (4 mg/kg) was administered i.v., and once muscles were paralyzed, the rat was placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA), and the tracheal catheter was connected to a mechanical rodent ventilator (7025 Stoelting) set to deliver 90 strokes per min (3 ml per stroke). All pressure and incision points were infiltrated with a long-lasting anesthetic (Xylocaine ointment 2%). All experiments were done in strict accordance with the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiologic Society.

The skull was opened and a burr hole was drilled above the VTA (2.2–2.4 mm anterioposterior; 0.3–0.5 mm lateral, from lambda) according to Paxinos and Watson (14). A glass microelectrode (4–6 MΩ) filled with a 2% pontamine sky-blue solution/0.5 M sodium acetate was lowered into the VTA. DA neurons were identified according to well-established electrophysiological features and by antidromic activation. Stimuli consisting of monophasic rectangular pulses (0.1–2.0 mA; 0.1–0.5 msec; 0.8 Hz) were generated by a Grass Instrument (Quincy, MA) stimulator (S88) and stimulus isolation unit (SIU5) and delivered to the nucleus accumbens (anterioposterior 8.8 mm; lateral 1.6 mm from lambda; ventral 7.1 mm from dura matter) (14) by means of a stainless-steel Formvar-coated, bipolar-stimulating electrode (250-μm tip diameter). Stimulating current was monitored on the oscilloscope. At the end of each recording session the recording site was marked by iontophoretic ejection (1–10 μA, negative current for 10 min) of 2%

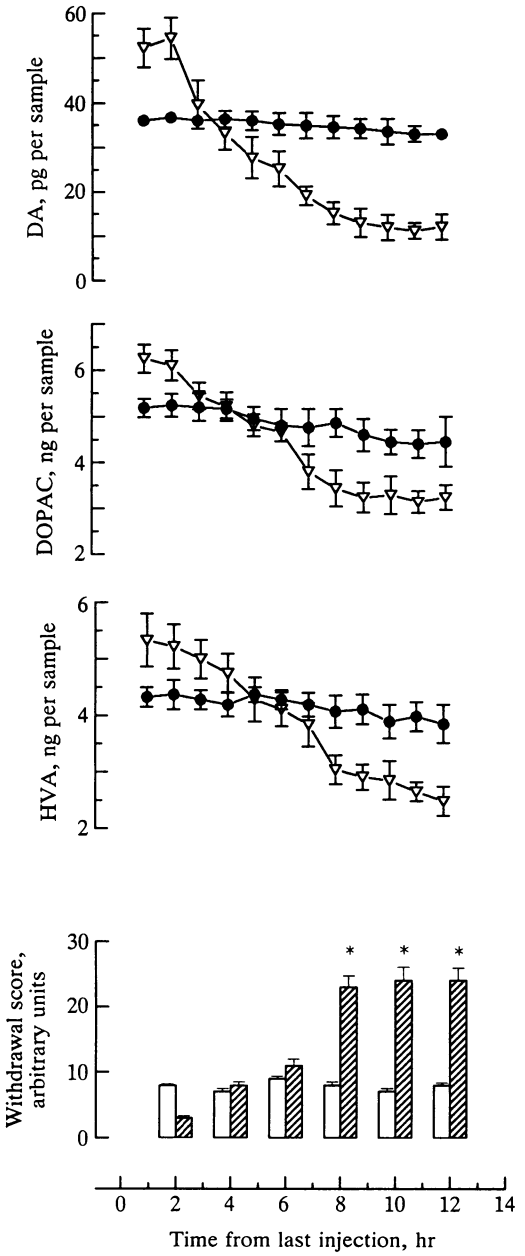

FIG. 1. (*Upper*) Effect of ethanol withdrawal on absolute extracellular concentrations of DA (*Top*), DOPAC (*Middle*), and HVA (*Bottom*) in the nucleus accumbens of ethanol-withdrawn (▽) and control rats (●). Values are means ± SEMs from six rats in each group. The two groups differ significantly ($P < 0.01$; two-way ANOVA). (*Lower*) Behavioral withdrawal scores are evaluated in the same rats as above. Control rats, open bars; ethanol-withdrawn rats, hatched bars. *, $P < 0.05$ by Mann–Whitney U test.

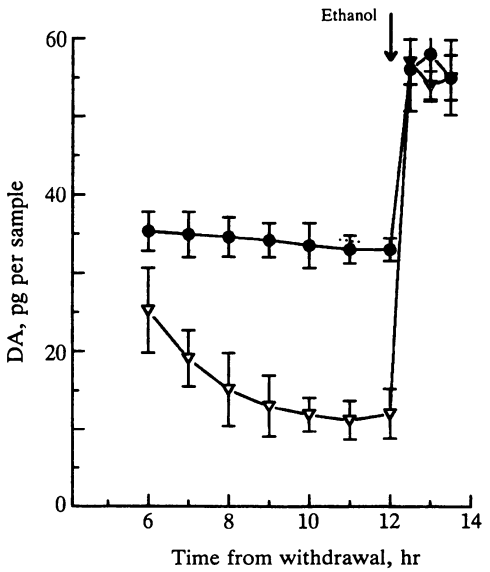

FIG. 2. Effect of a challenging dose of ethanol administered at arrow (5 g/kg *per os*) on DA outflow in the nucleus accumbens of ethanol-withdrawal (▽) and control rats (●). Values are means ± SEMs from six rats in each group. Ethanol-induced increases in DA concentrations in the two groups do not differ significantly ($P > 0.05$ by two-way ANOVA).

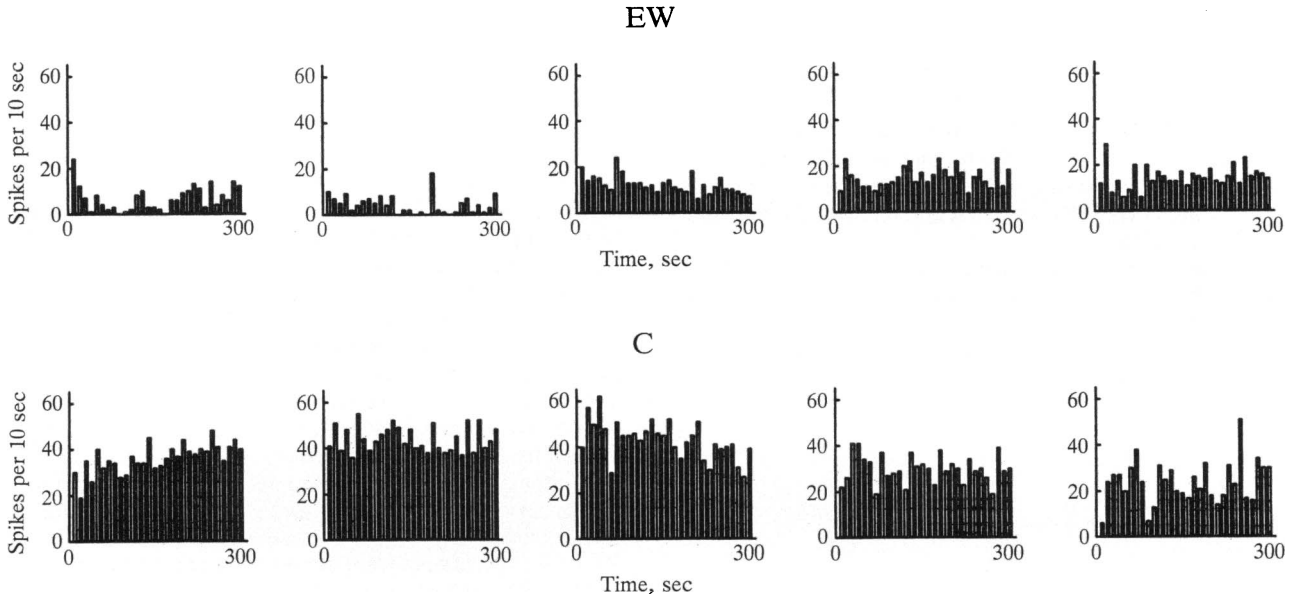

FIG. 3. Spontaneous activity of antidromically identified VTA-accumbens DA neurons encountered in an ethanol-withdrawn (EW) (*Upper*) and a control (C) rat (*Lower*). Each histogram represents the neuronal activity of a single neuron; note the difference in spontaneous activity between the two groups. Recordings were obtained in both cases 10–12 hr after the last ethanol and saline administrations, respectively.

pontamine sky-blue solution for histological verification. The extracellular neuronal signal from single neurons was amplified (Neurolog system) and displayed on a digital oscilloscope (Philips pm 3305) before storage on a magnetic tape for off-line data analysis. Firing rate and pattern analysis were done as already described (17).

The number of spontaneously active DA neurons was determined by passing the electrode eight times through four different areas within the VTA, according to the method proposed by Bunney and Grace (18). Coordinates of tracks were 2.2 and 2.4 mm anterior to lambda and 0.3 and 0.5 mm lateral to the midline. The electrode was lowered twice in each spot while the total number of active cells were counted and divided by the number of passes to obtain cells per track.

Refractory Period Measurement. Refractory periods were measured by using the twin-pulse test (19, 20): once the minimum stimulating current sufficient to evoke an antidromic response (threshold current) was determined, two stimuli were delivered at an interval that was progressively reduced. The delay at which the second antidromic response disappeared corresponded to the relative refractory period of that particular unit. Absolute refractory period was measured by using an identical procedure, but the stimulating current was increased to 2.5–3.0 times the threshold current.

RESULTS

Fig. 1 shows the absolute concentrations of DA, DOPAC, and HVA in the perfusates collected from nucleus accumbens of ethanol-withdrawn and control rats. Levels of DA [$F(90,1) = 15.56$, $P < 0.01$], DOPAC [$F(90,1) = 6.91$, $P < 0.01$], and HVA [$F(90,1) = 5.80$, $P < 0.01$] (two-way ANOVA) are significantly reduced in ethanol-withdrawn rats as compared with control animals. This decrease began ≈5 hr after the last ethanol administration and progressively decreased to 35% of control rats, 10–12 hr from withdrawal. Concomitantly, behavioral withdrawal scores increased to reach a plateau at 9–10 hr after the last ethanol administration (Fig. 1, *Lower*). A single administration of ethanol (5 g/kg) *per os* completely reversed withdrawal signs in ethanol-withdrawn rats, and DA outflow was stimulated to a similar extent in ethanol-withdrawn and control rats [$F(30,1) = 1.52$, $P > 0.05$] (Fig. 2).

Fig. 3 depicts the number and degree of spontaneous activity of DA neurons recorded from an ethanol-withdrawn rat and a control animal; as shown, the number of spontaneously active neurons encountered does not differ between the two animals. However, basal firing rate is markedly reduced in the ethanol-withdrawal group, and this is associated with a reduction in total burst firing and spikes per burst (Table 1). Furthermore, both absolute and relative refractory periods are elongated in the ethanol-withdrawal, as compared with the control group (Table 1). Intravenous administration of cumulative doses of ethanol to ethanol-withdrawn rats ($n = 6$), (0.25–2 g/kg) increases firing rate (Fig. 4).

DISCUSSION

Our results indicate that DA release in the nucleus accumbens is markedly reduced during ethanol-withdrawal syndrome. Furthermore, the effect of the last ethanol administration before withdrawal did not abate, indicating that no tolerance to this effect had developed. This result confirms our report (21) in which the ethanol-stimulating properties of the mesolimbic DA system displayed no tolerance.

The onset of the reduction of DA output parallels that of the withdrawal syndrome assessed behaviorally and is associ-

Table 1. Basal activity of mesoaccumbens DA neurons in ethanol-withdrawn rats

	EW	C
No. of cells	55	44
Cells per track	0.93 ± 0.1	0.98 ± 0.18
Firing rate, Hz	1.99 ± 0.21*	3.32 ± 0.24
Burst rate, burst per sec	0.41 ± 0.06*	0.71 ± 0.05
Spikes per burst	2.46 ± 0.19**	2.96 ± 0.15
ARP, msec	2.23 ± 0.14*	1.31 ± 0.09
RRP, msec	4.04 ± 0.33**	2.86 ± 0.29

Various electrophysiological parameters recorded from ethanol-withdrawn (EW) and saline treated-rats (C) are illustrated. Cells per track, number of cells responding to antidromic identification from nucleus accumbens divided by number of tracks; firing rate, number of spikes per sec (Hz); burst rate, number of bursts averaged for 1 sec; spikes per burst, number of action potentials per burst; ARP, absolute refractory period; RRP, relative refractory period. *, $P < 0.01$ by Student's t test for unpaired observation; **, $P < 0.05$.

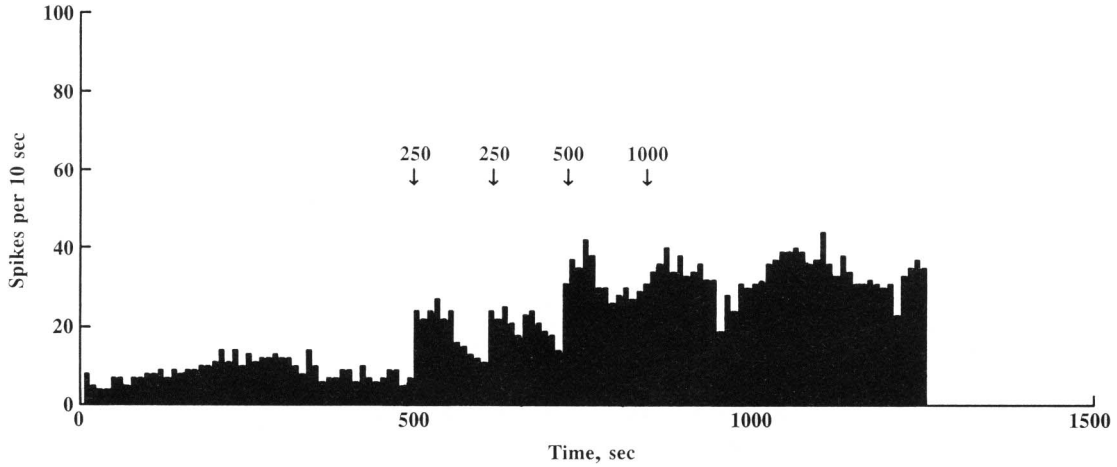

FIG. 4. Effect of cumulative doses of ethanol, administered i.v. at arrows, on an antidromically identified VTA-accumbens DA neuron recorded from an ethanol-withdrawn rat. Numbers above arrows indicate ethanol dosage expressed in mg/kg. Recording was done 10–12 hr after the last ethanol administration.

ated with a reduction of the spontaneous neuronal activity of mesoaccumbens dopaminergic neurons, as indicated by the recordings in unanesthetized rats. Indeed, the electrophysiological features of these neurons are uniformly reduced. The firing rate and burst firing are drastically decreased during ethanol-withdrawal syndrome. Considering that a burst of action potentials releases more DA in its projection areas than does the same number of evenly spaced action potentials (22, 23), the decrease in burst firing alone could substantially account for the reduced DA output in the nucleus accumbens. However, the number of spontaneously active DA neurons was not significantly different in treated rats as compared with controls. This result would indicate that dopaminergic neurons are not affected by depolarization inactivation but are rather more refractory to intrinsic and extrinsic depolarizing stimuli. The finding that absolute and relative refractory periods are prolonged during the ethanol-withdrawal syndrome is consistent with this possibility and suggests a possible alteration in the membrane machinery controlling the kinetics of ionic channels.

Regardless of the ionic species underlying these changes, which will require further clarification by intracellular recording experiments, our results indicate that dopaminergic mesolimbic activity is lowered during ethanol-withdrawal syndrome. Administration of ethanol restored electrophysiological and biochemical parameters, further confirming the causal role played by the suspension of ethanol after chronic treatment in the genesis of dopaminergic reduction.

If an increase in the mesolimbic dopaminergic tone is important in brain-reward mechanisms (3–5) and, more pertinently, in ethanol-induced reward (6), it is reasonable to suspect that a reduction of mesolimbic dopaminergic activity may form the basis of the dysphoria associated with the ethanol-withdrawal syndrome. The dysphoria, in turn, may trigger the drug-seeking behavior (craving) characteristic of abrupt cessation of chronic drug intake.

In conclusion, our experiments indicate that during ethanol-withdrawal syndrome a marked reduction of mesolimbic dopaminergic neuronal activity occurs, as indicated by biochemical and electrophysiological evidence. Although the ionic mechanism of this reduction remains to be elucidated, a lowered dopaminergic tone may provide a neurobiological correlate of the dysphoric effect of ethanol-withdrawal syndrome.

On a more general perspective, the analysis of various neurotransmitter systems involved in the ethanol-withdrawal syndrome, and possibly other withdrawal syndromes (24), may offer a model to study the neurobiological substrates of craving, which will help in the understanding of a condition that plays a major role in relapse.

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